# Monitoring Gene Therapy with Cytosine Deaminase: In Vitro Studies Using Tritiated-5-Fluorocytosine

Uwe Haberkorn, Franz Oberdorfer, Johannes Gebert, Iris Morr, Karin Haack, Klaus Weber, Markus Lindauer, Gerhard van Kaick and Hans Konrad Schackert

*Departments of Oncological Diagnostics and Therapy and Radiochemistry, German Cancer Research Center; and Department of Surgery, University of Heidelberg, Heidelberg, Germany*

Genetically modified mammalian cells that express the cytosine deaminase (CD) gene are able to convert the nontoxic prodrug 5-fluorocytosine (5-FC) to the toxic metabolite 5-fluorouracil (5-FU). PET with $^{18}$F-5-FC may be used for in vivo measurement of CD activity in genetically modified tumors. **Methods:** A human glioblastoma cell line was stably transfected with the *Escherichia coli* CD gene. After incubation of lysates of CD-expressing cells and control cells with $^{3}$H-5-FC high-performance liquid chromatography (HPLC) was performed. The uptake of 5-FC was measured after various incubation times using therapeutic amounts of 5-FC. In addition, saturation and competition experiments with 5-FC and 5-FU were performed. Finally, the efflux was measured. **Results:** We found that $^{3}$H-5-FU was produced in CD-expressing cells, whereas in the control cells only $^{3}$H-5-FC was detected. Moreover, significant amounts of 5-FU were found in the medium of cultured cells, which may account for the bystander effect observed in previous experiments. However, uptake studies revealed a moderate and nonsaturable accumulation of radioactivity in the tumor cells, suggesting that 5-FC enters the cells only through diffusion. Although a significant difference in 5-FC uptake was seen between CD-positive and control cells after 48 hr of incubation, no difference was observed after 2 hr of incubation. Furthermore, a rapid efflux could be demonstrated. **Conclusion:** 5-Fluorocytosine transport may be a limiting factor for this therapeutic procedure. Quantitation with PET has to rely more on dynamic studies and modeling, including HPLC analysis of the plasma, than on nonmodeling approaches.

**Key Words:** gene therapy; fluorocytosine; cytosine deaminase; PET; glioblastoma

**J Nucl Med 1996; 37:87–94**

Transfer and expression of suicide genes into malignant tumor cells represents an attractive approach for human gene therapy. Suicide genes typically code for nonmammalian enzymes that convert nontoxic prodrugs into highly toxic metabolites. Therefore, systemic application of the nontoxic prodrug results in the production of the active drug at the tumor site. Cytosine deaminase (CD), which is expressed in yeasts and bacteria but not in mammalian organisms, converts the antifungal agent 5-fluorocytosine (5-FC) to the highly toxic 5-fluorouracil (5-FU). In mammalian cells, no anabolic pathway is known that leads to incorporation of 5-FC into the nucleic acid fraction. Therefore, pharmacological effects (due to conversion of 5-FC to 5-FU by the intestinal microflora) are moderate and allow the use of high therapeutic doses (*1–3*). The distribution of 5-FC in mammalian tissues is uniform, and neither tissue-specific accumulation nor appreciable binding to plasma proteins has been observed (*3–5*). 5-Fluorouracil exerts its toxic effect by interfering with DNA and protein synthesis due to substitution of uracil by 5-FU in RNA and inhibition of thymidilate synthetase by 5-fluorodeoxyuridine monophosphate, resulting in impaired DNA biosynthesis (*6*).

The use of CD in cancer therapy has recently been described. Nishiyama et al. (*7*) implanted CD-containing capsules into rat gliomas and subsequently treated the animals by systemic application of 5-FC. They observed significant amounts of 5-FU in the tumors as well as a decrease in tumor growth rate and systemic cytotoxicity. This approach for local chemotherapy was expanded by Wallace et al. (*8*) for use in patients with disseminated tumor disease. They used monoclonal antibody (MAb)–enzyme conjugates to achieve a selective activation of 5-FC, thereby obtaining a sevenfold higher level of 5-FU in the tumor after administration of MAb-CD and 5-FC than with the systemic use of 5-FU.

Targeting the enzyme to the tumor site may also be achieved by the transfer and expression of the suicide gene by use of recombinant retroviral vectors. Encouraging results were initially obtained in rat gliomas using a retroviral vector system for transfer and expression of the herpes simplex virus thymidine kinase gene (*9,10*). Recently, in vitro and in vivo studies have further demonstrated the potency of the CD suicide system. Tumor cells that had been infected with a retrovirus carrying the CD gene showed a strict correlation between 5-FC sensitivity and CD enzyme activity (*11–14*). Although not all the tumor cells have to be infected to obtain a sufficient therapeutic response, repeated injections of the recombinant retroviruses may be necessary to reach a therapeutic level of enzyme activity in the tumor. Therefore, a prerequisite for gene therapy using a suicide system is monitoring of suicide gene expression in the tumor for two reasons: (a) to decide whether repeated gene transductions of the tumor are necessary and (b) to find a therapeutic window of maximum gene expression and consecutive 5-FC administration. Because 5-FC can be labeled with $^{18}$F and shows in vivo stability (*15*), PET can be used to assess enzyme activity in vivo (*16,17*). The objective of the present study was to evaluate uptake parameters for 5-FC in an in vitro model and to obtain data that may be useful for the development of future quantitative PET measurements.

## MATERIALS AND METHODS

### Cell Culture

The human glioblastoma cell line T1115 was stably transfected with the CD gene (Rowley S, et al., *in press*). Briefly, a polymerase chain reaction amplification was done using two specific oligonucleotides derived from the published sequence of the CD gene (*18*). The amplified CD gene differed from this sequence in the following respects: change of the GTG start codon to ATG; alteration of the sequence preceding the ATG to match more closely the Kozak (*19*) consensus sequence, which is necessary for efficient eukaryotic translation; and introduction of a *Hind*III restriction site at the

Received Dec. 8, 1994; revision accepted May 15, 1995.

For correspondence and reprints contact: Uwe Haberkorn, MD, Department of Oncological Diagnostics and Therapy, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.

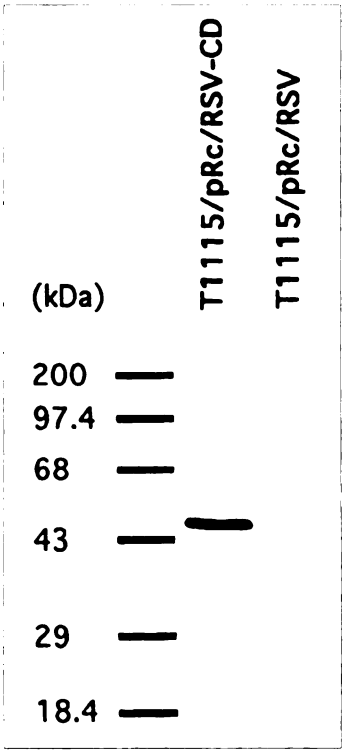


**FIGURE 1.** Western blot of T1115CD4 and T1115RSV cell lysates showing CD expression in T1115CD4 cells and no expression in T1115RSV cells.

