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Cytosine deaminase suicide gene therapy for peritoneal carcinomatosis

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Gene therapy is a novel therapeutic approach that might soon improve the prognosis of some cancers. We investigated the feasibility of cytosine deaminase (CD) suicide gene therapy in a model of peritoneal carcinomatosis. DHD/K12 colorectal adenocarcinoma cells transfected *in vitro* with the *CD* gene were highly sensitive to 5-fluorocytosine (5-FC), and a bystander effect could also be observed. Treating CD^+ cells with 5-FC resulted in apoptosis as detected by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling. *In vitro,* several human cell lines derived from ovarian or colorectal carcinomas, as well as the rat glioblastoma 9 L cell line, responded to CD/5-FC and showed a very strong bystander effect. 5-FC treatment of peritoneal carcinomatosis generated in syngeneic BDIX rats by CD-expressing DHD/K12 cells led to a complete and prolonged response and to prolonged survival. Our study thus demonstrated the efficacy of *CD* suicide gene therapy for the treatment of peritoneal carcinomatosis. **Cancer Gene Therapy (2000) 7, 20–26**

Key words: Cytosine deaminase; gene therapy; peritoneal carcinomatosis; colorectal carcinoma.

Gene therapy is a novel approach that might lead to improved treatments of some types of cancer. Although a number of clinical trials are currently being performed to evaluate the safety and the efficacy of such an approach in different settings, more experimental work is required to define the best models for gene therapy. Peritoneal carcinomatosis develops in an anatomical cavity and cannot be eradicated by conventional anticancer therapies. Therefore, it constitutes a suitable model to study the delivery of therapeutic genes and their preclinical and clinical efficiencies. We have reported previously that a herpes simplex virus (HSV)-thymidine kinase (*tk*)-based suicide gene therapy could improve the survival of rats with peritoneal carcinomatosis induced by colorectal adenocarcinoma cells.[1,2] Other investigators have also delivered therapeutic genes into anatomical cavities in which cancer had developed.[3–6].

Suicide gene therapy is based on the introduction in target cells of a gene coding for an enzyme that transforms a prodrug into a cytotoxic compound.[7,8] The gene for HSV-1 TK has been the subject of many investigations *in vitro* and *in vivo*.[9–14] The *Escherichia coli* gene coding for the cytosine deaminase (CD) enzyme is another suicide gene. CD transforms 5-fluorocytosine (5-FC) into cytotoxic 5-fluorouracil (5-FU).[15,16] This gene has already been tested *in vitro* as well as in several animal models, most of them being based on colorectal carcinoma cells,[17–25] and is now being considered for phase I clinical trials.[26]

The present report investigated the feasibility of a *CD*-based suicide gene therapy in a model of peritoneal carcinomatosis induced by colorectal carcinoma cells in syngeneic rats. It demonstrates that 5-FC treatment can eradicate the peritoneal carcinomatosis generated by colorectal carcinoma cells stably transfected with the *CD* gene. It also demonstrates that 5-FC induces apoptosis in CD^+ cells, and that several cell lines, including ovarian and colorectal carcinoma cell lines, are sensitive *in vitro* to the CD/5-FC cytotoxic effect.

September 11, 1998; February 27, 1999.

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MATERIALS AND METHODS

Plasmids

The pRSV-CD plasmid carrying the *E. coli* K12 *CD* gene (kindly provided by Dr. J. Gebert, Section of Molecular Diagnostics and Therapy, Chirurgische Universitäts-klinik, Heidelberg, Germany) has been described previously.[16] The pCMV-CD plasmid was constructed by inserting a 1.3-kb *Hin*dIII/*Bam*HI CD fragment from pRSV-CD into the pcDNA3 vector (Invitrogen, San Diego, Calif) at the *Hin*dIII/*Bam*HI sites. The pCMV-CD20 (cluster of differentiation 20 antigens) plasmid was kindly provided by Dr. Jim Koh (Laboratory of Molecular Oncology, Massachusetts General Hospital, Harvard Medical School, Charlestown, Mass).

Cell culture and transfections

The DHD/K12 colorectal cancer cell line was derived from a transplantable colon adenocarcinoma induced by 1,2-dimeth-

ylhydrazine in syngeneic BDIX rats.[27] DHD/K12 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, Md) supplemented with 5% fetal calf sera (FCS) (Life Technologies), 1% L-glutamine (200 mM), 1% *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (1 M), 1% L-arginine (0.55 mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL).

The 9 L glioblastoma cells and the MDA-MB-435 breast cancer cells were a gift from C. Grignet-Debrus (Laboratory of Fundamental Virology and Immunology, University of Liège). 9 L cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FCS, 1% sodium pyruvate (100 mM), 1% nonessential amino acids, penicillin (100 IU/mL), and streptomycin (100 μg/mL). MDA-MB-435 cells were grown in RPMI 1640 supplemented with 10 μg/mL insulin, 10% FCS, penicillin (100 IU/mL), and streptomycin (100 μg/mL).

