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Targeting of an inducible toxic phenotype in animal cells

(toxic vector/cell lineage/transgenic mice/thymidine kinase/acyclovir)

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ABSTRACT **We have developed a toxic, or suicide, vector whose action is based on the targeted expression of the herpes simplex virus 1 thymidine kinase gene product in cultured cells or transgenic animals. This protein is able to convert nucleoside analogs such as acyclovir and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) to toxic intermediates. The activation of these compounds disrupts cellular DNA replication, leading to rapid cell death. Neither acyclovir, FIAU, nor the herpes thymidine kinase alone is harmful to cells. This approach is simple and should have widespread applicability in studying lineage formation in cultured cells and transgenic animals.**

One problem in the study of vertebrate development is the absence of a genetic approach for the study of critical events in lineage formation and organogenesis. Various attempts have been made by both mechanical and chemical methods to specifically eliminate the function of a cell lineage in a living organism. Although some success has been achieved, the complex structures of the vertebrate systems do not allow an efficient destruction of a selective cell type by these methods.

The possibility of introducing a desired cloned gene into the mouse germ line has inspired the approach of expressing negative selectable markers in cells, which would either inhibit cell proliferation or lead to cellular degeneration (1–3). The knowledge that the cis-acting elements that specify tissue-specific gene expression reside in the promoter/enhancer regions of most genes (4) can be exploited to study lineage formation in cells in which they are active. One problem in designing a toxic vector is that the ablation of a cell line during embryonal life could lead to a lethal phenotype. Thus, a valuable alternative would be the manifestation of a toxic phenotype that is cell-specific and inducible.

One candidate of potential use in such an approach is the herpes simplex virus 1 thymidine kinase (HSV1-TK) (5). This enzyme alone is not harmful to cells, and in thymidine kinase-negative (TK^-) cells it can allow cell survival (6). The pathogenic effects of herpes simplex virus infection have led to the development of nucleoside analogs that selectively block viral spread as a consequence of their metabolism by the herpes enzyme but not its cellular counterpart (7–15). One analog, acyclovir {ACV, 9-[(2-hydroxyethoxy)methyl]guanine} (12, 13), has demonstrated clinical efficacy for the treatment of herpes infections in humans. Furthermore, in noninfected cells, ACV, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) (14), and related compounds display little cytotoxicity (12, 14, 15). These observations led us to examine whether the expression of the cloned HSV1-TK gene either in transfected cells or in a fibroblast line having a resident HSV1-TK gene would convert ACV and FIAU to cytotoxic intermediates. In this paper we show that the introduction of the HSV1-TK gene in cultured cells and in the immune system of transgenic animals, in conjunction with nucleoside analog treatment, leads to a drug concentration-dependent cytotoxicity and, consequently, to cell death.

MATERIALS AND METHODS

Gene Constructs. The structures of the recombinant DNAs used for transfection of COS cells (16) are shown in Fig. 1*a*. Plasmid pApS1 contains the mouse mammary tumor virus long terminal repeat (MTV-LTR) linked to the HSV1-TK gene (17). pRShGRα contains the Rous sarcoma virus long terminal repeat (RSV-LTR) linked to DNA encoding the human glucocorticoid receptor (hGRα) (18). pSV2cat contains the simian virus 40 (SV40) promoter region linked to the chloramphenicol acetyltransferase (CAT) gene (19). Plasmid pKHTK (see Fig. 3*a*) has been used to obtain transgenic mice. pKHTK was derived from pKCATH (20) by replacing the CAT gene with the HSV1-TK gene. In pKHTK, a 682-base-pair (bp) *Xba* I–*Eco*RI fragment of the μ heavy-chain enhancer (21, 22) linked to a 1.1-kbp fragment of the κ light-chain promoter (23) directs the transcription of a 1.5-kbp fragment of the HSV1-TK gene from position +2 relative to the TK transcription start site to 236 bp downstream of the TK polyadenylylation site.

