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Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: A negative selection system

(retroviral vector/gene therapy/suicide vector/5-fluorouracil)

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ABSTRACT **Expression of the bacterial gene for cytosine deaminase (CD; EC 3.5.4.1) in mammalian cells was evaluated as a negative selection system or suicide vector for potential use in gene transfer studies and therapies. Mammalian cells, unlike certain bacteria and fungi, do not contain the enzyme CD and do not ordinarily metabolize cytosine to uracil. Nor do they metabolize the innocuous compound 5-fluorocytosine to the highly toxic compound 5-fluorouracil. The *Escherichia coli* CD gene underwent PCR oligonucleotide-directed mutagenesis to enhance its expression in a eukaryotic system and it was then cloned into an expression vector, pLXSN, that also contains a neomycin-resistance gene. Murine fibroblast lines were transfected with the plasmid and subjected to brief selection in the neomycin analogue G418. Lysates from these cell populations exhibited significant CD activity detected by conversion of radiolabeled cytosine to uracil. In clonogenic assays transfected cells expressing CD were selectively killed by incubation in 5-fluorocytosine, whereas control cell lines were not. Dose–response studies evaluating [^{3}H]thymidine incorporation or cloning efficiency demonstrated profound inhibition at and above 65 μg of 5-fluorocytosine per ml. Mixed cellular assays showed that CD-positive cells could be eliminated without bystander killing of other cells. Retrovirus-mediated CD gene transfer into various tissues was also demonstrated. Thus CD, with its ability to produce the toxic antimetabolite 5-fluorouracil from 5-fluorocytosine, may be useful as a negative selection system for studies and treatments employing gene transfer techniques.**

The introduction of genes that confer a phenotype of resistance or sensitivity to various pharmacologic agents and thus allow positive or negative selection of cells has become a critically important technique in studies of molecular genetics and cell biology. Now as human gene therapies are developed applications of negative selection systems may be needed. Such applications could include the use of inserted genes to provide a "suicide" or "fail-safe" trait that would permit the destruction of gene-modified cells if their behavior became harmful to the host.

The enzyme cytosine deaminase (CD; EC 3.5.4.1) catalyzes the deamination of cytosine to uracil (1, 2). Mammalian cells do not ordinarily produce this enzyme, whereas many bacteria and fungi do. Microorganisms that express the CD gene convert 5-fluorocytosine (5-FCyt) to 5-fluorouracil (5-FUra), a highly toxic metabolite that is lethal to the cell (3). Since mammalian cells do not express significant amounts of CD they do not deaminate 5-FCyt (4, 5). This compound is nontoxic to them at concentrations that result in strong antimicrobial activity (6). However, 5-FUra has potent cytotoxic effects on mammalian cells and is widely used as a cancer chemotherapeutic agent. It is metabolized to 5-fluorouridine 5′-triphosphate and 5-fluoro-2′-deoxyuridine 5′-monophosphate, inhibiting both RNA and DNA synthesis and resulting in cell death (7, 8). Thus mammalian cells engineered to express CD should convert 5-FCyt to 5-FUra and be selectively sensitive to 5-FCyt compared to native, unmodified cells.

The bacterial gene for CD has recently been isolated and cloned (ref. 2; M.K., S. Danielsen, K. Barilla, and J. Neuhard, unpublished). In this report we describe the engineering of the bacterial CD gene into a eukaryotic expression vector, its transfer into and expression in mammalian cells, and the resultant sensitivity of such modified cells to 5-FCyt.

