Herpesvirus Saimiri Pathogenicity Enhanced by Thymidine Kinase of Herpes Simplex Virus

Christian Hiller,* Gültekin Tamgüney,* Nicole Stolte,† Kerstin Mätz-Rensing,† Dirk Lorenzen,† Simon Hör,* Mathias Thurau,* Sabine Wittmann,* Shimon Slavin,* and Helmut Fickenscher*†

*Institut für Klinische und Molekulare Virologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Schloßgarten 4, D-91064 Erlangen, Germany; †German Primate Center, Division of Virology and Immunology and Division of Primate Veterinary Medicine and Primate Husbandry, Kellnerweg 4, D-37077 Göttingen, Germany; and ‡Department of Bone Marrow Transplantation, Hadassah University Hospital, Jerusalem 91220, Israel

Received May 30, 2000; returned to the author for revision June 27, 2000; accepted September 24, 2000; published online November 16, 2000

Herpesvirus saimiri can be used as an efficient gene expression vector for human T lymphocytes and thus may allow applications in experimental leukemia therapy. We constructed recombinant viruses for the functional expression of the thymidine kinase (TK) of herpes simplex virus type 1 (HSV) as a suicide gene. These viruses reliably allowed the targeted elimination of transduced nonpermissive human T cells in vitro after the administration of ganciclovir. To test the reliability of this function under the most stringent permissive conditions, in this study we analyzed the influence of the produgs ganciclovir and acyclovir in common marmosets on the acute leukemogenesis induced by either wild-type herpesvirus saimiri C488 or by a recombinant derivative expressing TK of HSV. Antiviral drug treatment did not influence the rapid development of acute disease. In contrast, the presence of the HSV tk gene resulted in a faster disease progression. In addition, HSV TK-expressing viruses showed faster replication than wild-type virus in culture at low serum concentrations. Thus, HSV TK accelerates the replication of herpesvirus saimiri and enhances its pathogenicity. This should be generally considered when HSV TK is applied as a transgene in replication-competent DNA virus vectors for gene therapy. © 2000 Academic Press

Key Words: aciclovir; Callithrix jacchus; common marmosets; ganciclovir; herpes simplex virus; herpesvirus saimiri; pathogenicity; suicide gene; T-cell leukemia; thymidine kinase.

INTRODUCTION

Herpesvirus saimiri (Saimiriine herpesvirus type 2) is a γ2-herpesvirus (rhadinovirus) of South American squirrel monkeys (Saimiri sciureus) in which the virus persists without causing disease. However, in other New World monkey species such ascottontop tamarins (Saguinus oedipus) and in common marmosets (Callithrix jacchus), herpesvirus saimiri induces acute T-cell lymphomas leading to death within a few weeks after infection. The lymphoma is associated with massive virus replication and viral gene expression. The virus can be easily recovered by cocultivation of peripheral blood mononuclear cells (PBMCs) with permissive epithelial owl monkey kidney (OMK) cells (Daniel et al., 1976b; Duboise et al., 1998a; Knappe et al., 1998a,b).

After infection of human PBMC cultures with herpesvirus saimiri C488, the T lymphocytes are transformed to stable proliferation (Biesinger et al., 1992). Although growth transformed, the cells retain many essential functions of their nontransformed parental cells, including their MHC-restricted antigen specificity (Bröker et al., 1993; De Carli et al., 1993; Mittrücker et al., 1993; Weber et al., 1993). Stimulation induces cytotoxicity and the production of cytokines such as interferon-γ or tumor necrosis factor-α (TNF-α). Either CD4+ or CD8+ cells and either αβ-T or γδ-T cells can be transformed (Biesinger et al., 1992; Fickenscher et al., 1997; Klein et al., 1996; Pacheco-Castro et al., 1996; Yasukawa et al., 1995). In the growth-transformed human T lymphocytes that do not produce virion particles, only a few viral genes are expressed (Fickenscher et al., 1996; Knappe et al., 1997). One of them is the oncogene stpC, whose gene product interacts with cellular Ras and TNF-α receptor-associated factors, transforms rodent fibroblasts, and causes tumors in transgenic mice (Jung and Desrosiers, 1995; Jung et al., 1991; Lee et al., 1999; Murphy et al., 1994). Another one, Tip, is translated from the same bicistronic transcript and interacts specifically with the T-cell tyrosine kinase Lck (Biesinger et al., 1995). Both stpC and tip are necessary for T-cell transformation (Duboise et al., 1998a; Knappe et al., 1997).

