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# Drug resistance to 5-aza-2′-deoxycytidine, 2′,2′-difluorodeoxycytidine, and cytosine arabinoside conferred by retroviral-mediated transfer of human cytidine deaminase cDNA into murine cells

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**Abstract** *Purpose*: The hematopoietic toxicity produced by the cytosine nucleoside analogs is a critical problem that limits their effectiveness in cancer therapy. One strategy to prevent this dose-limiting toxicity would be to insert a gene for drug resistance to these analogs into normal bone marrow cells. Cytidine (CR) deaminase can deaminate and thus inactivate 5-aza-2′-deoxycytidine (5-AZA-CdR), 2′,2′-difluorodeoxycytidine (dFdC) and cytosine arabinoside (ARA-C). The aim of this study was to determine if gene transfer of CR deaminase into murine fibroblast cells confers drug resistance to these cytosine nucleoside analogs and if this resistance can be prevented by the CR deaminase inhibitor, 3,4,5,6-tetrahydrouridine (THU). *Methods*: NIH 3T3 murine fibroblast cells were transduced with retroviral particles containing the human CR deaminase cDNA. Assays measuring CR deaminase activity as well as the inhibitory action of 5-AZA-CdR, dFdC and ARA-C on colony formation, were performed in the presence of different concentrations of THU. *Results*: Retroviral-mediated transfer of the CR deaminase gene into 3T3 fibroblasts produced a considerable increase in CR deaminase activity. The transduced cells also showed significant drug resistance to 5-AZA-CdR, dFdC and ARA-C, as demonstrated by a clonogenic assay. This drug resistance phenotype and elevated CR deaminase activity were reversed by THU. *Conclusions*: These findings indicate that the CR deaminase gene can potentially be used in cancer gene therapy for protecting normal cells against the cytotoxic actions of different cytosine nucleoside analogs. In addition, the CR deaminase-transduced cells can be used as a model for screening different CR deaminase inhibitors in an intact cellular system.

**Key words** Cytidine deaminase · 5-AZA-2′-deoxycytidine · 2′, 2′-Difluorodeoxycytidine · Cytosine arabinoside · 3,4,5,6-Tetrahydrouridine

**Abbreviations** *3T3-CD3-V5* NIH 3T3 cells tranduced with MFG-CD virions · *5-AZA-CdR* 5-aza-2′-deoxycytidine · *ARA-C* cytosine arabinoside · *CR* cytidine · *dFdC* 2′,2′-difluorodeoxycytidine · *MDR* multiple drug resistance · *THU* 3,4,5,6-tetrahydrouridine

## Introduction

For many antineoplastic agents the dose-limiting toxicity is myelosuppression. Thus, gene therapy strategies devised to prevent the severe granulocytopenia associated with chemotherapeutic drugs would increase their curative potential [2]. There have been several reports on the use of retroviral-mediated gene transfer of different drug resistance genes into normal hematopoietic stem cells for protection against drug-induced toxicity. For instance, transfer of a mutant dihydrofolate reductase gene into marrow precursors provides hematopoietic chemoprotection from methotrexate in mice transplanted with these cells [6]. Likewise, drug resistance to alkylating agents in murine hematopoietic cells has been realized using retroviral gene transfer of $O^6$-alkylguanine-DNA alkyltransferase [1] or glutathione S-transferase [14]. Extensive gene transfer studies using the human MDR gene have shown that this gene conferred protection from the toxicity of MDR-responsive drugs in

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murine marrow cells [31, 34]. Administration of the cytotoxic drug, taxol, to mice transplanted with MDR-transduced hematopoietic cells results in the selective expansion of MDR-expressing cells [34].

Cytidine (CR) deaminase catalyzes the deamination of cytosine nucleosides and their analogs such as 5-AZA-CdR, dFdC and ARA-C resulting in a loss of their antineoplastic activity [3, 5, 25]. CR deaminase may be involved in clinical drug resistance to 5-AZA-CdR or ARA-C in some patients with leukemia since at the time of relapse after treatment the leukemic cells show elevated levels of this enzyme [27, 35]. ARA-C is a very effective chemotherapeutic agent against acute myeloid leukemia [11] whereas dFdC shows promising clinical antitumor activity [9, 15]. In clinical trials, 5-AZA-CdR has demonstrated antileukemic [21, 32] and interesting antitumor activity [24].