5′ end and mutations in codon 69 (change of CAA to CAC, which replaces glutamine by histidine) and in codon 179 (change of GAT to GAC, a silent mutation with no change in the amino acid sequence). After cloning of the 1.3-kb *Hind*III/*Xba*I CD fragment into the pRc/RSV expression vector (Invitrogen Corp., San Diego, CA), resulting in pRSVCD, T1115 cells were transfected with this vector or the parental pRc/RSVneo vector using Lipofectin (Gibco BRL, Eggenstein, Germany), and G418-resistant clones were selected. Two subclones, T1115RSV and T1115CD4, which had integrated the parental pRc/RSVneo and the pRc/RSV-CD vectors, respectively, were used for further analyses. These cell lines showed no difference in growth kinetics and morphology.

The cells were cultured in Eagle's basal medium (BME, Gibco BRL) supplemented with 10% fetal calf serum, 292 mg glutamine, 100,000 units penicillin and 100 mg streptomycin/liter and grown at 37°C with 5% $CO_2$. In all experiments, the cells were in the logarithmic growth phase, and all experiments were performed in triplicate.

### Western Blot Analysis

After centrifugation of T1115RSV and T1115CD4 cells, the pellets were resuspended in 100 m*M* Tris (pH 7.5/1 m*M* ethylenediaminetetraacetic acid (EDTA/1 m*M* dithiothreitol (DTT) and sonicated. Thereafter, the protein concentration of the cell lysates was determined by the BCA protein assay (*20*) (Pierce, Rockford, IL).

For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, we used a 10% separating and a 5% stacking gel and 1 × Laemmli buffer (25 m*M* Tris base/192 m*M* glycine/0.1% SDS). One hundred micrograms of total protein were loaded per lane, and a 25-mA electric current was applied. Blotting was performed for 1 hr using a nitrocellulose membrane (Hybond extra, Amersham, Braunschweig, Germany), high-molecular-weight buffer (48 m*M* Tris base/386 m*M* glycine/methanol/0.02% SDS) and the BioRAD wet blotting system (BioRAD Laboratories, Munich, Germany) with a 340-mA current. Thereafter, the membrane was incubated overnight with 1 × Tris-buffered saline (TBS) (137 m*M* NaCl/2.7 m*M* KCl/24.8 m*M* Tris [pH 7.4]/1% bovine serum albumin/5% powdered milk at 4°C. Antiserum was diluted in 1 × TBS/5% powdered milk/0.2% Tween-20. The polyclonal rabbit antiserum (generated by K.H.) was used in a dilution of 1:1000. As the second antibody, a goat anti-rabbit IgG antibody (1:7500) was used as the alkaline phosphatase conjugate (Dianova, Hamburg, Germany). As the substrate for the alkaline phosphatase, 5-bromo-4-chloro-3-inolyl phosphate *p*-toludine salt (165 μg/ml)/nitro blue tetrazolium (330 μg/ml) (Sigma, Deisenhofen, Germany) in diethylamine buffer (5 m*M* $MgCl_2$/0.1 *M* diethanolamine, pH 9) was used.

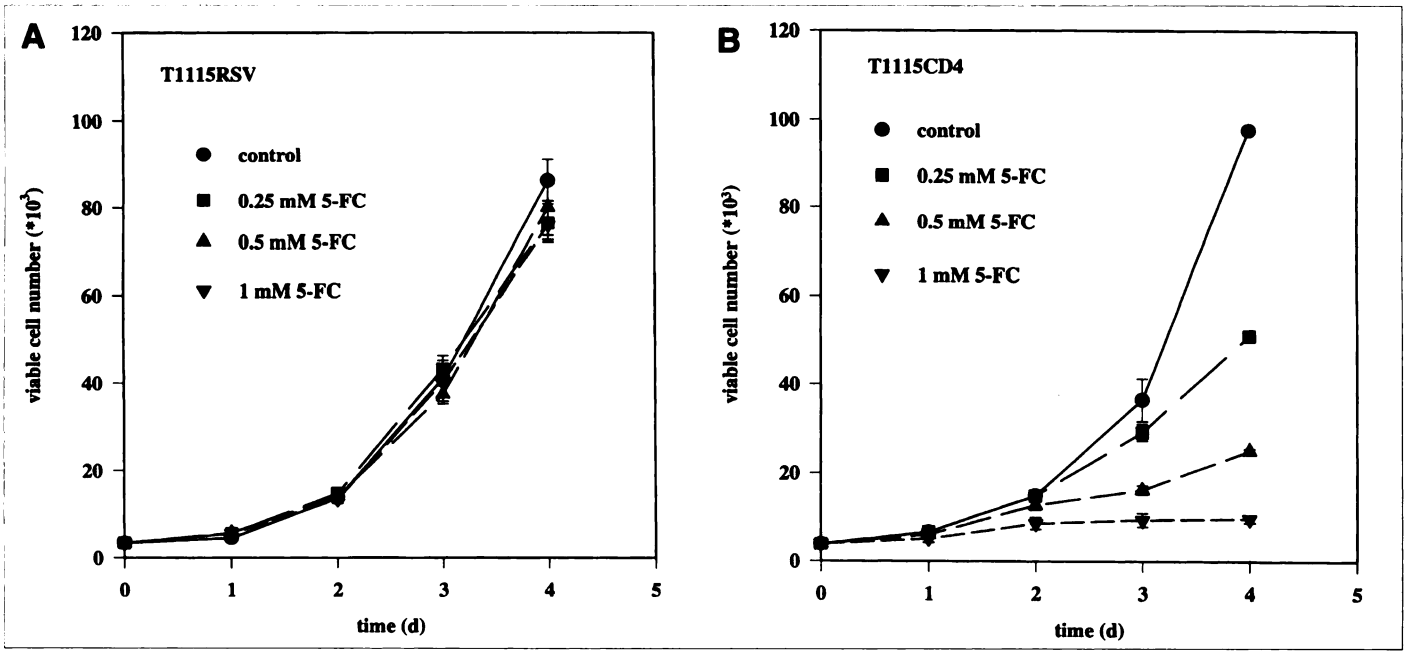


**FIGURE 2.** Growth curves (viable cells × $10^3$) of T1115RSV (A) and T1115CD4 (B) exposed to 0.25, 0.5 and 1 m*M* 5-FC. Mean values and s.d. are shown (n = 3).