The OVCAR-3 ovarian epithelial cancer cell line was maintained in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine (200 mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL). HCT116 human colon carcinoma cells were grown in McCoy's 5A modified medium supplemented with 10% FCS, 1% L-glutamine (200 mM), penicillin (100 IU/mL), and streptomycin (100 μg/mL).

For stable transfections, plasmids were linearized (*Xba*I for pRSV-CD and *Bgl*II for pCMV-CD) and transfected into DHD/K12 cells with the transfection reagent *N*-(1-[2,3-dioleoyloxy]propyl)-*N,N,N*-trimethylammonium methylsulfate (DOTAP) as recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). After selection for geneticin resistance (500 μg/mL of G418, active concentration, Boehringer Mannheim), 30 clones were isolated and analyzed for integration of the *CD* gene by polymerase chain reaction and for expression of the CD enzyme by immunoblot (anti-CD polyclonal rabbit antibody, a generous gift of Dr. Haack, Chirurgische Universitätsklinik, Heidelberg, Germany).

The transient transfections were also performed with the DOTAP method. Cells were cotransfected with pCMV-CD and pCMV-CD20 to estimate the transfection rate. We measured immunofluorescence with a fluorescence-activated cell sorter (FACS) using a monoclonal anti-CD20 antibody conjugated to phycoerythrin (Becton Dickinson, San Jose, Calif).

In vitro *cytotoxicity test*

Stably transfected cells or untransfected cells were seeded at a concentration of 1500 cells/well on 96-well, flat-bottom microplates in medium supplemented with 15% FCS. After 24 hours, cells were cultivated in 5-FC- (Sigma, St. Louis, Mo) or 5-FU-containing medium that was replaced every other day. After 6 days (5-FU) or 8 days (5-FC) of incubation with the drug, cell viability was measured by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Boehringer Mannheim).

For transiently transfected cells, the medium was removed the day after the DOTAP transfection and replaced by 5-FC- (2 mM) containing medium, which was replaced 48 hours later. Cell viability was measured after 96 hours of treatment by trypan blue exclusion and compared with control transfected cells grown in the absence of 5-FC.

The bystander effect was measured by coculturing different proportions of DHD/K12-CMV-CD (CD^+) and DHD/K12 (CD^-) cells. After seeding and treating the cells as mentioned above, their viability was measured by the WST-1 test.

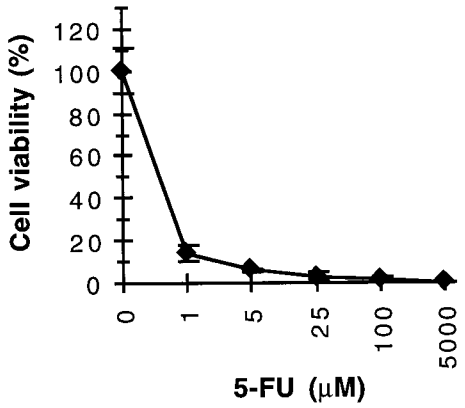

Figure 1. Cytotoxic effect of 5-FU on DHD/K12 cells *in vitro*. DHD/K12 cells were incubated for 6 days in the presence of increasing 5-FU concentrations as indicated. Cell viability was then measured with the WST-1 test.

Treatment of animals

At day 0, 10-week-old male BDIX rats were inoculated intraperitoneally (i.p.) with 10^6 DHD/K12-CMV-CD cells in 2 mL of serum-free medium. At day 14, the animals were treated with 5-FC (Ancotil, Roche, France) at 500 mg/kg/day or with normal saline buffer injected i.p. 5 days a week for 3 weeks. Two animals were sacrificed at 4 days after the last treatment, and the tumor evolution was evaluated by direct abdominal examination. Kaplan-Meier curves were established for eight animals and compared with the log-rank test.

Detection of apoptotic cells

DHD/K12 and DHD/K12-CMV-CD cells were cultured in medium with or without 5-FC (4 mM) for 48 hours. Cells were washed two times in phosphate-buffered saline, fixed in paraformaldehyde, permeabilized in methanol, and labeled by fluorescein-deoxyuridine triphosphate in the presence of terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) as recommended by the manufacturer (*In Situ* Cell Death Detection Kit, Boehringer Mannheim).

RESULTS

CD confers 5-FC sensitivity to DHD/K12 cells

The sensitivity of DHD/K12 colorectal carcinoma cells to 5-FU was evaluated by incubating the cells for 6 days in the presence of increasing 5-FU concentrations and testing cell viability with the WST-1 test. 5-FU killed DHD/K12 cells in a dose-dependent manner (Fig 1), thus indicating that these cells could be considered for *CD* gene therapy.

