

Cytosine Deaminase Gene as a Positive Selection Marker*

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Cytosine deaminase (EC 3.5.4.1), a non-mammalian enzyme, catalyzes the deamination of cytosine and 5-fluorocytosine to form uracil and 5-fluorouracil, respectively. Eukaryotic cells have been genetically modified with a bacterial cytosine deaminase gene to express a functional enzyme. When the genetically modified cells are combined with 5-fluorocytosine, it creates a potent negative selection system, which may have important applications in cancer gene therapy. In this paper, we introduce a novel positive selection method based upon the expression of the cytosine deaminase gene. This method utilizes inhibitors in the pyrimidine *de novo* synthesis pathway to create a condition in which cells are dependent on the conversion of pyrimidine supplements to uracil by cytosine deaminase. Thus, only cells expressing the cytosine deaminase gene can be rescued in a positive selection medium.

Bacterial cytosine deaminase (CDase)¹ catalyzes the deamination of cytosine to form uracil (1). CDase can also catalyze the deamination of 5-fluorocytosine (5-FC) to form 5-fluorouracil (5-FU), a widely used antitumor agent. Since CDase is present in bacteria and fungi, but not present in mammalian cells, the gene encoding CDase has been exploited in an enzyme/prodrug gene therapy approach to cancer treatment (2–5). As example, for metastatic colorectal carcinoma, an artificial gene composed of the carcinoembryonic antigen transcriptional regulatory domain has been linked to the coding domain of the CDase gene (4, 6). When infused into the liver, CDase is expressed in the carcinoembryonic antigen-positive metastatic tumor cells but not in the normal liver cells. Metabolic conversion of the non-toxic prodrug, 5-FC, to the potent antitumor anabolite, 5-FU, occurs selectively in the tumor cells. Most importantly, it has been demonstrated that only a very small percentage of tumor cells (2%–4%) in a tumor mass need express CDase to achieve significant antitumor effect (5, 7). This significant “bystander” effect results from the fact that 5-FU is produced at such high local concentrations (5) and 5-FU crosses biological membranes predominantly by non-facilitated diffusion (8).

The current challenge for the successful clinical exploitation of this approach is to routinely achieve CDase gene transfer at the required specific activity in a solid tumor mass. We are currently comparing and contrasting different viral and non-

viral gene delivery systems. For ease in manipulation, titrating, and evaluation of gene transfer, a dominant selectable marker gene, such as the neomycin resistance gene (Neo^R) is included with the therapeutic CDase gene. Despite widespread practice, it has been suggested that such a double gene system with neomycin phosphotransferase may cause gene instability and potentially lower viral titers (9, 10). It may be very desirable to use CDase as both a positive and negative selectable marker in a single gene system.

CDase has a very narrow range of substrates (11). There is no known toxic compound that can be directly detoxified by CDase for use in a positive selection scheme. As an alternative approach, we have attempted to make mammalian cells depend upon CDase activity by blocking *de novo* pyrimidine synthesis. Once blocked, these cells will then depend on the activity of CDase to convert extracellular cytosine into uracil for growth.

A *de novo* pyrimidine synthesis inhibitor, *N*-(phosphonacetyl)-L-aspartate (PALA), inhibits aspartate carbamyl transferase (Fig. 1) of the CADase complex (12–14). This PALA-induced blockade induces apoptosis, and as such, is lethal to mammalian cells in culture (15). However, the toxic effects of PALA can be completely circumvented by supplying uridine (15–18).

We have explored the combination of PALA and CDase as a positive selection method for cells expressing CDase. We now present the formula for this CDase-positive selection scheme and provide data for the efficacy of this system. Such a system makes it possible to use the CDase gene as a positive selection marker gene.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Cell Culture—JM-1 is a transformed and tumorigenic cell line established from a chemically induced liver carcinoma obtained from a Fischer rat (Ref. 19; provided by R. L. Jirtle, Duke University). PA317 is a retroviral packaging cell line derived from mouse embryo fibroblasts, 3T3 TK[−] (20). Both PA317 and 3T3 TK[−] cells were obtained from the American Type Culture Collection. JM-1/CD cells were created by electroporating a plasmid, pCMV/CD-1 (CDase gene transcriptionally controlled by a cytomegalovirus immediate early gene promoter; Ref. 4), and selecting for G418-resistant colonies. PA317/CD cells were made by electroporating a plasmid, pLNC-CD (containing the pCMV/CD-1 plasmid in a retroviral shuttle vector pLNCX backbone; Ref. 21), and selecting for G418-resistant colonies. Both JM-1/CD and PA317/CD cells have an integrated copy of the CDase gene and express a functional CDase enzyme as measured by Southern blots, PCR, and a CDase enzymatic assay.