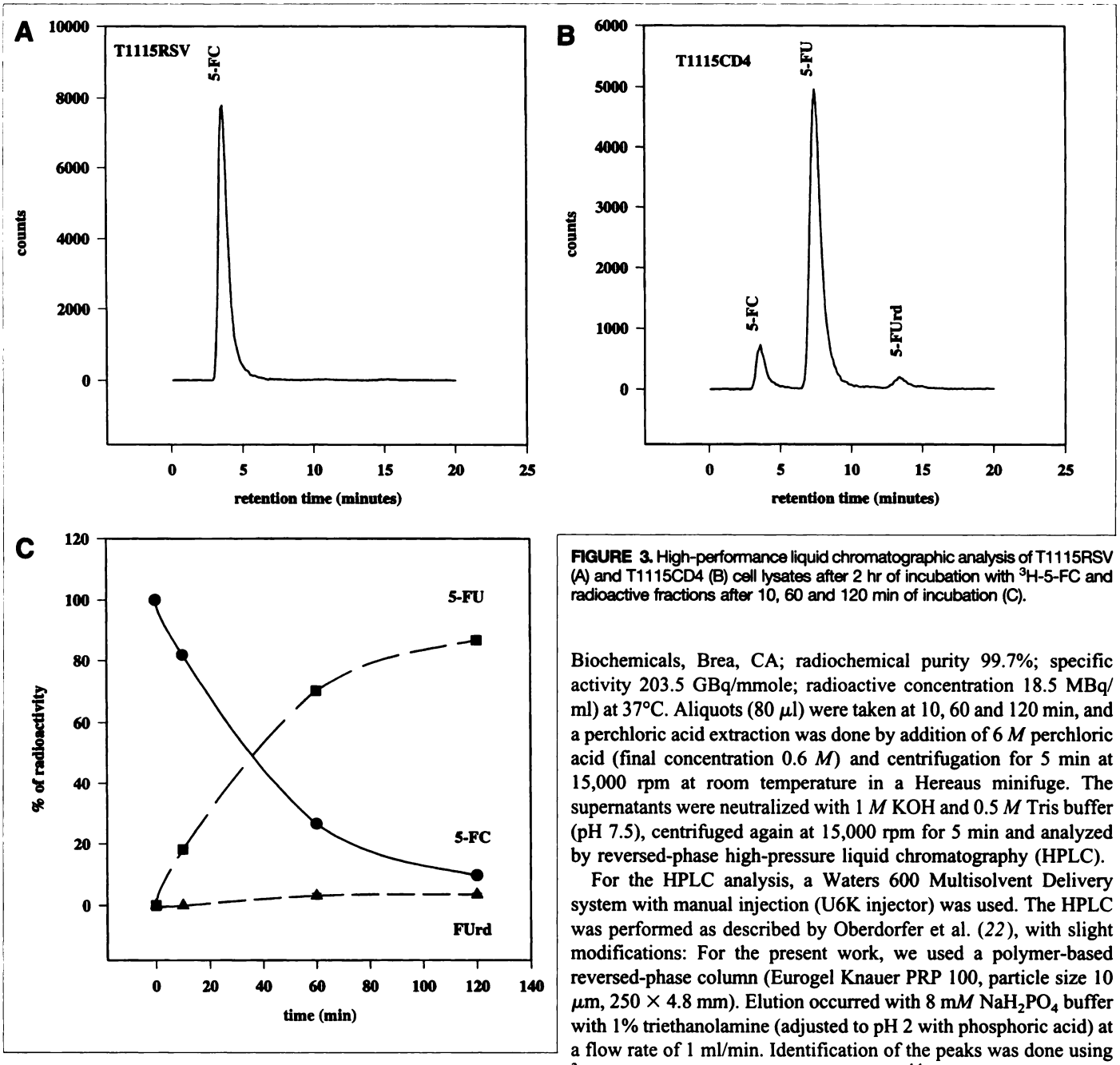


**FIGURE 3.** High-performance liquid chromatographic analysis of T1115RSV (A) and T1115CD4 (B) cell lysates after 2 hr of incubation with $^{3}$H-5-FC and radioactive fractions after 10, 60 and 120 min of incubation (C).

### Growth Inhibition

The T1115RSV and T1115CD4 cells were washed twice with phosphate-buffered saline (PBS) and incubated in supplemented BME medium without or with 0.25, 0.5 or 1 m*M* 5-FC. The viable cell number was determined on Days 1, 2, 3 and 4 after the onset of treatment using a Coulter ZM counter interfaced to a Coulter channelyzer (Coulter Electronics, Dunstable, UK) and the trypan blue method (*21*).

### Cytosine Deaminase Activity in Cell Lysates

The cells were trypsinized, washed with PBS and resuspended in lysis buffer (100 m*M* Tris, pH 7.5/1 m*M* EDTA/1 m*M* DTT) and lysed by freeze-thawing five times. The protein content of the lysates was determined by use of the bicinchoninic acid (BCA) protein assay (*21*) (Pierce).

For CD enzyme assays, 240 $\mu$l of the lysate (T1115RSV: 17.9 mg of total protein/ml; T1115CD4: 20.9 mg of total protein/ml) were incubated with 30 $\mu$l of 6-($^{3}$H)-5-FC (555 kBq) (Moravek Biochemicals, Brea, CA; radiochemical purity 99.7%; specific activity 203.5 GBq/mmole; radioactive concentration 18.5 MBq/ml) at 37°C. Aliquots (80 $\mu$l) were taken at 10, 60 and 120 min, and a perchloric acid extraction was done by addition of 6 *M* perchloric acid (final concentration 0.6 *M*) and centrifugation for 5 min at 15,000 rpm at room temperature in a Hereaus minifuge. The supernatants were neutralized with 1 *M* KOH and 0.5 *M* Tris buffer (pH 7.5), centrifuged again at 15,000 rpm for 5 min and analyzed by reversed-phase high-pressure liquid chromatography (HPLC).

For the HPLC analysis, a Waters 600 Multisolvent Delivery system with manual injection (U6K injector) was used. The HPLC was performed as described by Oberdorfer et al. (*22*), with slight modifications: For the present work, we used a polymer-based reversed-phase column (Eurogel Knauer PRP 100, particle size 10 $\mu$m, 250 × 4.8 mm). Elution occurred with 8 m*M* $NaH_2PO_4$ buffer with 1% triethanolamine (adjusted to pH 2 with phosphoric acid) at a flow rate of 1 ml/min. Identification of the peaks was done using $^{3}$H-5-FC (Moravek Biochemicals) and 2-($^{14}$C)-5-FC (Sigma; purity >98%; specific activity 1.117 GBq/mmole; radioactive concentration 35.15 MBq/ml) reference samples. Radioactivity was detected with a Canberra A250 flow-through detection system with a liquid scintillation flow cell (flow volume 500 $\mu$l). Retention time for 5-FC was 2.8 min (k′ = 0.6), whereas 5-FU eluted at 7.8 min (k′ = 3.3) on the present setup ($t_0$ was determined with urea).

In addition, production of 5-FU in the T1115CD4 lysate was determined after 10 and 20 min of incubation using 1 mg of total protein and a final concentration of 0.2 m*M* 5-FC (370 kBq $^{3}$H-5-FC). Specific CD activity was calculated from the difference of the 5-FU content after 10 and 20 min of incubation and expressed as picomoles per milligram of total protein per minute.

To determine the release of intracellularly produced 5-FU into the medium, 7 × $10^{6}$ T1115CD4 cells were incubated for 24 hr with 1.85 MBq of $^{3}$H-5-FC. Subsequently, the medium was centrifuged for 5 min at 1200 rpm in a Hereaus centrifuge to remove detached tumor cells. The cell-free medium was subjected to HPLC analysis after perchloric acid extraction.