MATERIALS AND METHODS

Molecular Techniques. The plasmid pMK116 was produced by one of us (M.K., S. Danielsen, K. Barilla, and J. Neuhard, unpublished) and contains a 1.6-kilobase (kb) DNA fragment isolated from *Escherichia coli* encoding the CD gene in the polycloning site of the vector pTZ18U (9). The plasmid pLXSN was a gift from A. Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle). It contains eukaryotic expression elements: (5′) Moloney murine sarcoma virus long terminal repeat (LTR) promoter, polycloning site, simian virus 40 early promoter, neomycin phosphotransferase gene, and Moloney murine leukemia virus promoter (3′) (10). The neomycin phosphotransferase gene was used as a dominant positive selectable marker conferring resistance to the neomycin analogue G418 (11). pMK116 was digested with the restriction enzymes *Hin*cII and *Bam*HI. pLXSN was digested with *Hpa* I and *Bam*HI. The 1.6-kb fragment from pMK116 and the 5.7-kb fragment from pLXSN were separated and isolated by electrophoresis in a low-melting-point agarose. The fragments were then ligated with T4 ligase, and plasmid preparations were produced employing standard techniques (12, 13). pCD1 represented the insertion of the unmodified bacterial CD sequence into pLXSN.

Oligonucleotide-directed mutagenesis of pCD1 was conducted in the following way. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by polyacrylamide gel electrophoresis (13). Oligonucleotides 5′-TGACGCGAATTCAGGCTAGCAATGTCG-3′ (corresponding to the 5′ end of the CD sequence in pCD1 but changing the start codon from GTG to ATG and inserting an *Eco*RI restriction site immediately upstream of the start codon) and 5′-CACACATTCCACAGCGGATCC-3′ (identical antisense to the 3′ region flanking the gene in pCD1) were used as primers and pCD1 was used as template in a

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Abbreviations: CD, cytosine deaminase; 5-FCyt, 5-fluorocytosine; 5-FUra, 5-fluorouracil; LTR, long terminal repeat.

PCR using a Perkin–Elmer/Cetus DNA thermal cycler. The resulting 1.6-kb fragment representing the modified CD gene and pLXSN were digested with *Eco*RI and *Bam*HI, electrophoretically isolated, and ligated. The resulting plasmid with the altered CD sequence is called pCD2. The 5′ region of the gene was sequenced by the dideoxynucleotide chain-termination method to verify the desired changes in sequence (13). Southern blots using the same probe were also performed on *Sac* I digests of 10-μg samples of purified genomic DNA from cell lines and a ^{32}P-labeled probe corresponding to the 1.6-kb CD gene found between the *Eco*RI and *Bam*HI sites in pCD2 (13). As a positive control, 10 μg of 3T3 DNA was combined with 30 pg of pCD2 representing five copies of the CD gene per cell.

Cellular Techniques. *Cell culture.* Cells were grown in D10 medium [complete medium based on Dulbecco's modified minimal essential medium (DMEM)—i.e., DMEM supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 2 mM glutamine, 50 units of penicillin per ml, and 50 μg of streptomycin per ml] and incubated at 37°C in 5% CO_2 in air. NIH 3T3 cells and PA317 cells are mouse fibroblast cell lines that have been described (14). 207-10 is a methylcholanthrene-induced murine sarcoma line and 38-2 is a dimethylhydrazine-induced colon adenocarcinoma line (both courtesy of S. A. Rosenberg, National Cancer Institute). PA317 has been derived from NIH 3T3 cells and contains a stably integrated replication-incompetent retroviral genome; it functions as a retroviral packaging line when transfected with plasmids containing a retrovirus-like sequence containing an intact packaging sequence (ψ). Plasmids pLXSN, pCD1, and pCD2 contain retroviral LTRs and an intact packaging sequence. Cells were transfected with purified plasmid DNA using a standard calcium phosphate precipitation method (13). The procedure for viral transduction has been described (15). Cells were exposed to supernatant from packaging lines and 5 μg of protamine per ml for 24 hr and then grown in D10 medium. Seventy-two hours after plasmid transfection or 48 hr after retroviral transduction, 1 mg of G418 per ml was added to the medium and cells were grown under these selection conditions for 7 days. Thereafter the cells were maintained in D10 medium only.

Clonogenic assay. Cells were diluted to 10^4 per ml, and 0.1 ml was placed into 4-cm (diameter) flat-bottomed wells of a six-well Costar tissue culture dish along with 5 ml of medium with 5-FCyt (United States Biochemical) and/or G418 (GIBCO) at concentrations described in the text and tables. They were incubated for 5 days, the wells were stained with Geimsa, and colonies of >25 cells were counted under 40× magnification.