The primary toxicity of these cytosine nucleoside analogs, limiting their dose-intensity, is bone marrow suppression. Retroviral gene transfer of CR deaminase into normal hematopoietic cells would be expected to render them resistant to these analogs and consequently allow dose escalation to improve their clinical effectiveness.

We have cloned and expressed the human CR deaminase complementary DNA (cDNA) [13] and used a retroviral plasmid expression vector to transfect this gene into ecotropic packaging cells [22]. We transduced normal murine hematopoietic and fibroblast cells and produced the ARA-C resistance phenotype in vitro [23]. In the present investigation, which confirms our preliminary study [8], we show that the CR deaminase-transduced fibroblast cells are not resistant uniquely to ARA-C, but also demonstrate cross-resistance to other cytosine nucleoside analogs such as 5-AZA-CdR and dFdC. In addition, we determined that this drug resistance phenotype and enhanced CR deaminase activity can be reversed by THU, a competitive inhibitor of CR deaminase [4].

## Material and methods

### Cell lines

Cells were grown in monolayers in Dulbecco's modified essential medium (Canadian Life Technologies, Burlington, Ontario) supplemented with 10% heat-inactivated fetal bovine serum (Wisent Technologies, St. Bruno, Quebec) and 5 μg/ml gentamicin (Canadian Life Technologies), and incubated at 37 °C in an atmosphere containing 7% $CO_2$. Murine ecotropic retrovirus-packaging cells GP + E-86 were obtained from A. Bank (Columbia University, New York) [16].

### Construction of retroviral vector and generation of virus-producing cell line

The plasmid expression vector pMFG-CD containing the human CR deaminase cDNA was constructed as described recently [22]. Briefly, the coding cDNA sequence for human CR deaminase [13] was cloned between the NcoI and BamHI sites of the pMFG retroviral plasmid (R. Mulligan, Harvard University, Cambridge, Mass.) to give the pMFG-CD construct. The plasmid pMFG-CD was transfected with pSV2 neo into GP + E-86 ecotropic packaging cells. Clones of cells resistant to G418 were isolated and further analyzed. The clone GP + E-86-CD3 was chosen for gene transfer studies since it demonstrated very high levels of CR deaminase expression, significant drug resistance to ARA-C, and a good viral titer [22].

### Transduction of murine fibroblast cells

Supernatant containing MFG-CD virions from GP + E-86-CD3 cells was placed in a flask of 3T3 fibroblast cells with 4 μg/ml polybrene. The cells were plated 3 days following infection in the presence of $10^{-6}$ *M* ARA-C and selected for 14 days. Clones of drug-resistant cells were isolated by ring cloning and thereafter maintained in medium without ARA-C. The 3T3-CD3-V5 clone showed increased CR deaminase expression and marked drug resistance to ARA-C [23].

### Clonogenic colony assay

Aliquots of 100 cells in 2 ml of medium were placed in six-well Costar tissue culture dishes and 18–20 h later, $10^{-7}$ *M* 5-AZA-CdR (Pharmachemie, Haarlem, The Netherlands), $10^{-7}$ *M* dFdC (Lilly Research Laboratories, Indianapolis, Ind.) or $10^{-6}$ *M* ARA-C (Upjohn, Canada) was added alone or with different dilutions of THU (Calbiochem, La Jolla, Calif.) for a 50-h drug exposure. After drug removal and an additional incubation of 10 days, colonies were stained with 0.5% methylene blue in 50% methanol and then counted. The average plating efficiency was 40–60%.

### Enzyme assay

CR deaminase activity was determined as described previously [19]. Briefly, monolayer cells ($2–5 \times 10^7$) were trypsinized, washed with phosphate-buffered saline and suspended in 5 m*M* Tris-HCl (pH 7.4) and 5 m*M* dithiothreitol. The cell suspension was freeze-thawed rapidly three times and centrifuged to obtain the cytosolic extract (supernatant). For enzyme assay, dilutions of the cytosol were placed in a reaction mixture with 50 m*M* Tris-HCl and 0.5 μCi $^3$H-cytidine (ICN Biomedicals, Irvine, Calif.), with or without THU. The mixture was incubated for 30 min at 37 °C and placed on Whatman P-81 phosphocellulose discs. The amount of radioactivity bound to the discs was assessed by scintillation counting. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the deamination of 1 nmol of cytidine per minute at 37 °C. The protein concentration was measured using the BioRad dye method with bovine serum albumin as the standard.