Next, we stably transfected DHD/K12 cells with expression vectors containing a viral eukaryotic promoter (Rous sarcoma virus or cytomegalovirus (CMV)) upstream of the *CD* gene. Two clones were selected that had integrated the *CD* gene and expressed the CD enzyme, as demonstrated by polymerase chain reaction and immunoblots, respectively (data not shown). These

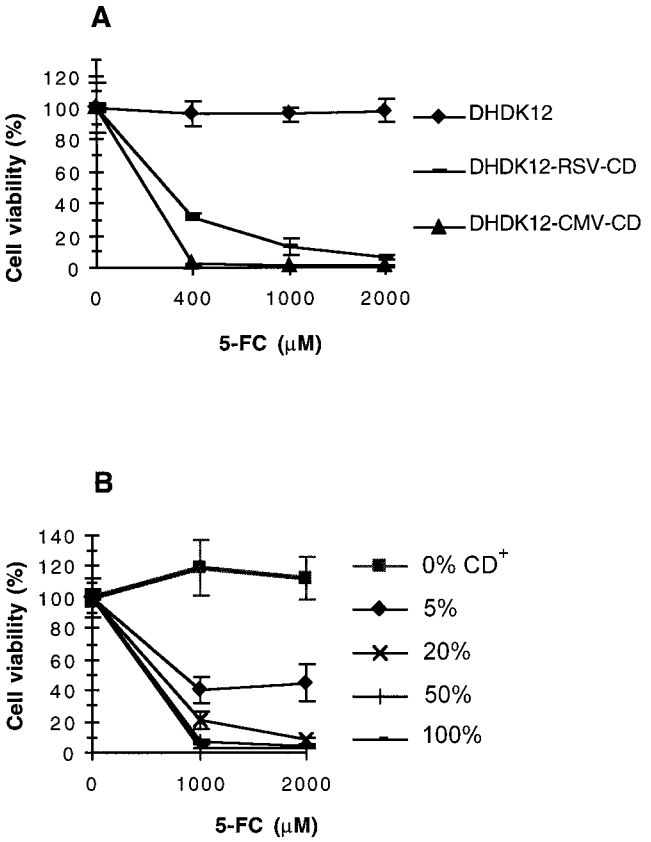

Figure 2. Cytotoxic effect of 5-FC on DHD/K12 CD^+ cells *in vitro*. **A:** stably transfected DHD/K12-RSV-CD and DHD/K12-CMV-CD cells as well as untransfected DHD/K12 cells were incubated *in vitro* for 8 days in the presence of increasing 5-FC concentrations as indicated. Cell viability was then measured with the WST-1 test. **B:** *In vitro* bystander effect. DHD/K12-CMV-CD cells and untransfected cells were cocultured in various proportions and incubated for 8 days in the presence of 5-FC (1 or 2 mM). The proportions of CD^+ cells were 0%, 5%, 20%, 50%, and 100%, respectively.

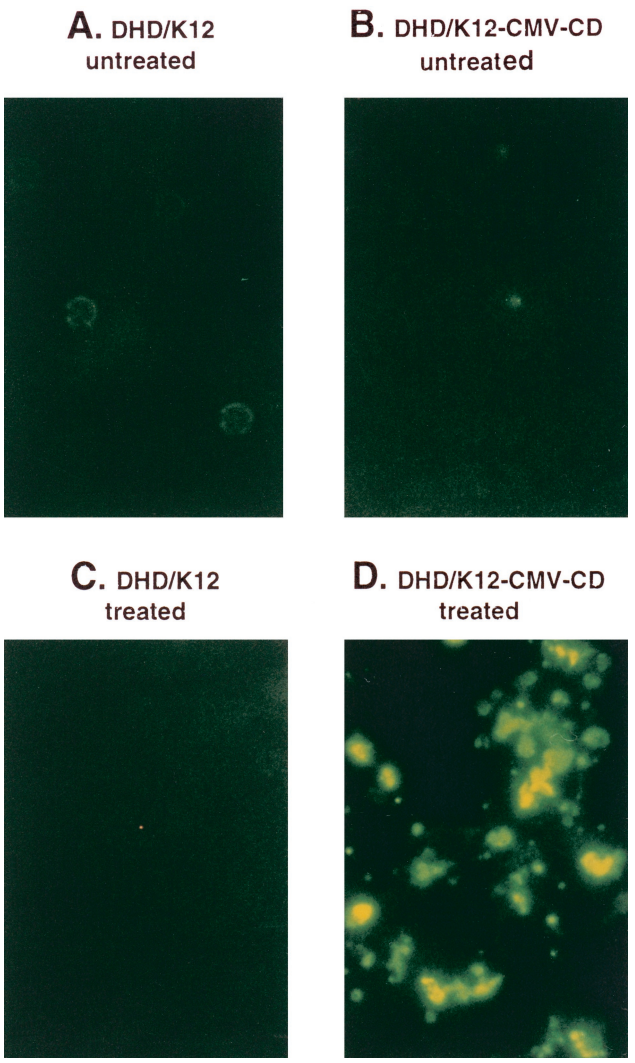

Figure 3. Induction of apoptosis by 5-FC in DHD/K12-CMV-CD cells. Untransfected DHD/K12 cells **(A,C)** or DHD/K12-CMV-CD cells **(B,D)** were left untreated or were treated with 5-FC (4 mM) for 48 hours. Treated cells were fixed, and apoptosis was evaluated by TUNEL.

clones, DHD/K12-RSV-CD and DHD/K12-CMV-CD, were incubated for 8 days in the presence of increasing concentrations of 5-FC. Measures of cell viability demonstrated that 5-FC concentrations ranging from 400 μM to 2 mM killed a large majority of the CD-transfected cells, whereas the viability of parental DHD/K12 cells was not affected (Fig 2A). Moreover, DHD/K12-CMV-CD clones were more sensitive to the prodrug than the DHD/K12-RSV-CD clones (Fig 2A), probably as a consequence of a better promoter activity.