Proliferation assay. Cells (10^3) were placed in flat-bottomed wells of a 96-well plate containing 0.2 ml of medium with additives as described in the tables and text. At the times indicated the wells were pulsed with 25 μl of [^{3}H]thymidine with an activity of 0.5 μCi/25 μl (1 Ci = 37 GBq). They were harvested 4 hr later with an automated cell harvester and assayed in a scintillation spectrometer. Twelve replicates of each condition were performed. These and the clonogenic assay data were analyzed by *t* test (16).

Enzyme assay. An *in vitro* assay for CD was performed using a modification of previously described methods (2). Cells (1×10^6) were washed once in normal saline and resuspended in 10 μl of 100 mM Tris, pH 7.8/1 mM EDTA/1 mM dithiothreitol. The cells were subjected to five cycles of rapid freezing and thawing and the material was then centrifuged 5 min in a tabletop microfuge. Ten microliters of cell lysate was combined with 10 μl of [^{3}H]cytosine (5 mM cytosine in 100 mM Tris, pH 7.8, with an activity of 0.5 μCi per 10 μl) and incubated for 4 hr. Ten microliters of sample and 10 μl of a marker solution containing unlabeled cytosine (0.4 mg/ml) and unlabeled uracil (0.4 mg/ml) in water were placed on thin-layer chromatography sheets (Kodak chromatogram sheet 13254) and developed in a mixture of 1-butanol (86%)/water (14%). After drying, bands corresponding to cytosine and uracil were cut out under short-wave UV illumination and assayed in a scintillation spectrometer. The radioactivity recovered from the cytosine and uracil bands accounted for essentially all of the label introduced to the sample, as judged by assaying the activity of 10 μl of label not subjected to chromatographic separation.

RESULTS

Rationale for Gene Cloning. Initially the entire unmodified coding region for CD from pMK116 was cloned into the polycloning site of pLXSN. The resulting construct was named pCD1. When transfected into 3T3 cells there was little evidence of gene expression (data not shown). Sequencing of the noncoding region immediately 5′ of the coding region of the CD gene revealed the following sequence: 5′ . . . CAATGTCGCATGTGGAGGCTAACAGTGTCG . . . 3′. Analysis of the protein in bacteria had previously revealed that translation began at the GTG codon (M.K., S. Danielsen, K. Barilla, and J. Neuhard, unpublished). Given the presence of upstream ATGs (at −15 and −22 5′ of the start codon) and the poor eukaryotic start signal proffered by the GTG, we reasoned that deletion of the upstream ATGs and mutation of the GTG to an ATG would improve expression of the CD gene in a eukaryotic cell. The 5′ upstream sequence was altered using oligonucleotide-directed mutagenesis to change the start codon from GTG to ATG and to delete the noncoding region between the *Eco*RI site and the start codon that contained the interfering ATGs. The modified gene was cloned into pLXSN under the LTR promoter upstream of the polycloning site. The resulting plasmid containing the engineered sequence is called pCD2. Sequencing of the 5′ region of the CD gene verified the correct sequence and the deletion of 88 base pairs upstream of the start site in the parental plasmid pCD1.

Transfection of Mammalian Cells with pCD2 Results in Expression of the CD Gene. 3T3 and PA317 cells were transfected with pCD2 and 72 hr later were placed in medium containing G418 (1 mg/ml). The cells were incubated in G418 for 7 days and then maintained in regular medium. Resistance to the neomycin analogue G418 allowed for enrichment of the cell population with those that had taken up and incorporated plasmid sequences. Line 3T3-CD represents a transfection derivative of 3T3; PA-CD-A and PA-CD-B represent separate transfection derivatives of PA317. Incorporation of the CD gene into the genome of these lines was demonstrated by Southern analysis (Fig. 1). A ^{32}P-labeled CD probe demonstrated the presence of the CD gene in DNA digested with *Sac* I. The positive control band (Fig. 1, 3T3 + pCD2) represented five copies of the CD gene per 3T3 cell; the bands for 3T3-CD, PA-CD-A, and PA-CD-B are less intense and likely represent an average gene copy number in these populations of one or two copies per cell.