All cells were grown in Dulbecco's modified essential medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 5% dialyzed fetal calf serum (complete medium). Cell culture selection was carried out by supplementing the complete medium with either 1 mg/ml G418 for neomycin selection or combinations of PALA, inosine, and cytosine for the positive CDase selection. Cell morphology was monitored daily under the microscope.

In Vitro Cytotoxic Assay—*In vitro* cytotoxic assays were performed as described previously (2) with slight modification. Briefly, cells were plated at 3000 cells/well in 96-well microtiter plates (day -1). The next day (day 0), media were replaced with 100 μ l/well of complete medium containing proper selection supplements. The media were changed every other day. On days 0, 3, 5, 7, and 10, cells in one set of plates were stained with 16 μ g/ml Hoechst's dye (bisbenzimidazole) in serum-free me-

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¹ The abbreviations used are: CDase, cytosine deaminase; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; CADases, carbamoyl-phosphate synthetase, aspartate transcarbamoylase, and dihydroorotase; PALA, *N*-(phosphonacetyl)-L-aspartate; TK, thymidine kinase.

dium at 130 μ l/well for 40 min at 37 °C. The plates were read in a Cytofluor fluorometer setting of 360/460 nm (excitation/emission) and sensitivity at 3.

Metabolite Diffusion Assay—PA317/CD cells were plated into the inserts of a 24-well Transwell™ plate at 5×10^4 cells/well. An equal number of the 3T3 TK⁻ cells were plated in the bottom wells of the 24-well plate. Cells in each well and insert were cultured overnight in 700 μ l of complete medium. The complete medium was replaced with 700 μ l of complete medium supplemented with 1 mM of PALA, 1 mg/ml inosine, and 1–8 mM of cytosine. The media were replaced every other day. At days 4 and 7, cell growth was determined by staining with 16 μ g/ml Hoechst's dye at 500 μ l/well or 700 μ l/insert as described above.

Cell Cycle Analysis—To determine cell cycle distribution of cells in the complete or selection media, cells were collected after 7 days of culturing in either medium and fixed in 70% ethanol at -20 °C. Flow cytometry analysis on these cells was done using the procedure described by Darzynkiewicz *et al.* (22).

RESULTS

The *in vitro* cytotoxicity of PALA on JM-1, JM-1/CD, PA317, and PA317/CD cells was first determined (Fig. 2). PALA was similarly toxic in all cell lines, with IC₅₀ and IC₉₅ being approximately 150 ± 20 μ M and 1.00 ± 0.02 mM, respectively.

PALA-induced cytotoxicity could not be reverted with cytosine alone (up to 5 mM), cytosine plus thymine (up to 2 mM

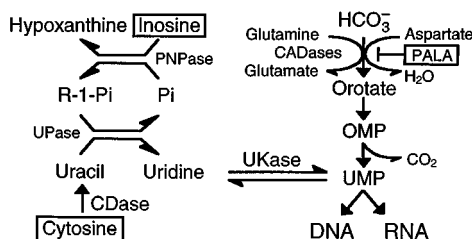
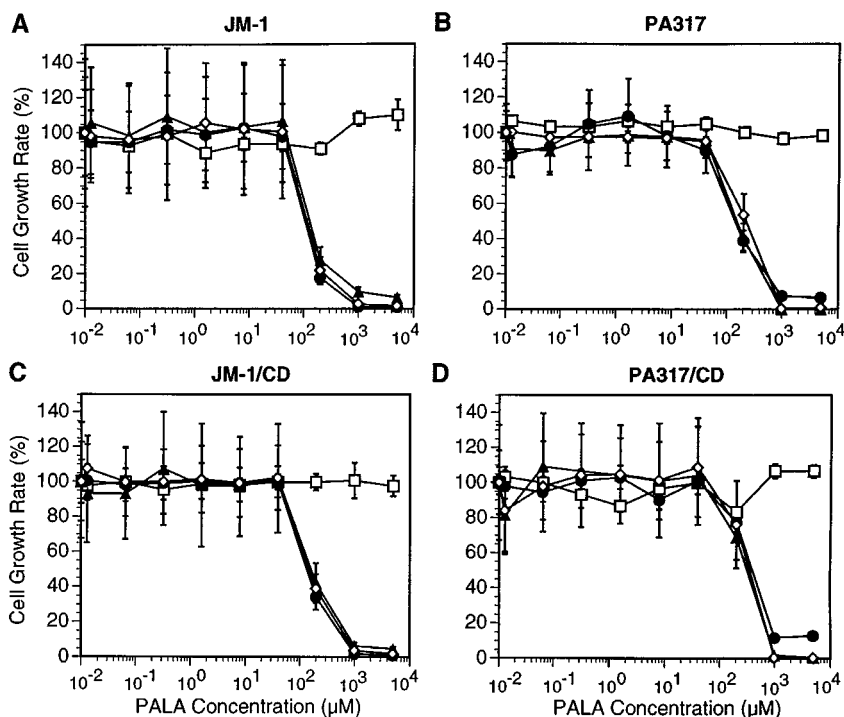