To test whether a bystander effect could be observed *in vitro*, various proportions of DHD/K12-CMV-CD and untransfected DHD/K12 cells were cocultured and challenged for 8 days with two different 5-FC concentrations, which killed 100% of CD-expressing cells but did not affect parental cells (Fig 2B). Under these experimental conditions, the presence of 20% of DHD/K12-CMV-CD cells was sufficient to induce a cytotoxic effect that killed 79% and 92% of the cells after incubation with 1 mM and 2 mM of 5-FC, respectively. These data also confirmed that the survival of untransfected DHD/K12 cells was unaffected by the 5-FC treatment.

Induction of apoptosis by 5-FC in DHD/K12-CD cells

We investigated whether the treatment of DHD/K12-CMV-CD cells with 5-FC induced cell death through apoptosis. Untransfected DHD/K12 and DHD/K12-CMV-CD cells were incubated for 48 hours with or without 5-FC (4 mM), fixed, and analyzed for apoptosis by TUNEL. Under these conditions, we did not observe any significant apoptosis in untransfected DHD/K12 cells treated with such a high 5-FC concentration or left untreated, or in the DHD/K12-CMV-CD cells incubated with the medium alone (Fig 3, A–C). However, the DHD/K12-CMV-CD cells displayed a significant apo-

Table 1. *In Vitro* CD-Induced Cytotoxicity and Bystander Effect

Transfection (5-FC)	Transfected cells (%)	Cell viability (%)			
		pcDNA3 (+)	CD20 (+)	CD20 + CD (−)	CD20 + CD (+)
HCT116	0.7	100	>100	90.2	7.8
MDA-MB-435	0.7	100	78.9	93.7	67.2
9 L	3	100	81.8	>100	5.1
OVCAR-3	10.5	100	>100	84.6	5.5

* HCT116, MDA-MB-435, 9 L, and OVCAR-3 cells were transiently transfected with expression vectors coding for the CD20 antigen, the CD enzyme, or a combination of both, or with the pcDNA3 empty expression vector. Transfection efficiencies were determined by FACS analysis of the percentages of CD20+ cells after transfection of the CD20 expression vector. These percentages of transfected cells were reproducible and were confirmed by two independent experiments. Transfected cells were then treated with 2 mM of 5-FC (+) for 96 hours or left untreated (−), and cell viability was measured with trypan blue. Cell viabilities were expressed as percentages of living cells compared with cells transfected with the control empty vector and treated with 5-FC. Data are representative of two independent experiments.

ptosis after 5-FC treatment, as shown by the high number of fragmented, fluorescent nuclei (Fig 3D).

In vitro *cytotoxicity and bystander effect with the* CD *suicide gene in adenocarcinoma cell lines*

Peritoneal carcinomatosis most frequently arises from digestive or ovarian carcinomas, and many of these tumors respond poorly to chemotherapy. Therefore, we investigated whether other cell lines derived from human colon (HCT116), ovarian (OVCAR-3), or breast (MDA-MB-435) carcinomas were also sensitive to the *CD* gene/5-FC cytotoxic effect. The 9 L rat glioblastoma cell line, known for its response to the HSV-*tk* suicide gene and its high bystander effect, was also included in this assay. The cell lines mentioned above were transiently transfected with the *CD* suicide gene and subsequently treated with 5-FC for 48 or 96 hours. The efficacy of the transient transfections was assessed by cotransfection of an expression vector coding for the B-cell-specific CD20 surface antigen and FACS counting of $CD20^+$ cells. Transfection efficiencies were low; only 0.7% of HCT116 cells, 0.7% of MDA-MB-435 cells, 3% of 9 L cells, and 10.5% of OVCAR-3 cells were positive for CD20 expression (Table 1). Despite this low transfection rate, significant cytotoxicity was observed in three of the four cell lines after transient transfection of the *CD* gene and 5-FC treatment for 48 hours (data not shown) or 96 hours (Table 1). Indeed, when compared with control cells transfected with the CD20 antigen expression vector alone or with an empty pcDNA3