## Results

The sensitivity of nontransduced (3T3) and CR deaminase-transduced (3T3-CD3-V5) cells to cytosine nucleoside analogs 5-AZA-CdR, dFdC, and ARA-C, was evaluated by clonogenic assay. To demonstrate a relationship between the drug response on 3T3-CD3-V5 cells and the activity of the CR deaminase gene, the CR deaminase inhibitor THU was also used.

As shown in Fig. 1, 5-AZA-CdR at a concentration of $10^{-7}$ *M* produced a substantial reduction in colony formation by the 3T3 cells, but had no considerable cytotoxic effect on the 3T3-CD3-V5 cells. The addition of $10^{-4}$ *M* THU restored drug sensitivity to 5-AZA-CdR by the transduced cells. In Table 1, the effect of different

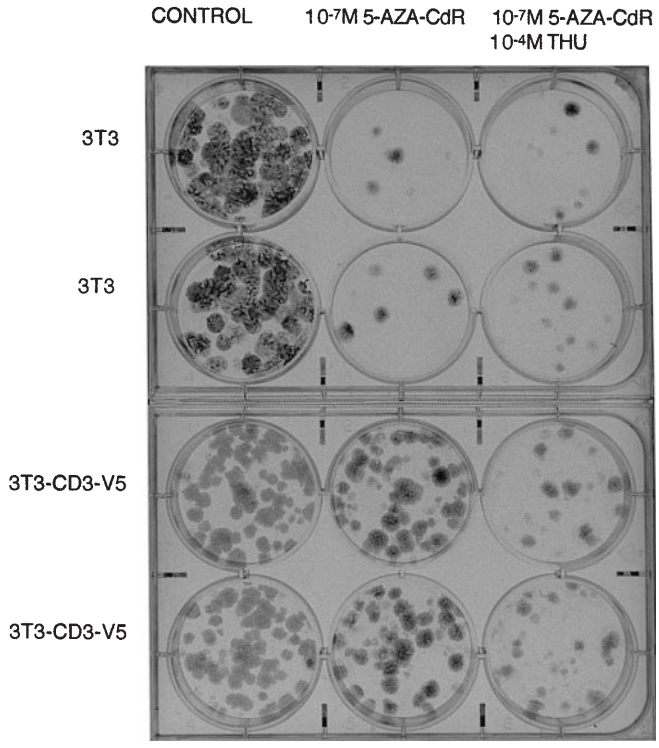


**Fig. 1** Effect of 5-AZA-CdR and THU on colony formation by murine fibroblasts. 3T3 or 3T3-CD3-V5 cells were plated at 100 cells per well. Drug exposure was 50 h

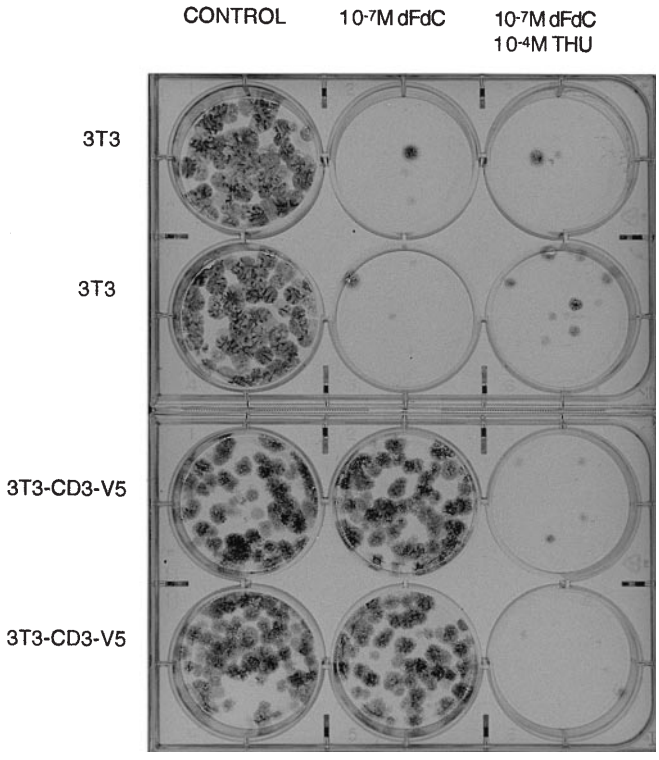


**Fig. 2** Effect of dFdC and THU on colony formation by murine fibroblasts. 3T3 or 3T3-CD3-V5 cells were plated at 100 cells per well. Drug exposure was 50 h

concentrations of THU on loss of clonogenicity by 3T3-CD3-V5 cells in the presence of $10^{-7}$ *M* 5-AZA-CdR is shown. THU at a concentration of $10^{-4}$ *M* increased the inhibitory action of 5-AZA-CdR on colony formation from 19.5% to 72%.