Gene transfer into human T cells and long-term expression of transgenes in these cells remains difficult by using classic methods, including retroviral vectors. Herpesvirus saimiri has three major features that are promising for vector development for human T cells. First, the virus does not integrate into the cellular chromosomes. The virus genome persists episomally in the nonpermissive human T cells in a high and stable copy number that precludes problems arising from chromosomal integra-

---

1 To whom reprint requests should be addressed. Fax: 49-9131-85-26493. E-mail: fickenscher@viro.med.uni-erlangen.de.
tion such as insertional mutagenesis and silencing on chromatin inactivation (Biesinger et al., 1992; Fickenscher et al., 1996, 1997). Second, as mentioned, the virus strain C488 is able to transform human T cells to stable growth in culture, which facilitates the amplification of functional T cells (Biesinger et al., 1992). These growth-transformed T cells appear to be nonmutagenic; if large cell numbers of autologous transformed T lymphocytes had been reinfused into the donor macaques, the cells were well tolerated and persisted over several months without side effects and without the occurrence of lymphoma or leukemia (Knappe et al., 2000). Third, the virus genome can be used for the functional long-term expression of foreign genes in human T cells (Hiller et al., 2000). Although virion production has never been observed for herpesvirus saimiri-transformed human T cells, possible therapeutic applications should include additional safety mechanisms such as an active elimination function mediated by a suicide gene. Moreover, packaging cell-dependent and replication-deficient vector variants would be required to preclude any virus replication in vivo.

Vectors based on herpesvirus saimiri C488 might allow novel developments in T-cell-mediated immunotherapy. One possible application of such T-cell vectors could be for the leukemia therapy mediated by allogeneic T cells. Persisting or relapsing residual leukemic disease and other hematological disorders can be treated efficiently by adoptive immunotherapy with allogeneic T cells after stem-cell transplantation (Collins et al., 1997; Kolb and Holler, 1997; Kolb et al., 1990, 1995; Slavin et al., 1988, 1995, 1996). Although the T-cell-mediated graft-versus-leukemia effect is highly active against tumor cells, the allogeneic T-cell therapy is hampered by the frequent side effect of graft-versus-host disease, which can hardly be controlled by conventional therapy. In the case of such complications, suicide gene expression has been applied for the targeted elimination of the grafted T cells. The herpes simplex virus type 1 (HSV) gene thymidine kinase (tk) is the prototype suicide gene that has been widely used in experimental oncology. TK activates ganciclovir (GCV) to a toxic metabolite inducing cell death. By the use of retroviral vectors, the tk gene was transduced into the donor T cells before transplantation (Bonini et al., 1997; Bordignon et al., 1995; Contassot et al., 1998; Munshi et al., 1997; Tiberghien et al., 1994, 1997). In three patients with graft-versus-host disease, the administration of GCV eliminated the grafted T cells and stopped the acute clinical complications (Verzeletti et al., 1998).

Our aim was to adapt herpesvirus saimiri for a possible application in allogeneic T-cell therapy. For this purpose, the prodrug-activating tk gene of HSV was inserted into the genome of herpesvirus saimiri by homologous recombination. A series of recombinant viruses efficiently expressed the transgene during lytic infection of permissive epithelial cells and during persistent infection of nonpermissive human T cells. In contrast to wild-type virus, the replication of the tk mutant herpesvirus saimiri was sensitive to GCV or acyclovir (ACV) treatment in culture (Hiller et al., 2000). After infection with wild-type or recombinant viruses, human T cells were transformed to stable growth. Nonpermissive, TK-expressing transformed T cells were efficiently eliminated in the presence of low concentrations of GCV. This elimination mechanism remained fully functional over an observation period of 1 year (Hiller et al., 2000). At any time during the course of a therapeutic application, such tk-transduced nonpermissive human T cells might be eliminated after the administration of GCV to remove the transfused allogeneic cells in the case of unwanted side effects such as acute graft-versus-host disease.

To study the degree of the functional reliability of this suicide gene system, we chose one of the most stringent systems available, namely leukemia induction by herpesvirus saimiri in susceptible New World primates. In this report, we analyzed the previously characterized mutant TK-2 in comparison to the wild-type virus C488 in common marmosets for its susceptibility to antiviral chemotheraphy and for its pathogenicity. Our unexpected results indicate that antiviral drugs do not act rapidly enough to interfere with the massive virus replication during acute viral leukemogenesis. In addition, the tk transgene accelerated virus replication and disease progression.