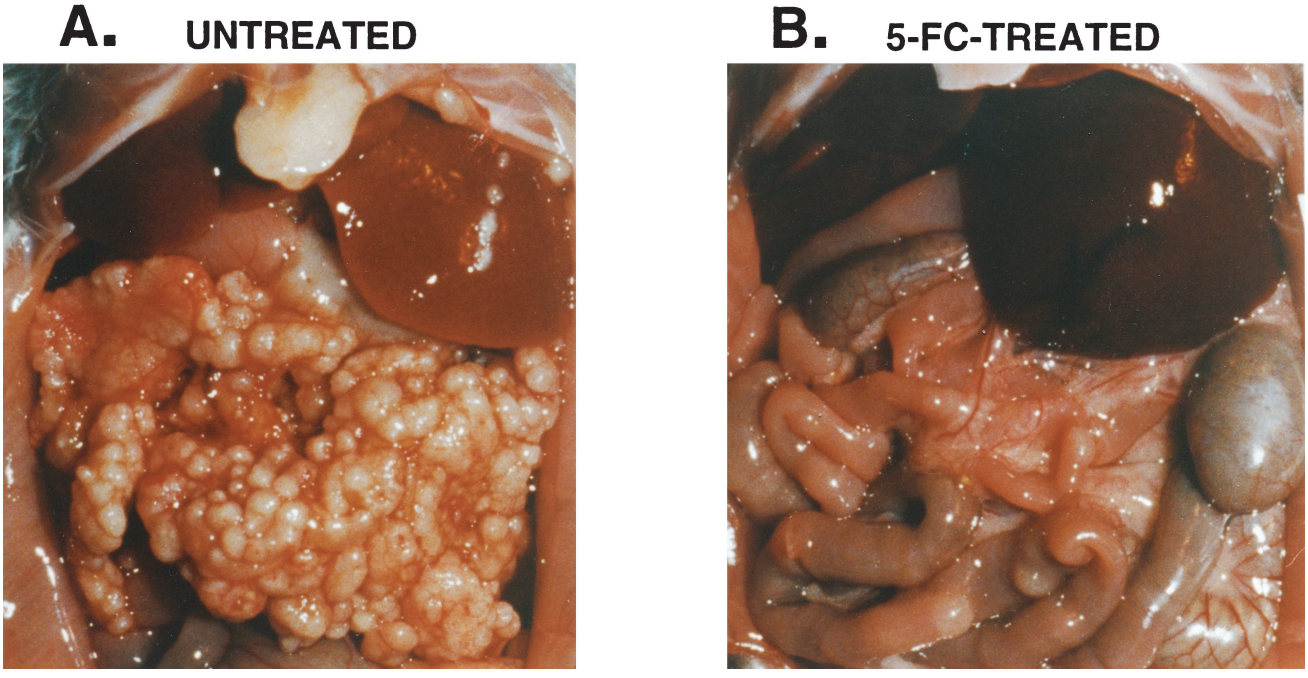

Figure 4. 5-FC treatment of rats injected with DHD/K12-CMV-CD cells. The figure shows the peritoneal cavity at day 36 of a rat injected with DHD/K12-CMV-CD cells and treated with normal saline buffer **(A)** and of a rat injected with DHD/K12-CMV-CD cells and treated with 5-FC (500 mg/kg/day) for 3 weeks starting at day 14 **(B)**. This experiment was performed on groups of two rats, and a representative picture is shown.

vector, CD-transfected HCT116, 9 L, or OVCAR-3 lines showed only 7.8%, 5.1%, and 5.5% of surviving cells, respectively, after treatment with 5-FC (Table 1). However, this cytotoxic effect was much less important in the MDA-MB-435 cell line, as 67% of cells were still alive after *CD* transfection and 5-FC treatment. Consequently, these data indicated that the *CD* gene is efficient *in vitro* against several cell lines other than DHD/K12, including colon and ovarian carcinoma cell lines, and that a significant bystander effect can be observed *in vitro*.

In vivo *response of DHD/K12-CD cells to 5-FC*

To demonstrate the *in vivo* feasibility of a *CD*-based suicide gene therapy, we used our model of peritoneal carcinomatosis induced by DHD/K12 cells in syngeneic BDIX rats. As described previously, injection of 10^6 DHD/K12 cells in the peritoneal cavity of these rats led to the development of macroscopic peritoneal tumor nodes within 10 days; all of the animals died of extensive peritoneal carcinomatosis before day 70.[1,2]

In an initial experiment, groups of two rats were injected either with untransfected DHD/K12 cells or with DHD/K12-CMV-CD cells. These animals were treated for 3 weeks (days 14–18, 21–25, and 28–32) with peritoneal injections of 5-FC (500 mg/kg/day) or normal saline buffer alone. The animals were sacrificed at day 36, and the reduction in tumor volume was assessed by direct abdominal examination. At day 36, all of the animals injected with untransfected DHD/K12 cells and treated with 5-FC or with saline buffer showed an extensive peritoneal dissemination (data not shown). Similarly, the two rats injected with DHD/K12-CMV-CD cells and treated for 3 weeks with saline buffer displayed a large number of tumor nodes disseminated in their peritoneal cavity (Fig 4A). However, animals injected with DHD/K12-CMV-CD and treated with 5-FC displayed a complete regression of the peritoneal carcinomatosis (Fig 4B).

To demonstrate whether this apparent excellent tumor response to CD/5-FC treatment translated into survival advantages, animals were injected with 10^6 DHD/K12-CMV-CD cells and treated for 3 weeks from day 14 with 5-FC at 500 mg/kg/day; next, survival curves were established. Animals injected with DHD/K12-CMV-CD cells and treated with normal saline buffer alone died between days 45 and 71 (Fig 5), as did animals that had not been treated or animals injected with parental DHD/K12 cells and treated with 5-FC (data not shown). However, rats injected with DHD/K12-CMV-CD cells and treated with 5-FC for 3 weeks showed a significantly improved survival, as six of eight animals were still alive at day 360 (Fig 5, $P < .001$).