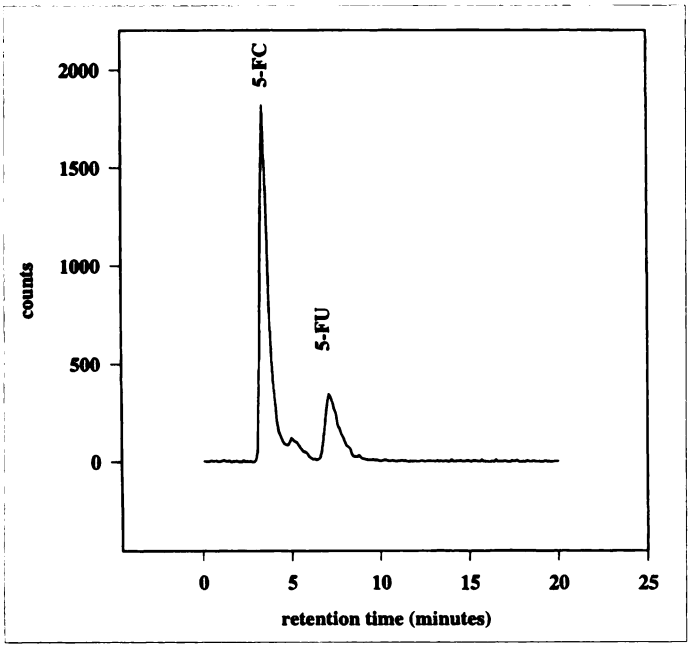


**FIGURE 4.** T1115CD4 cells incubated for 24 hr with $^{3}$H-5-FC. An HPLC analysis of the medium revealed that 22% of the radioactivity was 5-FU.

### Fluorocytosine Uptake Experiments

*Uptake under Therapeutic Conditions.* Three days after seeding of 6 × $10^4$ cells in six-well plates, the medium was replaced by 2 ml of fresh medium containing unlabeled 5-FC and 185 kBq $^{3}$H-5-FC/ml at a final concentration of 0.5 m*M*. After incubation for 30 min and 1, 2, 6, 24 and 48 hr, the cells were washed twice with ice-cold PBS and lysed with ice-cold 0.6 *M* perchloric acid and a cell scraper. The lysate was incubated on ice for 30 min, vortexed and centrifuged at 1500g for 5 min at room temperature. The pellet was washed again with ice-cold 0.6 *M* perchloric acid, centrifuged at 1500 × 1 g for 5 min and resuspended in 0.3 *M* NaOH. Subsequently, the radioactivity was counted in both fractions: the acid-insoluble (pellet) and the acid-soluble fraction (both supernatants). To exclude the possibility that the radioactivity in the acid-insoluble fraction was due to coprecipitation of proteins, the acid-insoluble fraction after 48 hr of incubation was extracted with 1 vol of phenol and centrifuged for 10 min at 10,000 rpm in a Heraeus minifuge. The supernatant was re-extracted with 1 vol of phenol/chlorophorm-isoamylalcohol (1:1), centrifuged again and counted.

### Saturation

Three days after seeding of 6 × $10^4$ cells in six-well plates, the cells were washed twice with and incubated in Earles balanced salt solution (EBSS) (Gibco BRL) for 30 min. Thereafter, the medium was removed, and 1 ml of EBSS containing 185 kBq $^{3}$H-5-FC/ml and cold 5-FC in final concentrations of 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 5 m*M* was added. After 10 min of incubation at 37°C, the cells were washed twice with PBS on ice and lysed with 0.3 *M* NaOH.

### Competition Experiments

The cells were preincubated in EBSS for 30 min. Thereafter, the medium was removed, and 1 ml EBSS was added containing 370 kBq $^{3}$H-5-FC/ml and cold 5-FC at a final concentration of 0.05 m*M*. After 10 min of incubation the cells were lysed with 0.3 m*M* NaOH. This uptake value was compared with those in the presence of 0.1 m*M* cytosine, 0.1 m*M* uracil or 0.1 m*M* hypoxanthine. Competition experiments with 5-FU were performed under the same conditions using 185 kBq $^{14}$C-5-FU/ml (0.05 m*M* final concentration) and 0.1 m*M* 5-FC, 0.1 m*M* uracil or 0.1 m*M* hypoxanthine, respectively.

### Efflux

Three days after seeding of 6 × $10^4$ cells in six-well plates, the cells were washed twice with EBSS and incubated for 30 min in 1 ml EBSS containing 740 kBq $^{3}$H-5-FC and cold 5-FC at a final concentration of 0.05 m*M*. The medium was removed, and the cells were washed twice with EBSS. Three wells for each cell line were used for immediate lysis of the cells. One milliliter of EBSS

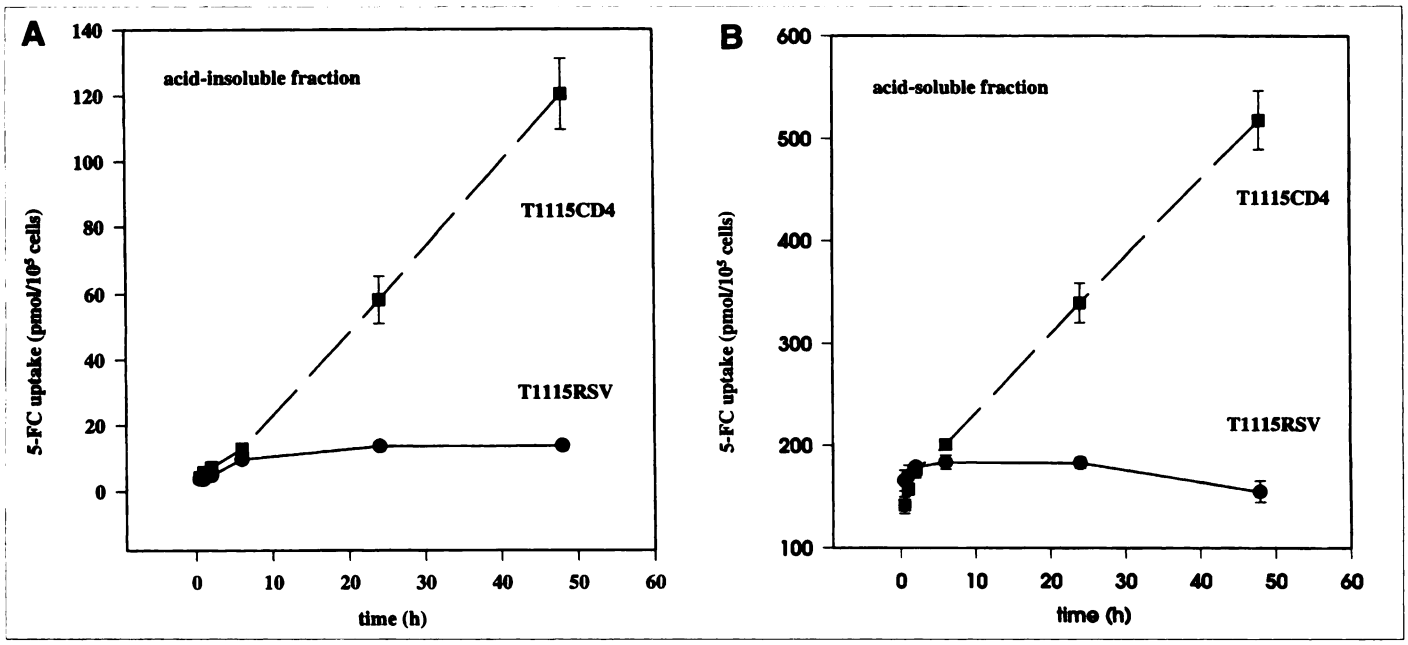


**FIGURE 5.** 5-Fluorocytosine uptake in acid-insoluble (A) and acid-soluble fractions (B) of T1115RSV and T1115CD4 cells. Total cell numbers: 3.7 × $10^5$ cells at 30 min and 1, 2 and 6 hr and 6.5 and 8.9 × $10^5$ cells after 24 and 48 hr for T1115RSV (T1115CD4: 3.1 × $10^5$ cells at 30 min and 1, 2 and 6 hr and 3.8 and 3.2 × $10^5$ cells at 24 and 48 hr). Mean values and s.d. are shown (n = 3).