Drugs. ACV (12, 13) and FIAU (14) are made by Burroughs Wellcome (Research Triangle Park, NC) and Bristol-Meyers (Syracuse, NY), respectively. We obtained them as a generous gift from D. Richman (Veterans Administration Hospital, San Diego, CA).

Tissue Culture Transfection. Fifty percent confluent COS cells (16) were transfected by the calcium phosphate technique (24) with 1 μg of pSV2cat, 5 μg of pApS1, and 1 μg of pRShGRα. The cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Dexamethasone (0.1 μM) was added before each experiment. When the efficiencies of the nucleoside analogs were to be tested, either ACV or FIAU (10 μM) was added to the medium. After 24 hr, the cells were detached by trypsin treatment, washed, and then replated in new medium containing the same concentrations of dexamethasone and ACV or FIAU as before. Twelve hours later the cells were harvested and the plasmid DNAs were recovered by the Hirt method (25). The recovered plasmid DNAs were digested by *Bam*HI, and the resulting restriction fragments were separated in a 0.8% agarose gel and then transferred to nitrocellulose paper. The immobilized DNA was then hybridized (26) to a nick-translated probe spanning the polyadenylylation site of SV40 (*Bam*HI–*Eco*RI fragment, SV40 positions 2533–1782) or with a pBR322 probe.

Cell Growth Assay. The cytotoxicity of the nucleoside analogs ACV and FIAU was determined by using a cell growth assay. Rat fibroblast line 208 (27) or Rat-2 clone 3B

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Abbreviations: ACV, acyclovir; FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouridine; TK, thymidine kinase; HSV1-TK, herpes simplex virus 1 TK; MTV-LTR, mouse mammary tumor virus long terminal repeat; SV40, simian virus 40.

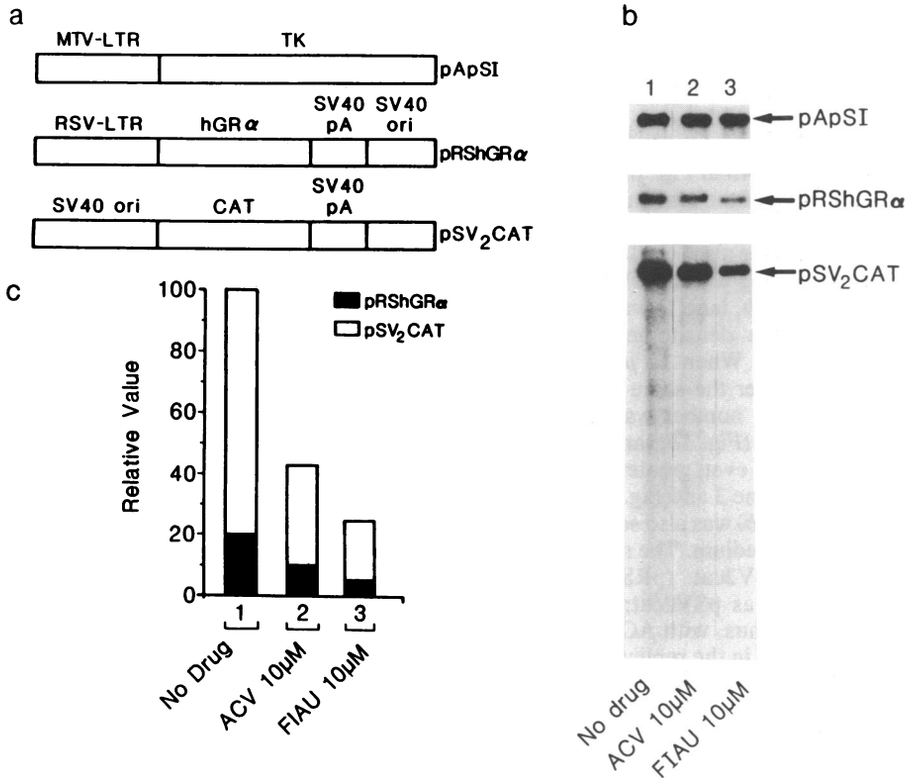

FIG. 1. HSV1-TK inhibition of plasmid DNA replication in transiently transfected cells. (*a*) Structure of the recombinants used for transfection of COS cells. ori, Origin of replication; pA, polyadenylylation site. See *Materials and Methods*. (*b*) Replication of pSV2cat and pRShGRα in COS cells in the presence of ACV or FIAU. Bands corresponding to each plasmid are indicated by arrows at right. (*c*) Densitometric scans of autoradiograms of Southern blot from the Hirt extracts. Open bars, pSV2cat (the intensity of its band in the absence of drug was taken as the 100% value); black bars, pRShGRα.