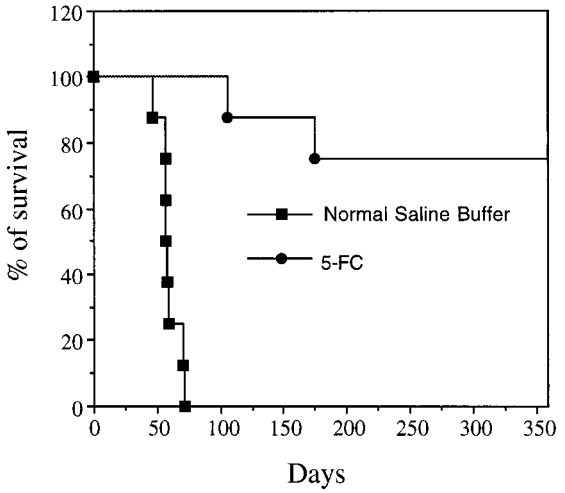

Figure 5. Survival of rats injected with DHD/K12-CMV-CD cells and treated with 5-FC. Two groups of eight rats were injected i.p. with 10^6 DHD/K12-CMV-CD cells and treated with saline buffer or with 5-FC (500 mg/kg/day) for 3 weeks starting at day 14. Survival curves for each group are shown. Six rats from the 5-FC-treated groups were still alive at day 360.

DISCUSSION

We had previously reported the efficacy of HSV-*tk* suicide gene therapy for peritoneal carcinomatosis.[1,2] The present report indicates that such a treatment could also be envisaged with the *E. coli CD* gene.

Interestingly, when animals are injected with cells transfected *in vitro*, the results obtained with the *CD* gene are better than those obtained with the *tk* gene. Indeed, after a peritoneal injection of TK-positive DHD/K12 cells and treatment with ganciclovir, the majority of the animals relapsed and died before day 140,[1] whereas in our model, the CD/5-FC approach apparently eradicated the tumor, or at least led to a prolonged remission in six of eight animals. These results confirm previous studies, which indicated a better cytotoxic effect of CD over TK *in vitro* or *in vivo*.[22,28] This superiority might be explained by a better bystander effect *in vivo*. The expression of a stably transfected gene might be very heterogenous, and some cells often loose this expression. We had shown previously that, after injection of TK-positive cells and treatment with ganciclovir, relapsing tumors expressed very low levels of the *tk* gene.[1] A strong bystander effect would allow a better eradication of tumor cells that have lost the transgene expression, and thus a more favorable outcome of the animals. However, the superiority of the *CD* gene over *tk* in our model might also simply be explained by vector differences. The *tk* gene had been inserted in a retroviral vector, and its expression was driven by the simian virus 40 promoter, whereas the *CD* gene was cloned in a CMV expression vector before stable transfection. The CMV promoter is probably stronger than the simian virus 40 promoter, and therefore allowed a better expression of the suicide gene. Moreover, our *in vitro* data with the Rous sarcoma virus and CMV promoters indicated that a better promoter led to a stronger bystander effect (data

not shown). These differences indicated that a careful choice of the promoter is critical for a better efficiency of *in vivo* gene therapy.

The *CD* gene allowed a very strong *in vitro* bystander effect with several cell lines, including ovarian and colorectal carcinoma cell lines and the well-studied glioblastoma 9 L cell line. These results confirmed that the *CD* gene could be a powerful tool for *in vivo* cancer gene therapy. The bystander effect generated by the CD/5-FC system is generally thought to be related to a diffusion of 5-FU from CD^+ to CD^- cells and has been reported to be very strong in different models.[22,28–30] However, it has been suggested recently that gap junctions could also play a role in this bystander effect,[31] indicating that the CD-generated bystander effect might be caused by several distinct mechanisms. Interestingly, in our set of cell lines, MDA-MB-435 cells do not show any bystander effect with the *tk* suicide gene[32] and do not communicate through gap junctions (F.P. et al, unpublished observations). Moreover, these cells are also sensitive to 5-FU (data not shown), and their poor response to 5-FC/CD is therefore not related to an intrinsic resistance to the cytotoxic drug. A strong bystander effect is required to observe any clinical benefit following *in vivo* gene transduction, as only a small proportion of tumor cells can be reached following *in vivo* treatment with viral or liposomal vectors. Our *in vitro* data thus suggest that *CD* gene therapy might be efficient on a number of cell types, including lines derived from ovarian and digestive neoplasms that are the most common cause of peritoneal carcinomatosis. However, some tumors might be resistant, and the identification of the mechanisms for such resistance is most important to design appropriate clinical trials.

An immunological reaction might also participate in the *in vivo* bystander effect and in the eradication of tumors after treatment with CD/5-FC. Indeed, it has been reported in other models that an immune response is required for the eradication of CD^+ tumors, and that the CD/5-FC therapy induces a protective immunity.[33,34] As our animals are immunocompetent and able to develop an immune reaction against cytokine-expressing DHD/K12 cells (C.L. et al, unpublished observations), it is possible that an immune reaction is involved in the observed favorable outcome of treated animals.