dFdC at a concentration of $10^{-7}$ *M* practically abolished colony survival by the parental 3T3 cells (Fig. 2). However, at this same concentration of dFdC, colony formation by the CR deaminase-transduced 3T3-CD3-V5 cells was not significantly altered. The addition of $10^{-4}$ *M* THU completely restored drug sensitivity to dFdC by the transduced cells. The effect of different concentrations of THU on colony formation by 3T3-CD3-V5 cells in the presence of $10^{-7}$ *M* dFdC is shown in Table 1. THU at concentrations of $10^{-6}$ *M* and $10^{-4}$ *M* increased the inhibitory effect of dFdC from 8.4% to 60.3% and 98.5%, respectively.

Treatment with $10^{-6}$ *M* ARA-C suppressed almost entirely colony formation by the control 3T3 cells whereas the 3T3-CD3-V5 cells showed nearly complete drug resistance to this concentration (Fig. 3). The addition of $10^{-4}$ *M* THU completely restored ARA-C sensitivity by these latter cells. In Table 1, the effect of different concentrations of THU on colony formation by 3T3-CD3-V5 cells in the presence of $10^{-6}$ *M* ARA-C is also shown. THU at concentrations of $10^{-5}$ and $10^{-4}$ *M* increased the inhibitory action of ARA-C from 5% to 76.2% and 89.5%, respectively.

Enzyme assays were performed on the cytosol obtained from the cells transduced with the MFG-CD retrovirus in order to compare the inhibitory activity of THU directly on the enzyme with the results obtained in intact cells. As revealed in Table 2, parental 3T3 cells showed low (< 3 units/mg) CR deaminase activity. In contrast, the enzyme activity of 3T3-CD3-V5 cells was 106.8 units/mg which represents an augmentation of over 30-fold in comparison with the 3T3 control cells. When the specific CR deaminase inhibitor THU was added to the cytosolic extract of 3T3-CD3-V5 cells, the level of enzyme activity declined in correspondence to the concentration of THU used. The CR deaminase activity in the transduced cells was inhibited by 52.7% with $10^{-6}$ *M* THU, 85.3% with $10^{-5}$ *M* THU, and

**Table 1** Effect of tetrahydrouridine (THU) on the antineoplastic action of 5-AZA-CdR, dFdC, and ARA-C on CR deaminase-transduced 3T3-CD3-V5 cells as determined by colony assay for a 50-h drug exposure. Data represent mean values ± SD ($n$ = 4–10). Loss of clonogenicity in the presence of $10^{-4}$ *M* THU alone was < 5%

| THU concentration (*M*) | Loss of clonogenicity (%) | | |
|---|---|---|---|
| | 5-AZA-CdR ($10^{-7}$ *M*) | dFdC ($10^{-7}$ *M*) | ARA-C ($10^{-6}$ *M*) |
| 0 | 19.5 ± 11.2 | 8.4 ± 9.9 | 5.0 ± 6.5 |
| $10^{-6}$ | 23.5 ± 28.6 | 60.3 ± 3.9 | 32.5 ± 11.3 |
| $10^{-5}$ | 52.2 ± 22.9 | 83.8 ± 22.4 | 76.2 ± 18.2 |
| $10^{-4}$ | 72.0 ± 10.0 | 98.5 ± 2.7 | 89.5 ± 8.7 |