(28), a TK^- cell line stably transformed to TK^+ by an MTV-LTR/HSV1-TK construction, were seeded at low density (3×10^4 cells per 10-cm culture dish) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum; in either the presence (0.1 μM) or absence of dexamethasone. Twelve hours later, various concentrations of ACV or FIAU were added to the cultures. After a total of 72 hr, cells were detached by trypsin treatment and resuspended in medium. Viable cells were recognized by trypan blue exclusion. Cell numbers were determined with a Neubauer hemocytometer.

Transgenic Mice. Several transgenic lines were obtained by the injection of the KHTK hybrid construct (see Fig. 3*a*). In this paper we describe line 686, whose pattern of RNA expression is similar to those of the other lines obtained. Transgenic and nontransgenic littermates were treated for 1 week with a 50-mg/ml solution of FIAU in phosphate-buffered saline released by a subcutaneously implanted miniosmotic pump (model 2002, Alzet, Alza Corp., Palo Alto, CA).

RNA Analysis. For RNA gel blot analysis, total RNA was isolated from various tissues of the transgenic line 686 by the LiCl/urea method (29), 1% agarose fractionated by electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose, and hybridized under stringent conditions (26) with the whole HSV1-TK gene as probe. The filter was autoradiographed at −70°C with an intensifying screen for 24 hr. S1 nuclease analysis was performed on 10-μg samples of total RNA from various tissues of the transgenic mice; the RNAs were hybridized in solution with a double-stranded probe (30) spanning from the *Ava* I site (+350) in the TK gene to the *Eco*RI site (−1100) indicated in Fig. 3*a*. After S1 nuclease treatment, the protected fragment, corresponding to RNA transcribed from the κ light-chain promoter, was 380 nucleotides long, in agreement with the expected size.

RESULTS

Inhibition of Plasmid Replication by Synergy Between the HSV1-TK Gene and ACV or FIAU. The toxic efficacy of antiherpetic drugs is presumably a consequence of the metabolism of these nucleoside analogs (such as ACV and FIAU) by HSV1-TK to phosphorylated derivatives that are incorporated into DNA by herpes DNA polymerases. This, in turn, leads to chain termination of the replicating viral template (refs 7–15 and refs. therein). The inability of the virus to replicate its genome appears to be directly related to the clinical benefit of these drugs. We wished to explore the possibility that HSV1-TK expressed from a transfected plasmid could metabolize antiherpetic nucleotides to substrates for the cellular DNA polymerase in uninfected cells. In principle, the incorporation of these activated drugs would lead to a net decrease in cellular DNA replication. Accordingly, a plasmid capable of replicating in COS cells (16), pSV2cat (Fig. 1*a*) (19), was cotransfected with a plasmid, pAPS1 (17), expressing the HSV1-TK gene under the control of the glucocorticoid-inducible promoter of the mouse mammary tumor virus (Fig. 1*a*). Here, the expression of the HSV1-TK gene is dependent on both the presence of glucocorticoids, such as the synthetic dexamethasone, and the glucocorticoid receptor to activate the MTV-LTR promoter. For this reason, a human glucocorticoid receptor expression vector, pRShGRα (18), was also cotransfected to ensure high levels of this trans-acting factor (Fig. 1*a*). pRShGRα itself contains the origin of replication of SV40 and therefore is able to replicate in COS cells.