**TABLE 1**
5-Fluorocytosine Uptake in Acid-Insoluble and Acid-Soluble Fractions after Incubation with 0.5 m*M* 5-Fluorocytosine

| Incubation time (hr) | 5-FC uptake in acid-insoluble fraction (pmol/$10^5$ cells) | | 5-FC uptake in acid-soluble fraction (pmol/$10^5$ cells) | |
|---|---|---|---|---|
| | T1115RSV | T1115CD4 | T1115RSV | T1115CD4 |
| 0.5 | 3.79 ± 0.32 | 4.23 ± 0.18 | 165.7 ± 9.9 | 142.2 ± 8.12 |
| 1 | 3.68 ± 0.45 | 5.8 ± 0.46 | 169.8 ± 10.7 | 157.5 ± 2.2 |
| 2 | 4.76 ± 0.62 | 7.27 ± 0.72 | 178.8 ± 1.47 | 175.8 ± 7.5 |
| 6 | 9.65 ± 0.28 | 12.82 ± 1.2 | 183.3 ± 6.78 | 200.7 ± 4.2 |
| 24 | 13.62 ± 0.57 | 58.16 ± 7.17 | 182.9 ± 5.17 | 339.7 ± 19.3 |
| 48 | 13.7 ± 0.96 | 120.37 ± 10.72 | 155.4 ± 10.19 | 517.7 ± 28.6 |

Results are mean ± s.d.
5-FC = 5-fluorocytosine.

was added to the other wells for further incubation. After 1, 2, 4, 6 and 10 min, three wells per cell line were washed again, and the cells were lysed. The radioactivity of the lysates as well as that of the medium was counted.

## RESULTS

### Cytosine Deaminase Activity in Cell Lysates

In contrast to T1115RSV cells, T1115CD4 cells express CD, as shown by Western blot analysis (Fig. 1). To determine sensitivity toward 5-FC, the human glioblastoma cell line T1115RSV and a CD-expressing clone T1115CD4 were grown in the presence of various 5-FC concentrations. No significant change in growth pattern was observed in T1115RSV cells within 4 days, even at 1 m*M* (Fig. 2A). T1115CD4 cells, however, showed 50% growth inhibition with 0.25 m*M* 5-FC after 4 days and almost complete inhibition when the cells were exposed to 1 m*M* 5-FC (Fig. 2B). Because we wanted to determine whether this inhibition was caused by CD-mediated 5-FU production, HPLC analysis of cell lysates that had been incubated with $^3$H-5-FC for various times was performed. The T1115RSV cells were unable to metabolize 5-FC to 5-FU (Fig. 3A), whereas in T1115CD4 cells significant amounts of 5-FU were observed (Fig. 3B). An additional metabolite was eluted from the column that could be identified as 5-fluorouridine (FUrd) (Fig. 3B). About 70% and 86% of the radioactivity was detectable as 5-FU after 1 and 2 hr, respectively (FUrd, 3.2% and 3.6%) (Fig. 3C). In the presence of 0.2 m*M* 5-FC, T1115CD4 lysates converted 4.3% and 8.5% of total 5-FC into 5-FU after 10 and 20 min of incubation, respectively. This corresponds to a specific enzyme activity of 168.5 pmol of 5-FU/mg of total protein/min. This value is within the range of values reported by others (*11–14*).

5-Fluorouracil, which is produced in CD-expressing tumor cells, may also be released into the extracellular space. Therefore, an HPLC analysis of the incubation medium was performed after 24 hr incubation of T1115CD4 with $^3$H-5-FC. We found that 22% of the radioactivity in the medium was 5-FU (Fig. 4).

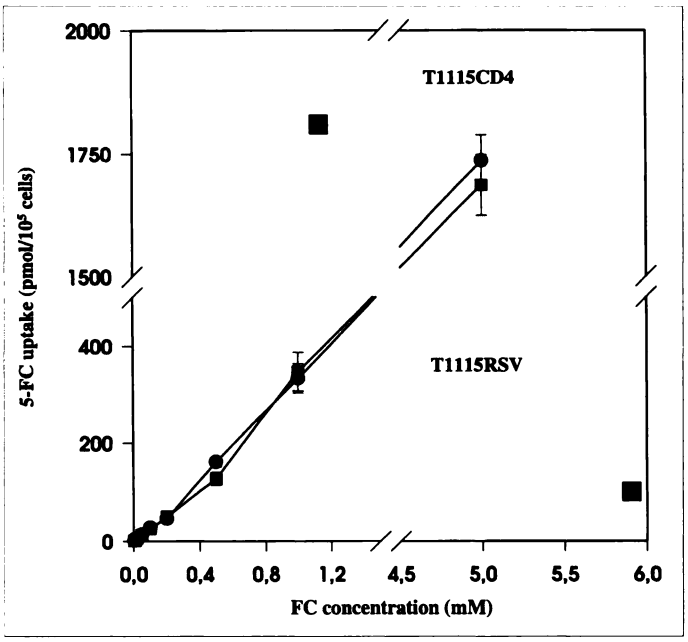


**FIGURE 6.** 5-Fluorocytosine uptake with increasing amounts of 5-FC in the medium and 10 min of incubation. Total cell numbers: 3.6 × $10^5$ cells for T1115RSV; 3.25 × $10^5$ cells for T1115CD4. Mean values and s.d. are shown (n = 3).