FIG. 1. The *de novo* pyrimidine synthesis pathway. *De novo* pyrimidine synthesis pathway and the mechanism for the positive selection using the CDase coupled with inosine and cytosine in the selection media to rescue PALA-induced apoptosis. *CADases*, carbamyl synthase, aspartate carbamyltransferase, and dihydroprotase; *CDase*, cytosine deaminase; *PNPase*, purine nucleoside phosphorylase; *UKase*, uridine kinase; *UPase*, uridine phosphorylase; *OMP*, orotate monophosphate; *PALA*, *N*-(phosphonacetyl)-L-aspartate; *P_i*, phosphate; *R-1-P_i*, ribose 1-phosphate; *UMP*, uridine monophosphate.

FIG. 2. *In vitro* cytotoxicity of PALA. Cells were cultured for 7 days in complete media supplemented with increasing amounts of PALA. Cytotoxicity was determined on days 0, 3, 5, and 7 as described under "Experimental Procedures." Data are represented as the percentage of cell growth in PALA-containing media compared to the cell growth in complete medium without PALA. □, day 0; ●, day 3; ▲, day 5; ◇, day 7.



each), cytidine (up to 2 mM), or cytidine and deoxycytidine (up to 2 mM each) in the CDase-positive cells.

In another approach, we synthesized orotate analogues 2-aminoorotate and 6-carboxycytosine. We hoped that CDase could convert these compounds into orotate so that the PALA inhibition on *de novo* pyrimidine synthesis could be bypassed

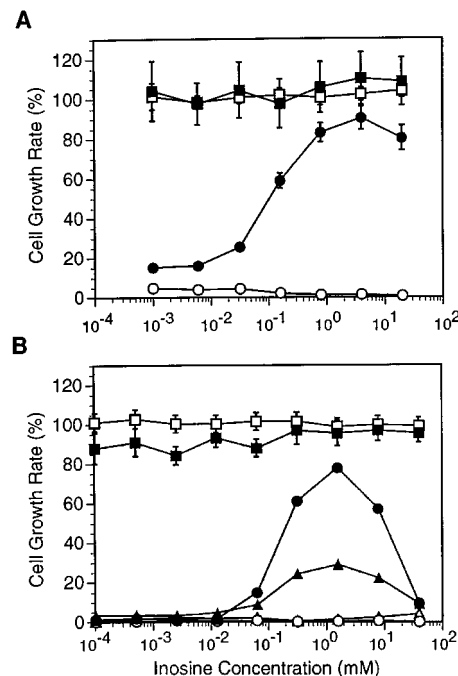


FIG. 3. Positive selection of CDase-positive cells using cytosine, inosine, and PALA. Cells were cultured in complete media supplemented with 1 mM PALA, 20 mg/liter cytosine, and increasing amounts of inosine. Cell survival was determined on days 0, 3, and 5 as described under "Experimental Procedures." Data are represented as the percentage of cell growth in PALA-containing media compared to the cell growth in complete medium. A, □, JM-1 at day 0; ○, JM-1 at day 5; ■, JM/CD at day 0; ●, JM/CD at day 5. B, □, PA317 at day 0; △, PA317 at day 3; ○, PA317 at day 5; ■, PA317/CD at day 0; ▲, PA317/CD at day 3; ●, PA317/CD at day 5.