RESULTS

Design of the infection and chemotherapy experiment

In comparison to the wild-type herpesvirus saimiri C488, the previously characterized HSV TK-expressing mutant virus TK-2 (Hiller et al., 2000) was tested in common marmosets (Callithrix jacchus) for its pathogenic properties and for its susceptibility to antiviral chemotheraphy by ACV and GCV. Ten healthy animals were intravenously infected either with C488 or with TK-2 at a tissue culture infectious dose (TCID)10 of 10^6 in 1 ml cell-free tissue culture supernatant. The high inoculum dose was applied to exclude limiting infection conditions. Drug therapy was performed at two different regimens: either early (days −3 to +14) or late (days +7 to +21) after infection. To ensure reliable drug concentrations, the drugs were subcutaneously injected in phosphate-buffered saline: ACV at a dosage of 30 mg/kg/d and GCV at a dosage of 15 mg/kg/d. This dosage is frequently used for other experimental and clinical applications. The individual treatment groups consisted of two animals each (Table 1).

Disease progression after infection

All infected animals developed signs of disease almost independently of the respective form of drug treat-
TABLE 1
Experimental Design and Results from Biopsy and Necropsy Materials

| Experimental conditions | Blood volume needed for virus isolation (µl) | Demonstration of virus DNA by tk- or orf75-specific PCR from T-cell lines established ex vivo from PBMC tk 75 PBMC d14 PBMC necropsy Thymus necropsy PBMC tk 75 PBMC d14 PBMC necropsy Thymus necropsy Survival time (days after infection) | Histological findings |
|-------------------------|---------------------------------------------|------------------------------------------------------------------------------------------------|--------------------|--------------------------------------------------|
| Blood volume needed     |                                             |                                                                                               |                    | Organ with lymphoma nodules                      |
| Animal                  |                                             |                                                                                               |                    |                                                  |
| Virus                   |                                             |                                                                                               |                    |                                                  |
| Drug                    |                                             |                                                                                               |                    |                                                  |
| Therapy period          |                                             |                                                                                               |                    |                                                  |
|                         | d7                                          |                                                                                               |                    |                                                  |
|                         | d14                                         |                                                                                               |                    |                                                  |
|                         | Necropsy                                   |                                                                                               |                    |                                                  |
| 8596                    | C488                                        |                                                                                               |                    |                                                  |
| 8532                    | ACV                                         |                                                                                               |                    |                                                  |
| 8598                    | Late                                        |                                                                                               |                    |                                                  |
| 8607                    | >100                                        |                                                                                               |                    |                                                  |
| 8569                    | >100                                        |                                                                                               |                    |                                                  |
| 8719                    | >100                                        |                                                                                               |                    |                                                  |
| 8632                    | >100                                        |                                                                                               |                    |                                                  |
| 8736                    | >100                                        |                                                                                               |                    |                                                  |
| 8666                    | >100                                        |                                                                                               |                    |                                                  |
| 8809                    | TK-2                                        |                                                                                               |                    |                                                  |
| 8533                    | >100                                        |                                                                                               |                    |                                                  |
| 8565                    | >100                                        |                                                                                               |                    |                                                  |
| 8564                    | >100                                        |                                                                                               |                    |                                                  |
| 8818                    | >100                                        |                                                                                               |                    |                                                  |
| 8821                    | >100                                        |                                                                                               |                    |                                                  |
| 8817                    | >100                                        |                                                                                               |                    |                                                  |
| 8767                    | >100                                        |                                                                                               |                    |                                                  |
| 8720                    | >100                                        |                                                                                               |                    |                                                  |
| 8742                    | >100                                        |                                                                                               |                    |                                                  |

>100, 100 µl blood was not sufficient for virus isolation.
The onset of disease was rapid, leading to apathy and pronounced diarrhea within a few hours. Necropsy was performed a few hours after the onset of symptoms. Although relevant drug-related differences were not encountered, the mutant virus TK-2 induced a more rapid disease progression (Fig. 1).

On necropsy and subsequent histological examination, all animals showed similar signs of peripheral T-cell}

FIG. 1. Survival curve of infected animals. Common marmosets were intravenously infected with $10^6$ TCID$_{50}$ of either wild-type virus C488 or mutant virus TK-2. Animals infected with TK-2 developed acute disease almost synchronously at 15–16 days postinfection (dpi). In comparison, the onset of disease was delayed after wild-type virus infection. The animal numbers are indicated in the survival curve.