Semiconfluent COS cells (16) were cotransfected by the calcium phosphate technique (24) with pSV2cat, pApS1, and pRShGRα and analyzed 36 hr later. The effect of ACV or FIAU on the replication of pSV2cat was gauged by the

reduction in the number of molecules of this plasmid extracted by the Hirt method (25) from transfected cells. Southern blot analysis (26) of *Bam*HI digests was performed by using as probe a nick-translated DNA fragment that specifically recognizes the polyadenylylation site of SV40, present in the plasmids of pSV2cat and pRShGRα (Fig. 1*a*). The results of this analysis are shown in Fig. 1*b*; in lane 1 the intensity of the band is indicative of the amount of linearized pSV2cat recovered from transfected COS cells when no drugs were added to the medium (see also Fig. 1*c*, lane 1). The intensity of the band corresponding to the pSV2cat replication in the absence of drug (Fig. 1*b*, lane 1) was normalized to 100% of relative value in the densitometric scanning analysis of the samples (Fig. 1*c*). When 10 μM ACV was added to the culture medium under the same experimental conditions, the total plasmid copy number was reduced by 40% relative to no drug treatment (Fig. 1*b*, lane 2, and Fig. 1*c*, open bar 2); the reduction was even greater (70%) when 10 μM FIAU was used (Fig. 1*b*, lane 3 and Fig. 1*c*, open bar 3). Replication of pRShGRα (Fig. 1*b*) was also sensitive to the addition of ACV or FIAU to the medium. The reduction was similar to that observed with pSV2cat. (pRShGRα never reached the same copy numbers as pSV2cat; we have no explanation for this difference.) Thus, with ACV and FIAU there was a 40% and 70% decrease in the replication of these plasmids. Plasmid pApS1, which does not replicate, served as an internal control. As expected, the number of copies was unchanged by any treatment (Fig. 1*b*). Finally, when either the nucleoside analogs were absent or the plasmid expressing TK was absent, no reduction in the amount of pSV2cat replication was observed (data not shown). These experiments indicate that the expression of the HSV1-TK gene is required for the conversion of the nucleoside analogs to toxic precursors.

Cytotoxic Effect of ACV or FIAU on a Cell Line Expressing the HSV1-TK Gene. To examine the effect on cellular DNA replication and survival, we used the rat fibroblast cell line 3B (28), which stably expresses the glucocorticoid-inducible MTV-LTR/HSV1-TK gene. Addition of dexamethasone to these cells leads to a 50-fold increase in HSV1-TK activity. We examined the sensitivity of these and control cells to various levels of ACV and FIAU. The effect of the drugs on the 3B cells was compared to the effects on the control rat fibroblast line 208 (27), which does not express HSV1-TK. The experiments were performed in the presence or absence of dexamethasone at 0.1 μM. The efficacy of the drugs was monitored as reduction in the number of viable cells. ACV at 0.1–1.0 μM had no detectable effect (Fig. 2*a*). ACV at 3.0 μM appeared to be slightly toxic (20% cell death), but only to 3B cells in the presence of dexamethasone. Its toxicity to 3B cells in the presence of dexamethasone was greater at higher concentrations: we observed 60% cell death at 10 μM ACV and 98% cell death at 100 μM. At 100 μM we observed nonspecific toxicity of ACV that was independent of the presence of dexamethasone. When FIAU was used, similar but more dramatic cytotoxic effects were observed (Fig. 2*b*). FIAU at 0.1 μM reduced viability by >60%, and at 3.0 μM 98% cell death was achieved in the absence of severe nonspecific toxicity. Thus, both drugs could reduce cell viability at doses that were apparently not harmful to the control 208 cells. These results indicate that the expression of the HSV1-TK gene confers upon cells a toxic potential that can be revealed by the addition of appropriate levels of nucleoside analogs.