(Fig. 1). However, supplementing the medium with these orotate analogues could not restore the growth of the CDase-positive cells. Subsequently, we have determined that neither 6-carboxycytosine or 2-aminooxalate are substrates for the bacterial CDase (data not shown).

However, PALA-induced toxicity could be reversed in CDase-positive cells if the culture media were supplemented with 20 mg/liter cytosine and 1–4 mM inosine (Fig. 3). In JM-1/CD, there was a 90% recovery when 4 mM inosine was present in the selection medium. In PA317/CD, approximately 80% recovery was obtained in 2 mM inosine containing medium. Thus, in the presence of inosine and cytosine in the medium, only CDase gene altered cells could overcome the toxic effects induced by PALA.

To effectively use media supplemented with PALA, cytosine, and inosine (selection medium) to positively select for cells that express CDase, it is important that the uridine metabolite cannot significantly diffuse out of the cells and subsequently rescue adjacent CDase-negative cells. To assess this potential, PA317/CD and 3T3 TK⁻ cells were plated in 24-well Transwell™ AA plates and grown in the selection medium. PA317/CD cells were plated into the inserts, while an equal amount of 3T3 TK⁻ cells were plated in the bottom wells. Metabolite diffusion was assessed on days 4 and 7 using cell growth of the control cells as an indicator (Fig. 4). Unmodified 3T3 TK⁻ cells at the bottom wells died, while modified PA317/CD cells in the inserts grew.

Taken collectively, these data indicate that cytosine and

inosine can rescue CDase-positive cells from the PALA-induced blockade in *de novo* pyrimidine synthesis. These data also showed that uridine or uracil, which was generated in the selection process, was insufficient to rescue CDase-negative cells.

Based on the above results, a positive selection medium was formulated by supplementing complete medium with 1 mM PALA, 1 mg/ml inosine, and 1 mM cytosine. This formulation was used in the following assays to validate its efficacy in the positive selection system.

Cell growth rates were compared in the positive selection medium and normal complete medium (Fig. 5). Unmodified parental cells did not grow in the positive selection medium. JM-1/CD grew similarly in either media, while PA317/CD grew relatively slower in the positive selection medium. This may result from the relative enzymatic activity of CDase in the two cell lines (approximately 50 nmol/min/mg in JM-1/CD and 17 nmol/min/mg in PA317/CD).

In the positive selection medium, cells expressing CDase showed no obvious morphological changes up to 5 days (Fig. 6H) when compared with cells cultured in normal complete medium (Fig. 6, E and F). However, CDase-negative cells showed significant PALA toxicity by day 3 (Fig. 6C) and showed characteristics of apoptosis (swollen nucleus and dissegmentation) by day 5 (Fig. 6G). CDase-positive cells grew similarly in either complete or selection media (Fig. 7, B and D), while CDase-negative cells were dead in positive selection medium (Fig. 7, A and C).

Cell cycle distribution studies confirmed that in the positive selection medium, PA317 cells (CDase-negative) showed typical distribution of apoptotic cells. However, PA317/CD cells (CDase-positive) were viable but showed a delay at G₀/G₁ stages, with 50% reduction of cells committing to either S or G₂ + M stages (Table I). This result was consistent with the observation that PA317/CD grew slower in the positive selection medium compared to that in the complete medium (Fig. 5B). However, there was no significant apoptosis presented for CDase-positive cells in selection medium.

DISCUSSION

Genetic manipulation of cells is an important tool in molecular cell biology. There are numerous procedures for genetically modifying cells and subsequently selecting for these modifications. Positive selection is one of the most popular means, since it takes advantage of the cells lacking certain detoxifying enzymes (*i.e.* Amp^R (ampicillin resistance), Tet^R (tetracycline resistance), Kan^R (kanamycin resistance), Neo^R (neomycin resistance), and Hyg^R (hygromycin B resistance)). Another means is complementation culture by providing certain enzymes (*i.e.* CADases, adenosine kinase, and TK). Both strategies consist of one toxin, one detoxification enzyme, or one complementation enzyme system. Such strategies do not work