### Uptake under Therapeutic Conditions

Incubation of the cells up to 48 hr under therapeutic conditions with 0.5 m*M* 5-FC resulted in a time-dependent increase in radioactivity in both (acid soluble and acid insoluble) fractions of CD-positive cells, whereas the control cells showed no increase (Fig. 5, Table 1). After 48 hr, 19% of the total intracellular radioactivity was present in the acid-insoluble fraction of T1115CD4 cells (T1115RSV 8%). The radioactivity in the CD-positive cells was 8.8-fold higher in the acid-insoluble fraction (Fig. 5A, Table 1) and 3.3-fold higher in the acid-soluble fraction (Fig. 5B, Table 1) than in the control cells. To exclude the possibility that the radioactivity in the acid-insoluble fraction was due to coprecipitation of proteins, the radioactivity was measured after phenol/chlorophorm-isoamylalcohol extraction. Because similar results were obtained after the phenol/chlorophorm-isoamylalcohol extraction, we conclude that most of the radioactivity was incorporated into the RNA/DNA fraction and does not coprecipitate with proteins. After 2 hr of incubation in T1115CD4 cells, only 4% of total radioactivity was present in the acid-insoluble fraction (T1115RSV 2.6%). Furthermore, total 5-FC uptake after 2 hr of incubation showed no difference in both lines (T1115RSV: 183.5 ± 5.4 pmole/$10^5$ cells; T1115CD4: 183.1 ± 7.5 pmole/$10^5$ cells). After 6 hr of incubation, a slight difference in total uptake was seen, with 213.5 ± 4 pmole/$10^5$ cells in T1115CD4 and 192.9 ± 6.8 pmole/$10^5$ cells in T1115RSV. Only very slow uptake was observed.

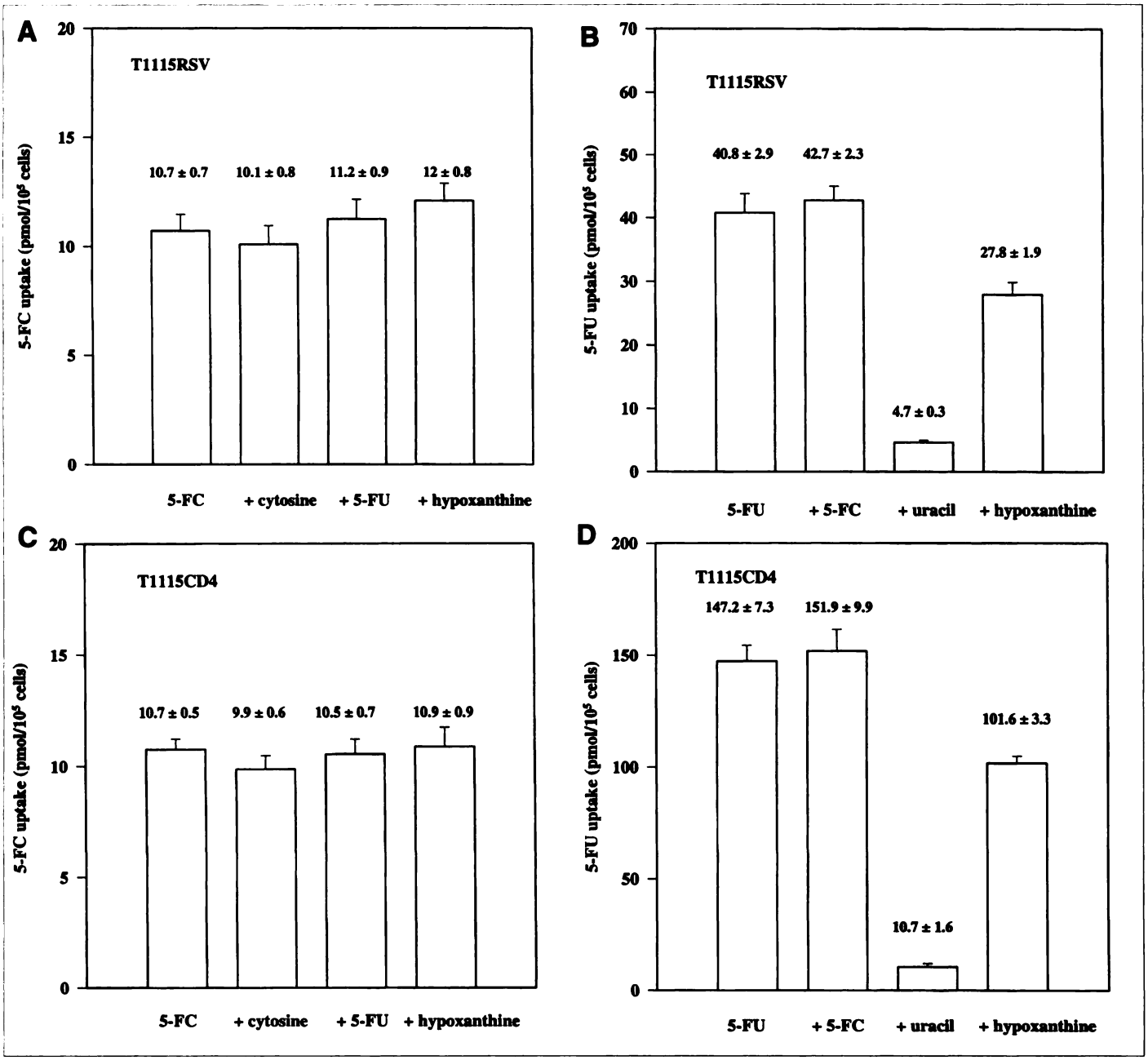


**FIGURE 7.** 5-Fluorocytosine uptake without and with a twofold molar excess of cytosine, 5-FU or hypoxanthine in T1115RSV (A) and T1115CD4 cells (C) and 5-FU uptake without and with a twofold molar excess of 5-FC, uracil or hypoxanthine (B and D). Total cell numbers: $3.0 \times 10^5$ cells for T1115RSV; $3.2 \times 10^5$ cells for T1115CD4. Mean values and s.d. (n = 3).

### Saturation and Competition

Kinetic criteria for a carrier-linked transport are saturation of transport activity with increasing substrate concentrations and transport selectivity. A high selectivity between chemically similar substrates indicates carrier-linked transport. Therefore, saturation and competition experiments were performed to characterize 5-FC uptake (uptake in this context denotes transport and metabolization). The experiments with increasing amounts of 5-FC in the medium are shown in Figure 6. Even at 5 m*M* 5-FC, no saturation of uptake could be observed. To test for selectivity of uptake, we performed competition experiments of 5-FC and 5-FU uptake. The 5-FC uptake was not inhibited by a twofold molar excess of cytosine, 5-FU or hypoxanthine in both cell lines (Fig. 7, A and C). In contrast, 5-FU uptake was specifically inhibited by uracil and to a lesser extent by hypoxanthine, but not by 5-FC (Fig. 7, B and D). In T1115CD4 cells, 5-FU uptake showed a 92.7% inhibition by uracil (T1115RSV 88.5%) and a 30.9% inhibition by hypoxanthine (T1115RSV 31.9%).

### Efflux

We performed an efflux experiment last. A rapid decrease in the lysates and a rapid increase of the radioactivity in the medium were observed, suggesting an efficient efflux mechanism (Fig. 8). There was no difference between the control cells and the CD-positive cells.