Specific Cell Killing in Transgenic Mice. We wished to establish whether the cell-specific expression of the HSV1-TK gene in transgenic mice would confer drug-dependent cytotoxicity as observed in cultured cells. As a model system we chose to target expression of the HSV1-TK gene to cells of the immune system. Accordingly, the immunoglobulin heavy-chain enhancer (21, 22) and light-chain promoter (23) were fused to the HSV1-TK gene to create the plasmid pKHTK. This promoter/enhancer combination has been shown to be active when transfected into lymphoid cells *in vitro* (20). The chimeric KHTK construct (Fig. 3*a*) was injected into fertilized mouse eggs, giving rise to a transgenic founder that established the 686 pedigree. RNA gel blot analysis of this pedigree revealed transcripts corresponding to the correct TK RNA in spleen and thymus (Fig. 3*b*). This pattern of expression is consistent with other studies in which

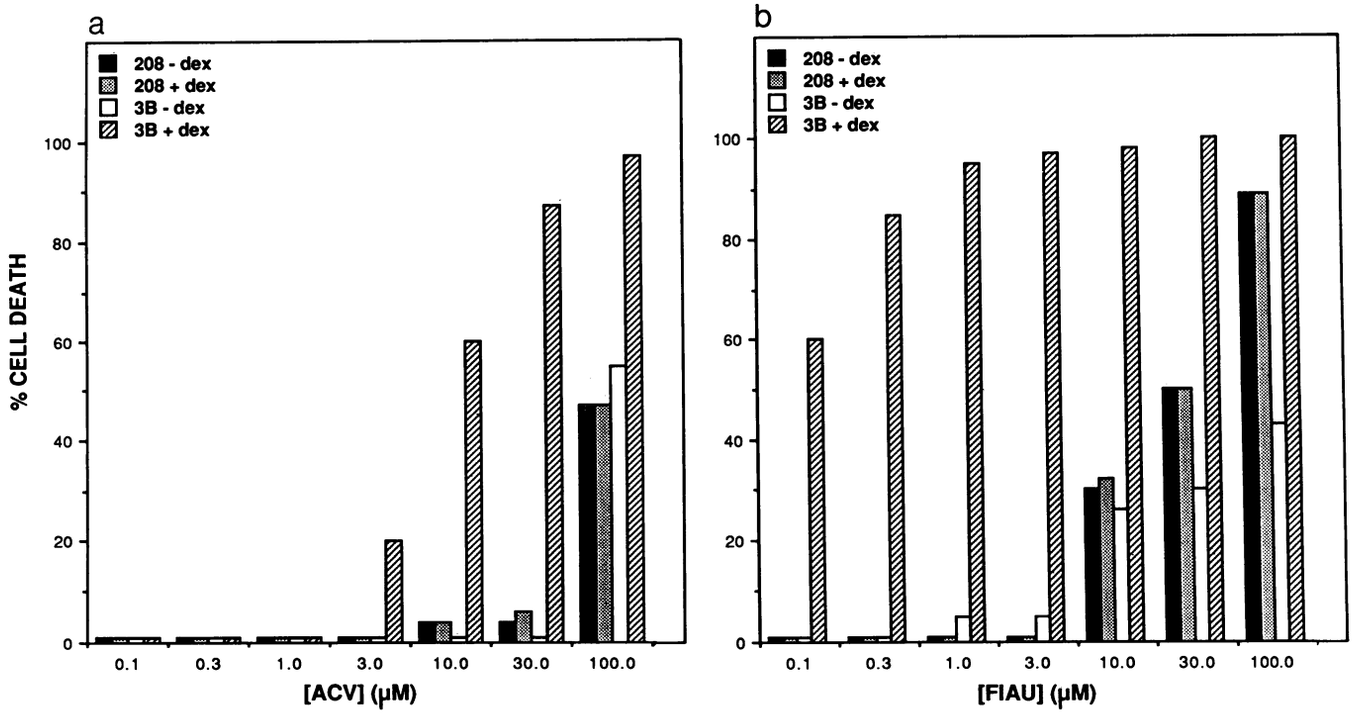

FIG. 2. Cytotoxic effect of ACV (*a*) or FIAU (*b*) on growth of control 208 cells and of 3B cells. dex, Dexamethasone.