The *in vitro* conversion of radiolabeled cytosine to uracil by lysates of cells was measured. Cell lines 3T3-CD, PA-CD-A, and PA-CD-B demonstrated CD activity by converting cytosine to uracil, whereas the nontransfected control lines did not (Table 1).

Cell Lines Expressing the CD Gene Are Sensitive to 5-FCyt Toxicity. Clonogenic assays were performed to assess the sensitivity of cells to 5-FCyt. Cells expressing the CD gene should not give rise to colonies in the presence of 5-FCyt as they would produce toxic 5-FUra. Cells not expressing the neomycin-resistance gene will not grow in the presence of G418. Assuming a sequence containing CD and neomycin-resistance genes has been integrated into the cell's genome and that both genes are expressed, no cell that can survive in

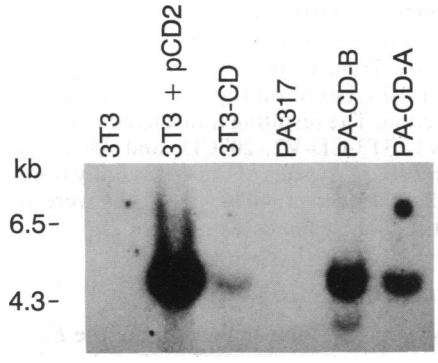

FIG. 1. Southern analysis for the presence of the CD gene in genomic DNA of transfected cells. Ten micrograms of cellular DNA was digested with *Sac* I, electrophoresed, transferred, and hybridized with a probe corresponding to the CD gene in pCD2. *Sac* I cuts through both LTR elements of pCD2. As a positive control, 30 pg of pCD2 DNA, the equivalent of five gene copies per cell, was added to 10 μg of 3T3 DNA (3T3 + pCD2) prior to digestion with *Sac* I.

G418 should survive in 5-FCyt and the relative cloning efficiency should drop to near zero. Table 2 demonstrates this for 3T3-CD, PA-CD-A, and PA-CD-B. Colony counts in G418 were generally greater than two-thirds of those in control medium containing no additives, indicating considerable enrichment of the population with cells that had incorporated plasmid. For cells treated with 5-FCyt alone the relative colony counts were 3–15% of control counts, indicating that the vast majority of the transfected cells were sensitive to 5-FCyt whereas control cells were not. In medium containing 5-FCyt and G418, almost no colonies of the CD-expressing lines were found. Fig. 2 summarizes the dose–response relationship between 5-FCyt concentration and inhibition of cell line PA-CD-A in clonogenic and proliferation assays. In line PA-CD-A, 5-FCyt profoundly inhibited [^{3}H]thymidine incorporation and colony counts over a concentration range of 62–500 μg/ml; below that range the effects were diminished somewhat but were still significant. Table 3 demonstrates the selectivity of 5-FCyt toxicity in mixed cell populations. Equal numbers of PA-CD-A cells (which contain the CD gene) and PA317 cells (which do not) were mixed in clonogenic assays. If the 5-FCyt toxicity were restricted to cells containing the CD gene, one would expect approximately one-half the cells to be eliminated from the mixed population, whereas nearly all of the cells in a pure PA-CD-A population and none of the cells in a pure PA317 population would be affected by 5-FCyt. However, if there were significant "bystander killing" by release of CD and/or 5-FUra into the medium, one would expect PA-CD-A and PA317 cells in the mixed population to be killed. The colony count in 5-FCyt was approximately one-half the colony count of the mixed population in nonselective medium, whereas control PA317 cells were unaffected by 5-FCyt and PA-CD-A cells were killed.