## DISCUSSION

In the present study using the human glioblastoma cell line T1115, we found that: (a) CD-expressing cells are sensitive to

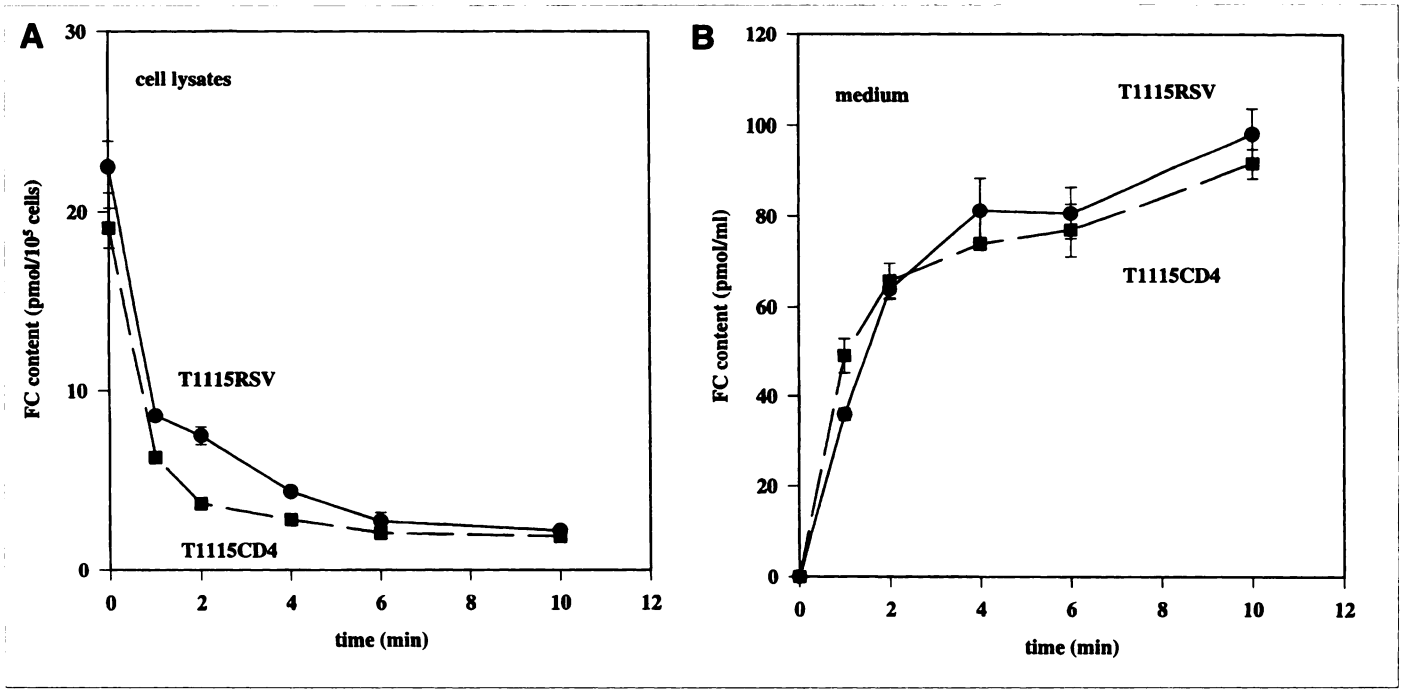


**FIGURE 8.** 5-Fluorocytosine content in cell lysates (A) and media (B) after different incubation times in 5-FC–free medium. Total cell numbers: $3.5 \times 10^5$ cells for T1115RSV; $3.8 \times 10^5$ cells for T1115CD4. Mean values and s.d. are shown (n = 3).

5-FC; (b) these cells are able to convert 5-FC into 5-FU; and (c) the 5-FU produced in CD-expressing cells is released into the incubation medium (Figs. 1–4). Uptake experiments with therapeutic concentrations of 5-FC revealed that after an extended incubation time (up to 48 hr), T1115CD4 cells incorporated significantly more radioactivity in both the acid-soluble (representing the cytoplasma) and the acid-insoluble fractions (representing RNA, DNA and proteins) than did the T1115RSV cells (Fig. 5). Similar results have been described for human colon carcinoma cells, which showed incorporation of 5-FU into the RNA fraction after 24 hr of incubation (*12*).

In patients with cancer undergoing gene therapy with the CD suicide system, PET with $^{18}$F-5-FC may be applied to evaluate the CD activity in vivo. Because of the half-life of $^{18}$F (109 min), the PET examination has to be performed within the first 2–3 hr after administration of the radiotracer. Therefore, shorter incubation times have to be evaluated to obtain a simulation for the PET study. We found a comparable total uptake of 5-FC after 2 hr in both cell lines in the therapy experiment, with most of the radioactivity in the acid-soluble fraction (Fig. 5), indicating that PET with standardization to body weight and applied dose may not be an appropriate quantitative approach.

We also found very slow uptake of 5-FC, which focused further experiments on the uptake mechanism. A transport system for cytosine in bacteria and yeasts has been described (*23,25*). Furthermore, in the yeast system, uptake of cytosine and uracil is mediated in part by the same carrier. However, there seem to exist different mechanisms for cytosine entry in prokaryotes and eukaryotes. Common uptake pathways between cytosine and pyrimidines as well as purines have been described in competition experiments for *Saccharomyces cerevisiae*, *Candida glabrata* and *Candida albicans* (*23*), with a lower affinity for 5-FC (*24,25*). In *C. glabrata*, Polak et al. (*25*) found two kinetic systems: one high affinity/low capacity and one low affinity/high capacity for cytosine transport.

Our results with the T1115 human glioblastoma cell line showed no saturation of 5-FC uptake up to 5 m*M* (Fig. 6). In addition, 5-FC uptake was insensitive to the presence of cytosine, 5-FU or hypoxanthine in a twofold molar excess (Fig. 7). Although, 5-FC did not affect uptake of 5-FU, a strong decrease in 5-FU uptake could be observed in the presence of 0.1 m*M* uracil and hypoxanthine, indicating a common transport system shared by purine nucleobases, as was suggested by Wohlhueter et al. (*26*) and others (*27,28*). We, however, observed a difference in 5-FU uptake between T1115CD4 and T1115RSV cells. Although we have no explanation for this phenomenon, we emphasize that there is no difference in growth behavior and morphology between CD-expressing cells and control cells (Fig. 2).

These findings are evidence that 5-FC enters human cells by diffusion rather than by a transport process (*29*). Similar results have been obtained in studies of the cytosine entry into human erythrocytes. Cytosine uptake has been shown to be much slower than the other nucleobases, and its entry appeared not to be mediated by a carrier, as indicated by the slow uptake kinetics, lack of saturation of uptake and the finding that the uptake was not affected by the presence of uracil, hypoxanthine or adenine (*29*).

The efflux experiments showed that the radioactivity was rapidly washed from the cells with no difference between CD-expressing cells and control cells (Fig. 8). The medium of cultured CD-positive cells contained 5-FU (Fig. 4), which may contribute to the bystander effect observed in separate experiments with these cells (Rowley S, et al., *unpublished results*) and also in human colon carcinoma cells expressing the CD gene (*12*). These studies suggest that 5-FU, released from CD-expressing cells into the medium, is capable of entering and finally killing neighboring cells, which may lack CD activity.