FIG. 2. Histopathological findings. The tissue sections were fixed in Formalin and stained with hematoxylin/eosin. The photographs were taken at 376× magnification. (A) Periportal infiltration of lymphoblasts in the liver. The infiltrate is mixed with eosinophilic granulocytes (liver, animal 8532). (B) Granulomatous infiltration of lymphoblasts in the liver. Several mitotic figures are marked with arrows. Again, the infiltrate is mixed with eosinophilic granulocytes (liver, animal 8532). (C) Severe granulomatous enteritis with mixed infiltrate and massive crypt abscess formation (duodenum, animal 8532). (D) Infiltrate composed of blast-like lymphocytes and granulocytes beneath the epithelium demonstrating the epitheliotropic character of the infiltration (ductus epididymidis, animal 8564).
lymphoma (Figs. 2A–2D). These consisted of marked lymphoproliferative alterations, primarily in the form of perivascular and interstitial lymphoid cell infiltrations with pleomorphic, blast- and mitosis-rich cell infiltrates, which also contained granulocytic, mainly eosinophilic cells (Figs. 2A and 2B). The lymphoid cell proliferations consisted of medium-sized to large blasts with vesicular nuclei and prominent nucleoli. Within the lymphoid cell proliferations, numerous mitoses with occasional atypical patterns were present (Fig. 2B). Such cell infiltrates were found in various stages in nearly all organs of all animals. The infiltrates were most pronounced in the liver, kidney, and spleen. Additional severe infiltrations were detectable in all organs with epithelial components, including bile ducts and gallbladder, pancreatic ducts, epididymis ducts (Fig. 2D), saliva ducts, and areas of trachea and bronchi, and they resulted in severe inflammatory alterations. The lung parenchyma itself was less affected.

Characteristic alterations of the lymphatic system were apparent in differing severity in all animals. Lymph nodes tended to be enlarged and showed an expansion of sinal areas and enlargement of T-cell zones in paracortical areas, which can be attributed to the lymphoproliferative blast-like cell infiltrates described above. Corresponding lymphoproliferative cell infiltrates were also present in the gastrointestinal tract, primarily in the submucosal areas and radiating from there (Fig. 2C). All animals showed a gradual decrease in gastrointestinal alterations from proximal to distal, with cecum and colon generally showing the least alterations. The central nervous system also showed alterations caused by infiltrates, although they were less pronounced. In conclusion, all animals showed diffuse systemic lymphoproliferative organ infiltration.

The alterations were classified as low, medium, and severe, according to the severity of the histological changes. Obvious large tumors were not observed, although nodular, tumor-like cell aggregates were beginning to form in individual animals (Table 1). The morphological changes in the TK-2-infected animals were of intermediate severity and were not influenced by drug therapy. In contrast, the extent of histological anomalies was generally reduced by any form of drug treatment in the wild-type virus-infected animals. However, in many wild-type virus-infected animals (7 of 10), a formation of tumor nodules was encountered. This was hardly found in mutant virus-infected monkeys (1 of 10).

Monitoring of infection and leukemogenesis

Blood samples were taken before infection, at weekly intervals (days +7 and +14), and on necropsy. The blood samples were tested for virus load by cocultivation of permissive OMK cells with 3, 10, 30, and 100 μl whole blood (Table 1). Blood samples drawn after 1 week from
mutant virus-infected animals (4 of 10) already released virus, whereas this was not the case at day 7 after wild-type virus infection. Two weeks after infection, all mutant virus-infected marmosets had high virus load, whereas only 5 of 10 wild-type virus-infected animals released measurable amounts of virus from their blood cells. At the late stage of disease (day 14), 3 ml blood was sufficient for virus isolation in all mutant virus-infected monkeys, but only in 3 of 10 wild-type virus-infected animals.

Primary cells were purified from blood and various organs (thymus, spleen, and lymph nodes). Samples of these fresh cell preparations were used for the direct demonstration of virus DNA by PCR. Both the mutant and the wild-type virus DNAs were detected with a PCR for the viral structural gene orf75 (primers HF596 and HF597). The mutant virus DNA was specifically demonstrated with a PCR for the HSV tk gene (primers HF344 and HF370). Further samples of freshly isolated cells were cocultivated with permissive OMK cells to reisolate the virus. Other samples were cultivated in complete T-cell medium without interleukin-2 to isolate lymphoma cell lines. In each individual case, the virus reisolation and the generation of tumor cell lines were successful from tissue or blood samples taken during necropsy.

In almost all mutant virus-infected animals, virus DNA was detectable in PBMCs from day 7 postinfection (Table 1, Fig. 3). In contrast, virus DNA was detectable in PBMCs from wild-type virus-infected animals at day 14 for the first time. Virus DNA was demonstrated in PBMC samples of all terminally sick animals. Stably proliferating T-cell lines were isolated from late PBMC and necropsy tissue samples (thymus, spleen, and lymph nodes). The resulting T-cell lines were genotypically confirmed by orf75- and tk-specific PCR (Fig. 3, lower panels). The respective virus strain, wild type or mutant, was reisolated by cocultivating fresh cells from PBMCs or organs from necropsy samples in each individual case. The genotype of these viruses was confirmed by PCR and Southern blot hybridization of PCR products.