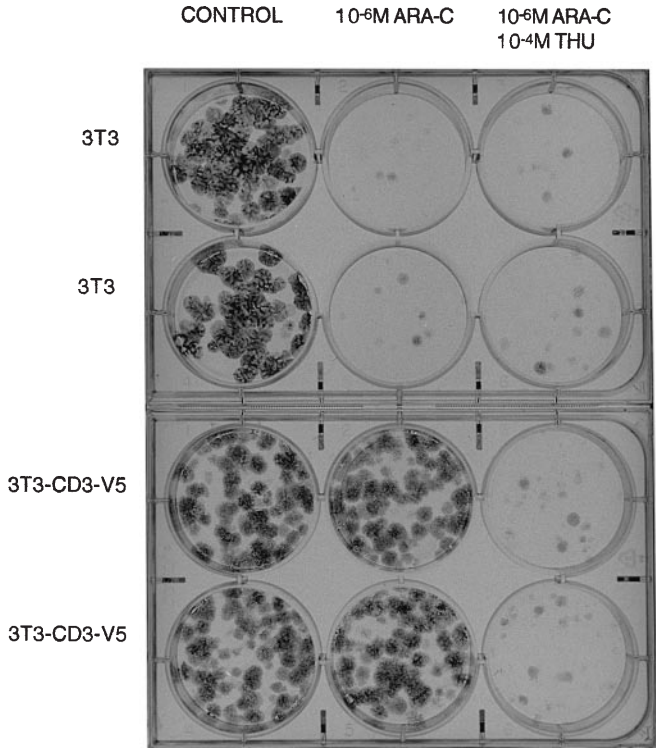


**Fig. 3** Effect of ARA-C and THU on colony formation by murine fibroblasts. 3T3 or 3T3-CD3-V5 cells were plated at 100 cells per well. Drug exposure was 50 h

**Table 2** Effect of different concentrations of THU on CR deaminase activity in murine cells. CR deaminase activity was measured in cell extracts of the different cell lines in the presence of the indicated concentrations of THU

| Cell line | THU Concentration (M) | CR deaminase activity (units/mg)[a] | Enzyme inhibition (%) |
|---|---|---|---|
| 3T3 | 0 | $<3$ | 0 |
| 3T3 | $10^{-4}$ | $<3$ | $<1$ |
| 3T3-CD3-V5 | 0 | $106.8 \pm 8.3$[b] | 0 |
| 3T3-CD3-V5 | $10^{-6}$ | $50.5 \pm 14.0$[b] | 52.7 |
| 3T3-CD3-V5 | $10^{-5}$ | $15.7 \pm 3.3$[b] | 85.3 |
| 3T3-CD3-V5 | $10^{-4}$ | $<3$ | $>97$ |

[a] units defined as nanomoles CR deaminated per minute

[b] mean ± SD(n = 4-7)

$>97\%$ with $10^{-4}$ *M* THU. The correlation coefficient between THU inhibition of enzyme activity and its ability in transduced cells to restore drug sensitivity was $>0.96$ for dFdC and ARA-C, and $>0.87$ for 5-AZA-CdR.

## Discussion

The new cytosine nucleoside analogs have considerable promise in cancer therapy. dFdC exerts its cytotoxic effect primarily by inhibiting DNA synthesis [30]. This clinically active cytosine nucleoside analog has demonstrated good response rates in tumor therapy [9, 15]. The experimental agent 5-AZA-CdR has the novel action of inhibiting DNA methylation which can result in an induction of differentiation of neoplastic cells [10, 18] and the activation of silent tumor suppressor genes [7, 17, 29]. In clinical studies, 5-AZA-CdR has shown favorable activity against several types of hematological malignancies [21, 32, 36]. In a pilot study in stage IV metastatic non-small-cell lung cancer, 5-AZA-CdR produced some very interesting responses, including one patient still alive $>6$ years post-treatment [24].

Current conventional chemotherapy of most advanced metastatic cancers is disappointing with low response rates and short life expectancies. Both dFdC and 5-AZA-CdR have the potential to be very effective antitumor agents if their dose-limiting hematotoxicity [9, 24] could be circumvented. The degradative enzyme CR deaminase can convert by hydrolytic deamination 5-AZA-CdR, dFdC and ARA-C to pharmacologically inactive compounds [3, 5, 25]. One approach to overcome the hematopoietic toxicity produced by these cytosine nucleoside analogs would be to introduce the CR deaminase gene into normal marrow precursors to render them resistant to their cytotoxic action.

Several studies utilizing different drug resistance genes have shown that chemoprotection of hematopoietic cells is feasible. Gene transfer of dihydrofolate reductase, glutathione S-transferase and MDR into murine hematopoietic progenitors has been shown to confer drug resistance to methotrexate, alkylating agents and MDR drugs, respectively [6, 14, 31, 34]. A clinical trial in breast cancer patients utilizing MDR gene transfer to bestow chemoprotection has been initiated [28].