Finally, expression of the CD gene had no obvious deleterious effects on cells in the absence of 5-FCyt, as judged by cloning efficiency, proliferation *in vitro*, growth rates in culture, or microscopic morphology.

Table 1. Conversion of cytosine to uracil *in vitro* by lysates of cell lines containing the CD gene

Cell line lysate	*n*	CD activity, pmol of uracil per 10^9 cells per min
Buffer only	5	1.2 ± 0.2
3T3	4	2.2 ± 1.2
3T3-CD	3	35.7 ± 11.3
PA317	2	1.0 ± 0
PA-CD-A	4	157.7 ± 8.8
PA-CD-B	2	172.0 ± 11.0
3T3-CD-V1	1	175
3T3-CD-V2	1	164
207CD	1	316
38CD	1	742

Values are presented as average ± SEM and represent conversion of radiolabeled cytosine to uracil by cell lysates *in vitro*. *n*, Number of experiments.

Table 2. Clonogenic potential in G418 and/or 5-FCyt of cell lines transfected with the pCD2 plasmid or retroviral vector and expressing the CD and neomycin-resistance genes

Cell line	Medium additive*	Average colony count Exp. 1	Exp. 2
3T3	None	171 ± 8.7	153 ± 10.4
	G418	0 ± 0	0 ± 0
	5-FCyt	160 ± 4.7	138 ± 6.8
	G418 + 5-FCyt	0 ± 0	0 ± 0
3T3-CD	None	131 ± 4.6	130 ± 1.7
	G418	123 ± 3.5	100 ± 6.8
	5-FCyt	14 ± 1.4†	17 ± 1.4†
	G418 + 5-FCyt	0 ± 0†	0 ± 0†
PA317	None	84 ± 8.1	33 ± 2.3
	G418	0 ± 0	0 ± 0
	5-FCyt	80 ± 2.5	40 ± 1.3
	G418 + 5-FCyt	0 ± 0	0 ± 0
PA-CD-A	None	187 ± 5.8	137 ± 0.9
	G418	123 ± 7.7	77 ± 1.2
	5-FCyt	3 ± 1.4†	5 ± 1.5†
	G418 + 5-FCyt	0 ± 0†	0 ± 0†
PA-CD-B	None	161 ± 6.0	80 ± 2.9
	G418	112 ± 7.9	38 ± 3.9
	5-FCyt	11 ± 1.7†	15 ± 1.2†
	G418 + 5-FCyt	0 ± 0†	0 ± 0†
3T3-CD-V1	None	143 ± 1.5	ND
	G418	127 ± 4.9	
	5-FCyt	9 ± 0.3†	
	G418 + 5-FCyt	1 ± 0.3†	
3T3-CD-V2	None	154 ± 5.2	ND
	G418	135 ± 4.9	
	5-FCyt	4 ± 1.2†	
	G418 + 5-FCyt	3 ± 0.7†	
38-2	None	31 ± 3.8	ND
	G418	0 ± 0	
	5-FCyt	31 ± 2.5	
	G418 + 5-FCyt	0 ± 0	
38CD	None	59 ± 5.0	ND
	G418	25 ± 5.0	
	5-FCyt	16 ± 0.6†	
	G418 + 5-FCyt	0 ± 0†	
207-10	None	47 ± 1.7	ND
	G418	0 ± 0	
	5-FCyt	37 ± 2.7	
	G418 + 5-FCyt	0 ± 0	
207CD	None	46 ± 1.7	ND
	G418	34 ± 3.5	
	5-FCyt	4 ± 1.4†	
	G418 + 5-FCyt	1 ± 0.6†	

Cells (10^3) were inoculated into tissue culture plates containing medium with the designated additives. After 5 days the plates were stained and colonies were counted. Triplicates of each condition were performed in each experiment. ND, not done.

*G418 was used at a concentration of 1 mg/ml except for cell lines 38-2, 38CD, 207-10, and 207CD, where it was 0.3 mg/ml. 5-FCyt was used at a concentration of 0.5 mg/ml except for cell lines 38-2, 38CD, 207-10, and 207CD, where it was 0.125 mg/ml.