Despite these encouraging results, it becomes clear from a number of reports that therapy with a single suicide gene will often lead to partial, and sometimes minor, responses. A combination of several genes should increase the efficiency of the therapy. Given the low toxicity of the suicide genes, it is certainly conceivable to combine several suicide genes such as the HSV-1-*tk* gene and the *E. coli CD* gene.[35–38] Also, several teams are now evaluating a combination of a suicide gene with cytokine genes to boost the immunological component of the bystander effect.[4,39,40] The present model, as described in this paper and in previous publications,[1,2,4] is based on immunocompetent animals and is suitable for the evaluation of such therapies with several genes.

ACKNOWLEDGMENTS

We thank Dr. J. Gebert for the pRSV-CD vector, Dr. Haack for the CD antibody, and Dr. Jim Koh for the pCMV-CD20 vector. We are most thankful to J-P. Cheramibien and G. Rocoux for their help with the *in vivo* experiments, K. Bajou and F. Kebers for the TUNEL protocol, N. Jacobs and R. Greimers for the FACS analysis, and W. Dewé for his help with statistical analysis. A.-C.H. is supported by a Télévie fellowship and F.P. is supported by an FRIA fellowship. V.B. and M.-P.M. are Research Associates at the National Fund for Scientific Research (Belgium). This research was supported by grants from Télévie, from the "Centre Anti-Cancéreux" (Liège, Belgium), and from "Concerted Action Program, convention 97/02-214," Communauté Française de Belgique.

REFERENCES

1. Lechanteur C, Princen F, Lo Bue S, et al. HSV-1 thymidine kinase gene therapy for colorectal adenocarcinoma-derived peritoneal carcinomatosis. *Gene Ther.* 1997;4:1189–1194.
2. Princen F, Lechanteur C, Lopez M, et al. Repeated cycles of retrovirus-mediated HSV-TK gene transfer plus ganciclovir increase survival of rats with peritoneal carcinomatosis. *Gene Ther.* 1998;5:1054–1060.
3. Aoki K, Yoshida T, Sugimura T, et al. Liposome-mediated in vivo gene transfer of antisense K-ras construct inhibits pancreatic tumor dissemination in the murine peritoneal cavity. *Cancer Res.* 1995;55:3810–3816.
4. Coll J-L, Mesnil M, Lefebvre M-F, et al. Long-term survival of immunocompetent rats with intraperitoneal colon carcinoma tumors using herpes simplex thymidine kinase/ganciclovir and IL-2 treatments. *Gene Ther.* 1997; 4:1160–1166.
5. Smythe WR, Kaiser LR, Hwang HC, et al. Successful adenovirus-mediated gene transfer in an in vivo model of human malignant mesothelioma. *Ann Thorac Surg.* 1994; 57:1395–1401.
6. Smythe WR, Hwang HC, Elshami AA, et al. Treatment of experimental human mesothelioma using adenovirus transfer of the herpes simplex thymidine kinase gene. *Ann Surg.* 1995;222:78–86.
7. Freeman SM, Whartenby KA, Freeman JL, et al. In situ use of suicide genes for cancer therapy. *Semin Oncol.* 1996;23:31–45.
8. Moolten FL, Wells JM, Heyman RA, et al. Lymphoma regression induced by ganciclovir in mice bearing a herpes thymidine kinase transgene. *Hum Gene Ther.* 1990;1:125–134.
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 KW, Walbridge S, et al. *In situ* retroviral-mediated gene transfer for treatment of brain tumors in rats. *Cancer Res.* 1993;53:83–88.
11. Rainov NG, Kramm CM, Aboody-Guterman K, et al. Retrovirus-mediated gene therapy of experimental brain neoplasms using the herpes simplex-thymidine kinase/ganciclovir paradigm. *Cancer Gene Ther.* 1996;3:99–106.
12. Oldfield EH, Ram Z, Culver KW, et al. Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. *Hum Gen Ther.* 1993;4:39–69.
13. Ram Z, Culver KW, Walbridge S, et al. Toxicity studies of