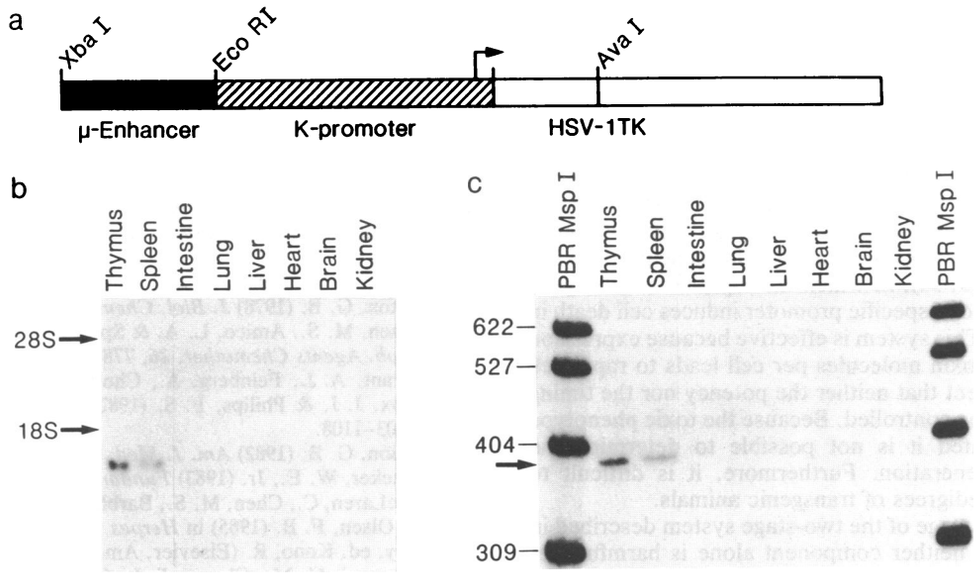

FIG. 3. Expression in transgenic mice of the KHTK hybrid construct. (*a*) Structure of pKHTK (see *Materials and Methods*). (*b*) RNA gel blot analysis of HSV1-TK mRNA from transgenic mice. Twenty micrograms of total RNA was used in each lane. Migration positions of the ribosomal RNAs are indicated as size markers. (*c*) S1 nuclease analysis of HSV1-TK mRNA from transgenic mice. *Msp* I-digested pBR322 provided size markers.

the entire μ heavy-chain gene (31) or a construct containing a similar promoter/enhancer combination (32) was expressed in both B and T cells. Very low levels of expression of the transgene were detected in other tissues, including liver, intestine, lung, and brain, after long autoradiographic exposure of the blot (data not shown). The expression in lung and intestine may result from the presence of lymph nodes in these tissues; the significance of TK transcripts in liver and brain is unclear. S1 nuclease analysis was used to verify the location of the transcription start site in the transgenic tissues. This analysis revealed a correct initiation of transcription from the hybrid promoter in the same tissues already shown to contain detectable levels of TK RNA by RNA gel blot analysis (Fig. 3*c*). Further, to test whether the TK protein was biologically active, TK assays were performed on extracts from these tissues. Enzyme activity was detectable only in thymus and spleen (data not shown).

To evaluate the efficacy of our system *in vivo*, an experiment was performed in which transgenic and nontransgenic littermates were implanted subcutaneously with a miniosmotic pump containing FIAU at 50 mg/ml. FIAU was chosen because it was most efficient in blocking DNA replication *in vitro*. The dose was calculated to elevate serum levels of the drug to concentrations shown to be effective in cell culture. One week after the pump was implanted, the animals were sacrificed. Since the mass of the thymus and of the spleen is determined primarily by their content of T cells and of T and B cells, respectively, selective toxicity should have a direct impact on the size and cell content of these organs. Autopsy revealed a completely normal anatomy in the control mouse, with no apparent toxic effects after 1 week of FIAU treatment (Fig. 4). In the transgenic mouse, all organs were normal with the exception of the spleen and thymus, which displayed decreased weight and were visually atrophied (Fig. 4). Reduction of total spleen weight was 30%, while that of total thymus was 50%. This decrease of total net weight is the apparent consequence of a massive loss of lymphocytes. Cytometric analysis revealed an 83% depletion of cells in the thymus and a 57% loss of cells in the spleen. Analysis of four additional animals, which were similarly treated, showed 80–98% cell death in the thymus and 50–85% cell death in the spleen. Fluorescent cell sorting analysis indicated a 60–70% decrease of circulating B and T cells.