†$P < 0.01$ for comparison of G418 + 5-FCyt vs. G418 or 5-FCyt vs. None.

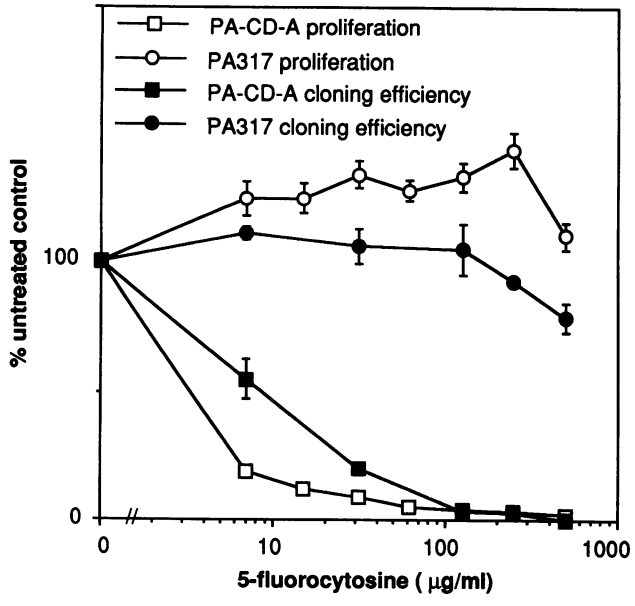

FIG. 2. Dose–response relationship between 5-FCyt concentration and inhibition of PA-CD-A proliferation and cloning efficiency. Simultaneous clonogenic assays and [^{3}H]thymidine incorporation assays were performed with PA-CD-A and PA317 cells in various concentrations of 5-FCyt. Results are expressed as "% untreated control," which is defined for proliferation as [(average cpm in 5-FCyt/average cpm in control medium alone) × 100] and for clonogenic assays as [(average number of colonies in 5-FCyt/average number of colonies in control medium alone) × 100]. The proliferation assay contained 12 replicates and the clonogenic assay contained 3 replicates. Brackets represent SEM; where not visible, SEM < 2%.

Retrovirus-Mediated Gene Transfer Results in Successful Expression of the CD Gene in Various Cell Types. Because of the complementarity of the pCD2 sequences and the replication-incompetent retrovirus sequences stably integrated into the parental PA317 cell line (14), the supernatants of PA-CD-A and PA-CD-B contain replication-incompetent but infectious retroviral vector particles containing the CD gene. 3T3 fibroblasts, murine sarcoma cells (line 207-10), and a murine colon adenocarcinoma line (line 38-2) were transduced with these retroviral vector-containing supernatants and selected by exposure to G418 as described in *Materials and Methods*. Transduction efficiencies in these experiments averaged 1–10% based on the population of cells surviving G418 selection. The resulting transduced lines are designated 3T3-CD-V1, 3T3-CD-V2, 207CD, and 38CD, respectively. As seen in Table 1, lysates of the retrovirally transduced lines converted cytosine to uracil *in vitro*. They were also sensitive to 5-FCyt in clonogenic assays (Table 2).

Table 3. 5-FCyt selectively eliminates cells expressing the CD gene from mixed populations *in vitro*

Cells	Medium additive*	Average colony count	
		Exp. 1	Exp. 2
PA-CD-A	None	122 ± 5.6	126 ± 1.5
	G418	90 ± 4.5	85 ± 2.3
	5-FCyt	7 ± 0.6	14 ± 3.0
	G418 + 5-FCyt	0.7 ± 0.3	1 ± 0.0
PA317	None	83 ± 2.0	74 ± 1.5
	G418	0 ± 0	0 ± 0
	5-FCyt	85 ± 3.0	71 ± 5.7
	G418 + 5-FCyt	0 ± 0	0 ± 0
PA-CD-A + PA317	None	164 ± 5.5	140 ± 5.6
	G418	83 ± 2.7	72 ± 3.2
	5-FCyt	84 ± 1.5†	72 ± 1.5†
	G418 + 5-FCyt	0.7 ± 0.3	1 ± 0.3

Cells were inoculated into wells as follows: 10^3 PA-CD-A, 10^3 PA317, or 10^3 PA-CD-A plus 10^3 PA317. Values represent average ± SEM of three replicates.