retroviral-mediated transfer for the treatment of brain tumors. *J Neurosurg.* 1993;79:400–407.
14. Kun LE, Gajjar A, Muhlbauer M. Stereotaxic injection of herpes simplex thymidine kinase vector producer cells (PA317–G1Tk1SvNa.7) and intravenous ganciclovir for treatment of progressive or recurrent primary supratentorial pediatric malignant brain tumors. *Hum Gene Ther.* 1995;6:1231–1255.
15. Austin EA, Huber BE. A first step in the development of gene therapy for colorectal carcinoma: cloning, sequencing, and expression of *Escherichia coli* cytosine deaminase. *Mol Pharmacol.* 1993;43:380–387.
16. Rowley S, Lindauer M, Gebert JF, et al. Cytosine deaminase gene as a potential tool for the genetic therapy of colorectal cancer. *J Surg Oncol.* 1996;61:42–48.
17. Ohwada A, Hirschowitz EA, Crystal RG. Regional delivery of an adenovirus vector containing the *Escherichia coli* cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum Gene Ther.* 1996;7:1567–1576.
18. Kanai F, Lan K-H, Shiratori Y, et al. In vivo gene therapy for α-fetoprotein-producing hepatocellular carcinoma by adenovirus-mediated transfer of cytosine deaminase gene. *Cancer Res.* 1997;57:461–465.
19. Evoy D, Hirschowitz EA, Naama HA, et al. In vivo adenoviral-mediated gene transfer in the treatment of pancreatic cancer. *J Surg Res.* 1997;69:226–231.
20. Lan K-H, Kanai F, Shiratori Y, et al. In vivo selective gene expression and therapy mediated by adenoviral vectors for human carcinoembryonic antigen-producing gastric carcinoma. *Cancer Res.* 1997;57:4279–4284.
21. Li Z, Shanmugam N, Katayose D, et al. Enzyme/prodrug gene therapy approach for breast cancer using a recombinant adenovirus expressing *Escherichia coli* cytosine deaminase. *Cancer Gene Ther.* 1997;4:113–117.
22. Trinh QT, Austin EA, Murray DM, et al. Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. *Cancer Res.* 1995;55:4808–4812.
23. Hirschowitz EA, Ohwada A, Pascal WR, et al. In vivo adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther.* 1995;6:1055–1063.
24. Huber BE, Austin EA, Good SS, et al. In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. *Cancer Res.* 1993;53:4619–4626.
25. Topf N, Worgall S, Hackett NR, et al. Regional "prodrug" gene therapy: intravenous administration of an adenoviral vector expressing the *E. coli* cytosine deaminase gene and systemic administration of 5-fluorocytosine suppresses growth of hepatic metastasis of colon carcinoma. *Gene Ther.* 1998;5:507–513.
26. Hirschowitz E, Lieberman M, Crystal RG. Phase I study of direct administration of a replication-deficient adenovirus vector containing the *E. coli* cytosine deaminase gene to metastatic colon carcinoma of the liver in association with the oral administration of the pro-drug 5-fluorocytosine. *Hum Gene Ther.* 1997;8:985–1001.
27. Martin F, Caignard A, Jeannin JF, et al. Selection by trypsin of two sublines of rat colon cancer cells forming progressive or regressive tumors. *Int J Cancer.* 1983;32:623–627.
28. Hoganson DK, Batra RK, Olsen JC, et al. Comparison of the effects of three different toxin genes and their levels of expression on cell growth and bystander effect in lung adenocarcinoma. *Cancer Res.* 1996;56:1315–1323.
29. Huber BE, Austin EA, Richards CA, et al. 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.
30. Lawrence TS, Rehemtulla A, Ng EY, et al. Preferential cytotoxicity of cells transduced with cytosine deaminase compared to bystander cells after treatment with 5-fluorocytosine. *Cancer Res.* 1998;58:2588–2593.
31. Mesnil M, Duflot-Dancer A, Piccoli C, et al. The bystander cell killing effect mediated by a combination of cytosine deaminase and uracil phosphoribosyl transferase genes *in vitro*: contribution of membrane diffusion and gap junctions. *Proc Am Assoc Cancer Res.* 1998;39:515.
32. Grignet-Debrus C, Calberg-Bacq C-M. Potential of *Varicella zoster* virus thymidine kinase as a suicide gene in breast cancer cells. *Gene Ther.* 1997;4:560–569.
33. Consalvo M, Mullen CA, Modesti A, et al. 5-Fluorocytosine-induced eradication of murine adenocarcinomas engineered to express the cytosine deaminase suicide gene requires host immune competence and leaves an efficient memory. *J Immunol.* 1995;154:5302–5312.
34. Mullen CA, Coale MM, Lowe R, et al. 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.
35. Aghi M, Kramm CM, Chou T-C, et al. Synergistic anticancer effects of ganciclovir/thymidine kinase and 5-fluorocytosine/cytosine deaminase gene therapies. *J Natl Cancer Inst.* 1998;90:370–380.
36. Blackburn RV, Galoforo SS, Corry PM, et al. Adenoviral-mediated transfer of a heat-inducible double suicide gene into prostate carcinoma cells. *Cancer Res.* 1998;58:1358–1362.
37. Freytag SO, Rogulski KR, Paielli DL, et al. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther.* 1998;9:1323–1333.
38. Uckert W, Kammertons T, Haack K, et al. Double suicide gene (cytosine deaminase and herpes simplex virus thymidine kinase) but not single gene transfer allows reliable elimination of tumor cells in vivo. *Hum Gene Ther.* 1998;9:855–865.
39. Chen S, Li Chen XH, Wang Y, et al. Combination gene therapy for liver metastasis of colon carcinoma *in vivo*. *Proc Natl Acad Sci USA.* 1995;92:2577–2581.
40. Nanni P, De Giovanni C, Nicoletti G, et al. The immune response elicited by mammary adenocarcinoma cells transduced with interferon-γ and cytosine deaminase genes cures lung metastases by parental cells. *Hum Gene Ther.* 1998;9:217–224.