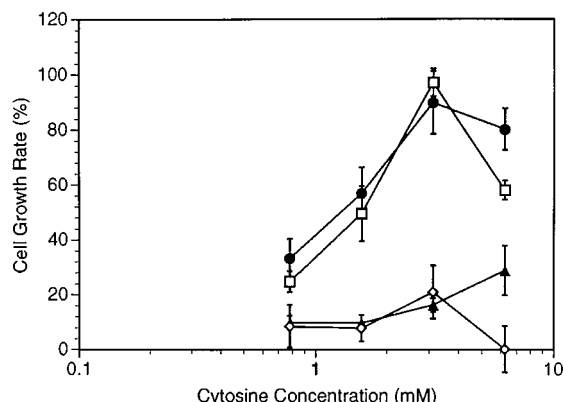
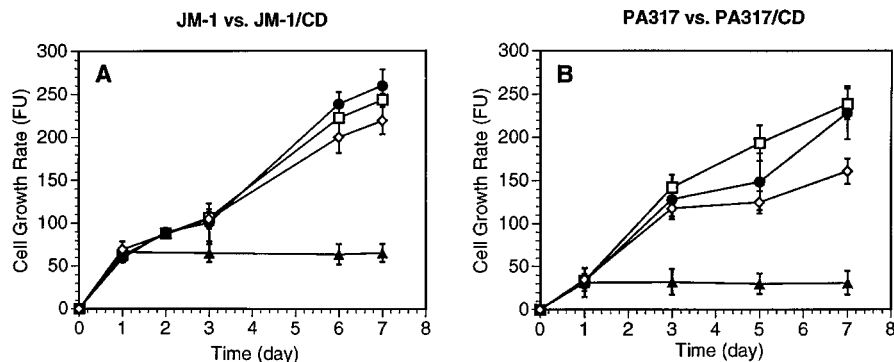


FIG. 4. **Metabolic diffusion assay.** Cells were cultured in complete media supplemented with 1 mM PALA, 1 mg/ml inosine, and increasing amounts of cytosine. PA317/CD cells were plated in the inserts of the 24-well Transwell™ plates, while an equal amount of 3T3 TK⁻ cells were plated in the bottom wells. Cytotoxicity was determined at days 4 and 7 as described under "Experimental Procedures." Data are represented as the percentage of cell growth in PALA-containing media compared to the cell growth in complete medium without PALA. □, PA317/CD at day 4; ●, PA317/CD at day 7; ▲, 3T3TK⁻ at day 4; ◇, 3T3TK⁻ at day 7.

FIG. 5. **Comparison of cell growth rates in either complete or positive selection medium.** Equal amount of cells were seeded and cultured in either complete medium or the positive selection medium (complete medium supplemented with 1 mM PALA, 1 mg/ml inosine, 1 mM cytosine). Cell growth rate was determined as described under Experimental Procedures. □, complete/parental; ●, complete/CD; ▲, selection/parental; ◇, selection/CD.