*D10 medium with the following additives: None; G418, 1 mg/ml; 5-FCyt, 125 μg/ml; G418 + 5-FCyt, 1 mg/ml and 125 μg/ml, respectively.

†Values do not differ significantly from PA317 colony counts in unmodified medium or medium containing 5-FCyt.

DISCUSSION

This report describes the engineering of the *E. coli* CD gene into a eukaryotic expression vector, its successful expression in mammalian cells that normally lack endogenous CD activity, and the subsequent sensitivity of cells expressing the gene to the toxic effects of 5-FCyt, an agent nontoxic to unaltered mammalian cells. This provides the basis for development of negative selection systems or suicide vectors for use in gene transfer studies and therapies.

The gene for CD has recently been isolated and cloned from *E. coli* (M.K., S. Danielsen, K. Barilla, and J. Neuhard, unpublished). We modified the bacterial gene to optimize eukaryotic expression (17). Introduction of the gene by transfection or retroviral vectors resulted in the ability of the genetically modified cells to convert cytosine to uracil. When such cells were exposed to 5-FCyt they exhibited arrested proliferation in [^{3}H]thymidine uptake assays and failed to produce colonies in clonogenic assays. Selective killing of CD-containing cells in mixed populations of cells was demonstrated in mixed clonogenic assays.

The ability to render cells selectively susceptible to the toxic effects of 5-FCyt is important for several reasons. (*i*) It provides a tool potentially useful in basic research as it gives a convenient way to selectively eliminate subpopulations of cells *in vitro* for studying temporal relationships in interactions between cell types. Similarly, it could be used *in vivo* in studies of development; certain tissues could be engineered to contain the gene and then selectively eliminated at certain points in development, an approach that has already been used with the aid of the herpes thymidine kinase gene (18). It could also be used in studies of homologous recombination (19, 20) as an easily assayed target in "knock-out" experiments—i.e., successful homologous recombination in the region of the CD gene could be recognized as loss of sensitivity to 5-FCyt. (*ii*) The gene could be used in gene transfer therapies as a "suicide vector." Such a vector could contain a desired therapeutic gene and also the CD gene, allowing elimination with 5-FCyt of cells containing the vector. Such a maneuver would be useful for purposes of safety and for regulation of therapeutic gene doses. Employment of this CD system would allow some measure of negative control over the effects of gene expression in the host by allowing one to eliminate or reduce in size the population of genetically engineered cells in the host. This could be especially useful if one wanted to limit the time the host was exposed to the gene product. Similarly, if the therapeutic gene were overexpressed and/or were producing undesired and unforeseen effects (e.g., unwanted metabolic effects or neoplastic transformation of the altered tissues) one could selectively eliminate the tissues with an agent that would not be harmful to the host. The herpes thymidine kinase gene could also be used in this manner as a suicide vector since it confers sensitivity to the antiviral agents ganciclovir and acyclovir (18, 21). However, these drugs are commonly used in the treatment of viral infections in patients with immunodeficiencies and cancer, the very patients who may benefit from new gene transfer therapies. In this situation, necessary treatment of the viral infection could destroy the genetically altered therapeutic cells. The CD system

would provide a superior alternative as 5-FCyt is not often used clinically and many alternative antifungal agents are available for treatment of infections. Alternatively, the CD system may be used to complement thymidine kinase as a suicide vector, providing cells with two unique pathways through which they could be controlled. This may be useful as genetically altered cells can, through various processes, cease to express an alien gene; should a cell lose sensitivity to one it may retain sensitivity to another. (*iii*) The system may be of use in the development of live vaccines by providing a method for producing "attenuated" or controllable pathogens as immunogens.

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