The human CR deaminase cDNA has been cloned and expressed in our laboratory and has been shown to encode a 146-amino-acid protein of 48.7 kDa [13]. The small size of the CR deaminase cDNA facilitates its genetic manipulation. Cells transduced with CR deaminase show marked ARA-C resistance in vitro and a large increase in enzyme activity [23]. In a preliminary investigation [8], we have observed that transduced fibroblast cells are also cross-resistant to other cytosine nucleoside analogs (5-AZA-CdR and dFdC). The present study confirmed and extended our initial study. We demonstrated by colony formation assays that the CR deaminase gene transfer into 3T3 cells conferred substantial drug-resistance to 5-AZA-CdR and dFdC. To illustrate the cause-effect relation between the overexpression of the CR deaminase gene and the drug-resistance phenotype, we utilized the CR deaminase inhibitor, THU. The inhibitory effect on CR deaminase and hence reversal of the drug resistance became increasingly evident as the concentration of THU was increased.

Colony formation by the CR deaminase-transduced 3T3-CD3-V5 cells was not considerably inhibited by 5-AZA-CdR, whereas this analog produced a pronounced cytotoxic effect on the non-transduced 3T3 cells (Fig. 1). When 5-AZA-CdR was added simultaneously with THU, the inhibitory activity of this analog in

the 3T3-CD3-V5 cells was restored depending on the concentration of the CR deaminase inhibitor (Table 1). Treatment with dFdC practically did not affect colony formation by 3T3-CD3-V5 cells whereas the control 3T3 cells were very sensitive to this analog (Fig. 2). THU enhanced the inhibitory action of dFdC on colony survival by transduced cells (Table 1). Likewise, ARA-C did not noticeably alter colony formation by 3T3-CD3-V5 cells, but almost abolished cell survival by parental cells (Fig. 3). Colony formation by transduced cells was markedly reduced by ARA-C in the presence of THU (Table 1). These studies with THU clearly indicate that the enhanced level of CR deaminase activity in the transduced cells is primarily reponsible for drug resistance to these cytosine nucleoside analogs. It should be noted that drug resistance to cytosine nucleoside analogs can also occur by several other mechanisms [20].

To determine whether the reversal of drug resistance in the transduced cells by THU correlated with its inhibition of CR deaminase, we prepared cytosol extracts from these cells and investigated the effect of this CR deaminase inhibitor directly on the enzyme. The CR deaminase activity measured in 3T3-CD3-V5 cells was over 30-fold higher than that noted in the 3T3 cells (Table 2). When increasing concentrations of THU were added to the cytosol extracts from transduced cells, enzyme activity progressively fell, reaching a level equivalent to that in non-transduced cells (Table 2). Since in the case of dFdC and ARA-C, $10^{-4}$ *M* THU showed almost complete reversal of drug resistance in the CR deaminase-transduced cells and inhibited deaminase activity in these cells by $>97\%$, the correlation between the effects of this enzyme inhibitor on the cells and on the enzyme is very good. THU was the first inhibitor of CR deaminase to be identified [4]. Its clinical importance was shown when it was noted that THU in combination with ARA-C significantly increases the plasma level of ARA-C in patients with solid tumors [12].

Neff and Blau [26] have also demonstrated, using a different retroviral vector (LCDSN), that gene transfer of CR deaminase into 3T3 cells confers ARA-C resistance. They employed a growth assay to show a 4.5-fold increased resistance to ARA-C by $ID_{50}$, compared with cells transduced with a control vector. The enzyme activity measured in transduced fibroblast cells was 7-fold lower than that determined in our present study with clone 3T3-CD3-V5. These investigators also transduced the lymphoid leukemic cell line CCRF-CEM and showed some drug resistance to the growth inhibitory effects of ARA-C and dFdC. They observed that the resistance to growth inhibition by ARA-C could be reversed by $2.5 \times 10^{-4}$ *M* THU.

Shröder et al. [33] transfected the CR deaminase cDNA into murine fibroblast cells, using a mammalian expression vector and demonstrated an augmentation in CR deaminase activity and ARA-C resistance of about threefold. They determined as well that $CD34^+$ selected human peripheral blood progenitor cells express a low level of CR deaminase activity.

In conclusion, if our in vitro results that CR deaminase gene transfer can make cells resistant to different cytosine nucleoside analogs can be extended in vivo to normal human hematopoietic stem cells, it may be possible to improve tumor therapy with dFdC and 5-AZA-CdR. The protection of the hematopoietic stem cells by gene therapy will permit the use of more intensive therapy with these analogs to increase their clinical effectiveness against tumor cells without encountering the problem of severe myelosuppression. Furthermore, the CR deaminase-transduced cells can be used as a model for screening different CR deaminase inhibitors in an intact cellular system.

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