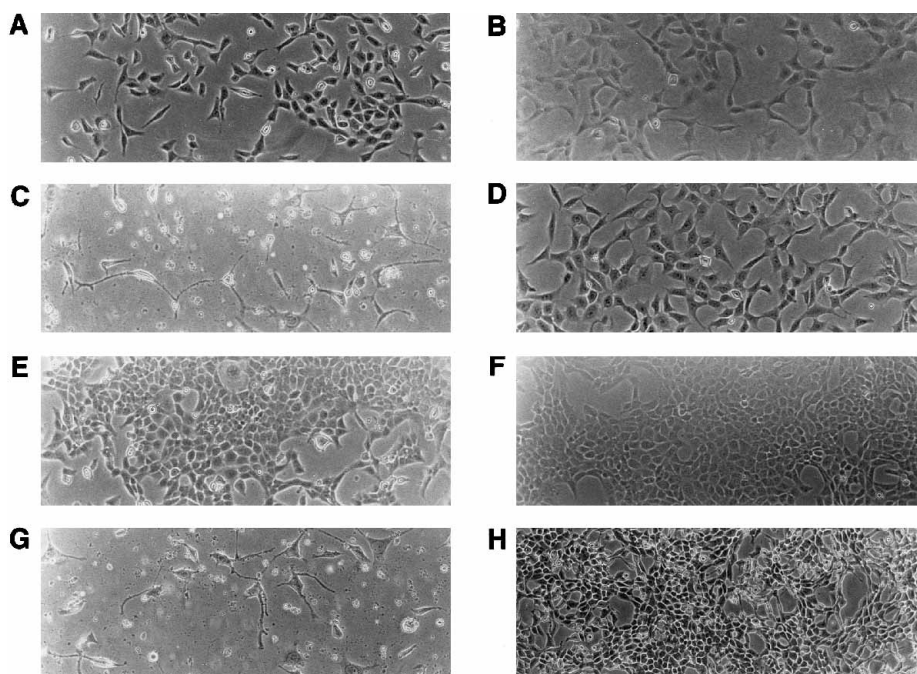


FIG. 6. **Morphological characteristics of cells in either complete or positive selection medium.** Equal amounts of PA317 (A, C, E, and G) and PA317/CD (B, D, F, and H) cells were seeded and cultured in either complete medium (A, B, E, and F) or positive selection medium (complete medium supplemented with 1 mM PALA, 1 mg/ml inosine, 1 mM cytosine). Cell morphological appearance was photographed at day 1 (A and B), day 3 (C and D), and day 5 (E, F, G, and H).

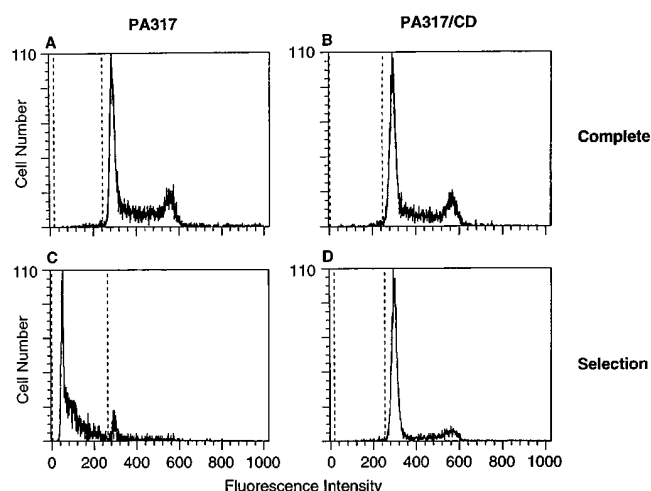


FIG. 7. **Cell cycle distribution in either complete or positive selection medium.** PA317 (A and C) and PA317/CD (B and D) cells were cultured in either complete medium (A and B) or positive selection medium (C and D) for 7 days. After fixation in 70% ethanol, cell cycle distribution was determined by flow cytometry analysis as described under "Experimental Procedures."

TABLE I
Cell cycle distribution

	PA317		PA317/CD	
	Complete media	Selection media	Complete media	Selection media
G ₀ /G ₁	44.3	11.11	52.7	77.5
S	42.2	<2.00	32.4	14.6
G ₂ + M	13.5	<1.00	14.9	7.90
G ₂ + M/G ₁	1.93	<1.00	1.93	1.90
Apoptosis	1.50	85.32	1.88	1.59

with CDase since there is no toxic compound known to be selectively detoxified by CDase.

To solve this problem, we have employed PALA to block the *de novo* synthesis pathway of pyrimidines and force the cells to rely on a CDase dependent salvage pathway (Fig. 1). Because of the narrow substrate range of CDase, cytidine, 2'-deoxycyti-

dine, and the orotate analogs 6-carboxycytosine and 2-amino-orotate were not converted to useful anabolites by the enzyme. Although CDase effectively converts cytosine into uracil, the intrinsic equilibrium between uracil and uridine is clearly in favor of uracil. It became obvious that supplying cytosine alone would not allow for rescue of CDase-positive cells in the presence of PALA.

To circumvent the problem, the equilibrium between uracil and uridine had to be altered. To accomplish this goal, inosine was used to increase the cellular concentration of ribose 1-phosphate, thereby shifting the equilibrium between uracil and uridine toward uridine (Fig. 1). For JM-1/CD cells, the rescue of the CDase-positive cells by cytosine and inosine was approximately 100%. However, the rescue of the PA317/CD cells was approximately 80%. This may result from the intracellular enzymatic activity of the CDase.

Although growth recovery differs from cell line to cell line, CDase gene-modified cells grown in the positive selection medium do not undergo significant cell death. Their growth rates may slightly decreased depending on the enzymatic activity of CDase, but their morphological characters are not altered (Fig. 6).

In summary, the bacterial CDase gene can be used as a positive selection marker in combination with PALA, cytosine, and inosine in the positive selection medium. This positive selection is safe and effective in manipulating cells altered by or carrying bacterial CDase gene. In combination with the negative selection using 5-FC, this positive selection makes it more effective and attractive for using the bacterial CDase in human cancer gene therapy.

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