DISCUSSION

We have described the generation of a toxic, or suicide, vector whose action is based on the controlled expression of the HSV1-TK gene product. The expression of this product alone seems to have no beneficial or obvious deleterious effects on cultured cells. The rationale of this approach is based on the ability of this enzyme to convert ACV, FIAU, and related compounds to toxic nucleotide intermediates. The activation of these compounds as a consequence of HSV1-TK expression disrupts cellular DNA replication, leading to cell death. It is not clear what extent of chromosomal damage must be absorbed to manifest cytotoxic effects, but it is clear that the toxicity is proportional both to the levels of HSV1-TK expression and to the concentration of ACV or FIAU to which the cells are exposed. The toxic effects exerted by this system on cultured cells were so striking that it seemed likely that this technique would be useful for studying cell lineage in transgenic animals. Such a

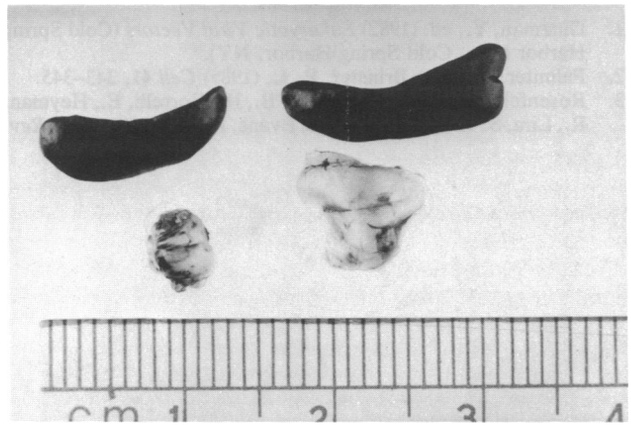

FIG. 4. Comparison of transgenic TK-expressing (*Left*) and nontransgenic (*Right*) tissues after treatment of mice with the nucleoside analog FIAU. (*Upper*) Spleen. (*Lower*) Thymus.

system would exploit the ability of tissue-specific promoters to target restricted expression of HSV1-TK to desired cell types. Since the expression of HSV1-TK alone is not harmful, one can generate stable transgenic pedigrees prior to conducting ablation studies. The ability to control drug dose and delivery should allow manipulation of the relative extent of the toxic effects and thus allow not only study of the consequence of lineage ablation but analysis of the plasticity of residual stem cells and their capacity for regeneration.

Recently, another toxic system with the same purpose has been described (33, 34), in which the diphtheria toxin gene under the control of a specific promoter induces cell death in transgenic mice. This system is effective because expression of one or a few toxin molecules per cell leads to rapid cell death. It is apparent that neither the potency nor the timing of cell death can be controlled. Because the toxic phenotype cannot be regulated it is not possible to determine the potential for regeneration. Furthermore, it is difficult to generate stable pedigrees of transgenic animals.

The major advantage of the two-stage system described in this paper is that neither component alone is harmful, yet together they yield a highly toxic phenotype. This achieves an important level of control not possible for the diphtheria toxin system. One limitation is that toxicity depends on cellular DNA replication and the identification of functional promoters for the desired target cell.

In cell culture it was possible to optimize drug treatment to achieve virtually complete cytotoxicity. In animals the toxicity, although severe, was not absolute. It is likely that the protocol used in this study to induce toxicity in animals was not optimal. It should be possible to achieve greater toxicity by increasing the concentration of FIAU, by extending the time of treatment, or by a combination of both. By controlling these parameters, it may be possible to progress from mild cellular degeneration to nearly complete destruction of a specific cell lineage and thus provide valuable animal models to study lineage formation and cell function.

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