## CONCLUSION

After CD-mediated conversion of 5-FC to 5-FU, catabolization and efflux occur, as in the case of systemic 5-FU administration with the difference that 5-FU is produced in the tumor cells and also is released from CD-expressing cells to enter the

neighboring cells, which have no CD activity. Although, the enzyme activity proved sufficient for growth inhibition of tumor cells in vitro, transport may be the limiting factor for therapeutic application. We observed a slow entry into the tumor cells and presented evidence that 5-FC does not enter the cells through active or facilitated transport; rather, it enters by diffusion, and we also found rapid efflux. These phenomena may be compensated for by the fact that large doses of 5-FC (usually daily doses of 150 mg/kg body weight in patients with mycosis) can be given. Furthermore, it may be possible to increase the 5-FU level in the tumor cells by modulation of the efflux or by modulating 5-FU metabolism to increase the fraction in the acid-insoluble fraction (*30,31*).

We observed significant differences in 5-FC uptake between CD-expressing cells and control cells after 48 hr of incubation. There was, however, no difference in total uptake after a 2-hr of incubation, suggesting that in a clinical situation quantitation of PET data using the body weight and injected dose may not be appropriate. Rather, a modeling approach for the evaluation of kinetic constants for 5-FC entry and CD enzyme activity, including HPLC analysis of plasma levels of 5-FU released from the tumor, may be necessary. Therefore, in a next step to clinical application of suicide gene transfer into human tumors, animal studies with $^{18}$F-5-FC will be necessary to assess whether PET can be used for the in vivo measurement of CD activity in solid tumors. Furthermore, in these experiments, we will have to address the question of whether modulation of 5-FU transport and metabolism may enhance tracer accumulation.

## REFERENCES


1. Scholer HJ. Flucytosine. In: Speller DCE, ed. *Antifungal chemotherapy*. New York: Wiley; 1980:35–106.
2. Harris BE, Manning BW, Federle T, Diasio RB. Conversion of 5-fluorocytosine into 5-fluorouracil by human intestinal microflora. *Antimicrob Agents Chemother* 1986;29: 44–48.
3. Polak A, Eschenhof E, Fernex M, Scholer HJ. Metabolic studies with 5-fluorocytosine-6-$^{14}$C in mouse, rat, rabbit, dog and man. *Chemotherapy* 1976;22:137–153.
4. Block ER, Bennett JE, Livotti LG, Klein WJ, McGregor RR, Henderson L. Flucytosine and amphotericin B: hemodialysis effects on the plasma concentration and clearance. *Ann Intern Med* 1974;80:613–617.
5. Koechlin BA, Rubio F, Palmer S, Gabriel T, Duschinsky R. The metabolism of 5-fluorocytosine-2-$^{14}$C and of cytosine-1-$^{14}$C in the rat and the disposition of 5-fluorocytosine-2-$^{14}$C in man. *Biochem Pharmacol* 1966;15:435–446.
6. Myers CE. The pharmacology of the fluoropyrimidines. *Pharmacol Rev* 1981;33:1–15.
7. Nishiyama T, Kawamura Y, Kawamoto K, et al. Antineoplastic effects of 5-fluorocytosine in combination with cytosine deaminase capsules. *Cancer Res* 1985;45:1753–1761.
8. Wallace PM, MacMaster JF, Smith VF, Kerr DE, Senter PD, Cosand WL. Intratumoral generation of 5-fluorouracil mediated by an antibody-cytosine deaminase conjugate in combination with 5-fluorocytosine. *Cancer Res* 1994;54:2719–2723.
9. Culver KW, Ram Z, Walbridge S, et al. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* 1992;256: 1550–1552.
10. Ram Z, Culver WK, Walbridge S, et al. In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res* 1993;53:83–86.
11. Huber BE, Austin EA, Good SS, Knick VC, Tibbels S, Richards CA. In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. *Cancer Res* 1993;53:4619–4626.
12. Huber BE, Austin EA, Richards CA, Davis ST, Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proc Natl Acad Sci USA* 1994;91:8302–8306.
13. Mullen CA, Kilstrup M, Blaese M. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. *Proc Natl Acad Sci USA* 1992;89:33–37.
14. Mullen CA, Coale MM, Lowe R, Blaese RM. Tumors expressing the cytosine deaminase suicide gene can be eliminated in vivo with 5-fluorocytosine and induce protective immunity to wild type tumor. *Cancer Res* 1994;54:1503–1506.
15. Visser GWM, Boele S, Knops GHJN, Herscheid JDM, Hoekstra A. Synthesis and biodistribution of ($^{18}$F)-5-fluorocytosine. *Nucl Med Commun* 1985;6:455–459.
16. Harris JD, Gutierrez AA, Hurst HC, Sikora K, Lemoine NR. Gene therapy for cancer using tumour-specific prodrug activation. *Gene Ther* 1994;1:170–175.
17. Gutierrez AA, Lemoine NR, Siroka K. Gene therapy for cancer. *Lancet* 1992;339: 715–721.
18. Danielsen S, Kilstrup M, Barilla K, Jochimsen B, Neuhard J. Characterization of the Escherichia coli codBA operon encoding cytosine permease and cytosine deaminase. *Mol Microbiol* 1992;6:1335–1344.
19. Kozak M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 1986;44:283–292.
20. Smith PK, Krohn RJ, Hermanson GT, et al. Measurement of protein using bicichoninic acid. *Anal Biochem* 1985;150:76–85.
21. Doyle A, Griffiths JB, Newell DG, eds. *Cell and tissue culture: laboratory procedures*. New York: Wiley; 1993.
22. Oberdorfer F, Hofmann E, Maier-Borst W. Preparation of $^{18}$F-labeled 5-fluorouracil of very high purity. *J Labd Comp Radiopharm* 1989;27:137–145.
23. Fasoli MOF, Kerridge D. Uptake of pyrimidines and their derivatives into Candida glabrata and Candida albicans. *J Gen Microbiol* 1990;136:1475–1481.
24. Polak A, Grenson M. Evidence for a common transport system for cytosine, adenine and hypoxanthine in Saccharomyces cerevisiae and Candida albicans. *Eur J Biochem* 1973;32:276–282.
25. Polak A. Transport of the antimycotic 5-fluorocytosine and related substances in fungi. *J Antimicrob Chemother* 1990;26:465–468.
26. Wohlhueter RM, McIvor RS, Plagemann PGW. Facilitated transport of uracil and 5-fluoruracil, and permeation of orotic acid into cultured mammalian cells. *J Cell Physiol* 1980;104:309–319.
27. Aronow B, Ullman B. Role of the nucleoside transport function in the transport and salvage of purine nucleobases. *J Biol Chem* 1986;261:2014–2019.
28. Beck JT, Ullman B. Genetic demonstration of bidirectionality in the high affinity purine base transporter of mutant mouse S49 cells. *J Biol Chem* 1987;262:2392–2397.
29. Plagemann PGW, Wolffendin C, Puziss MB, Wohlhueter RM. Purine and pyrimidine transport and permeation in human erythrocytes. *Biochim Biophys Acta* 1987;905:17–29.
30. Grem JL, Fischer PH. Alteration of fluorouracil metabolism in human colon cancer cells by dipyridamole with a selective increase in fluorodeoxyuridine monophosphate levels. *Cancer Res* 1986;46:6191–6199.
31. Kamm YJL, Rietjens IMCM, Vervoort J, et al. Effect of modulators on 5-fluorouracil metabolite patterns in murine colon carcinoma determined by in vitro $^{19}$F magnetic resonance spectroscopy. *Cancer Res* 1994;54:4321–4326.