Reisolated viruses at TCID 50 of 10^6/ml were tested for drug sensitivity by end point titration in the presence of increasing concentrations of GCV. Although wild-type virus C488 did not react significantly, there was a strong reduction in virus titers of the reisolated TK-2 virus in presence of GCV (Table 2). Thus the TK-2 virus did not escape the drug treatment by inactivation of the tk gene.

Enhanced replication of the TK-2 mutant virus in vitro

To confirm these observations in vitro, the replication of the wild-type C488 virus and the TK expression mutant was compared in OMK cells in presence of either 2% or 10% FBS (Fig. 4). Subconfluent cells (175 cm^2, 50-ml volume) were infected with equal titers of either virus (250 ml of 10^4 TCID 50/ml). Every other day, 1-ml samples were taken from the supernatants of the infected cultures. These samples were serially diluted and tested for their limiting dilution titer each in six parallel cultures in 48-well plates. During the first 6 days, the mutant virus replicated faster than the wild-type virus under reduced serum concentrations (2% FBS), resulting in a titer differ-

### Table 2

<table>
<thead>
<tr>
<th>Animal</th>
<th>Virus</th>
<th>Drug treatment</th>
<th>Therapy period</th>
<th>GCV (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8596</td>
<td>C488</td>
<td>None</td>
<td>None</td>
<td>&gt;10^3</td>
</tr>
<tr>
<td>8532</td>
<td>C488</td>
<td>None</td>
<td>None</td>
<td>&gt;10^3</td>
</tr>
<tr>
<td>8809</td>
<td>TK-2</td>
<td>None</td>
<td>None</td>
<td>&gt;10^3</td>
</tr>
<tr>
<td>8533</td>
<td>TK-2</td>
<td>None</td>
<td>None</td>
<td>&gt;10^3</td>
</tr>
<tr>
<td>8817</td>
<td>TK-2</td>
<td>GCV</td>
<td>Early</td>
<td>&gt;10^3</td>
</tr>
<tr>
<td>8700</td>
<td>TK-2</td>
<td>GCV</td>
<td>Early</td>
<td>&gt;10^3</td>
</tr>
<tr>
<td>8720</td>
<td>TK-2</td>
<td>GCV</td>
<td>Late</td>
<td>&gt;10^3</td>
</tr>
</tbody>
</table>

Titers in pfu/ml.

![Graph](image1.png)

**Fig. 4.** Replication of wild-type and recombinant viruses. Permissive OMK cells were infected with equal titers (TCID 50 = 10^4/ml) of either wild-type C488 or tk-mutant TK-2 virus in presence of either 2% or 10% FBS. Every second day, samples of the supernatants were titrated on subconfluent OMK layers in microtiter plates. Early after infection, the mutant virus had an obvious replication advantage, which was even more pronounced under low FBS concentrations.
ence of up to 100-fold. Under conventional 10% FBS concentration, the replication advantage was detectable for 4 days only (Fig. 4). Finally, all of the parallel cultures yielded a titer of $10^6$/ml.

DISCUSSION

In common marmosets (*Callithrix jacchus*), the TK-expressing mutant virus TK-2 (Hiller et al., 2000) was tested in comparison to the wild-type herpesvirus saimiri C488 for pathogenicity and for sensitivity to antiviral chemotherapy. Despite antiviral treatment with ACV or GCV, all animals developed acute disease after infection and were finally subjected to necropsy (Fig. 1). The lack of drug effects was not due to escape mutation of the *tk* gene, because the reisolated viruses remained strongly susceptible to GCV treatment in vitro (Table 2). Histologically, all animals showed signs of acute peripheral T-cell lymphomas (Fig. 2). When we were planning the experiment, we expected TK expression to allow control of the mutant virus-induced lymphomas by activation of GCV to the toxic derivative. The unusually rapid progression of herpesvirus saimiri-induced T-cell lymphoma-like lesions in common marmosets seems to be the reason for the unexpected outcome. In contrast to the rapid lymphoma development, the therapeutic effects of GCV require at least 1 week of treatment in most in vitro and in vivo studies (Bush et al., 1998; Cohen et al., 1997; Munshi et al., 1997). In this context, it is conceivable that the spread of infection is much more rapid in this system than the slow onset of antiviral activity of GCV in the individual cell. Only in the wild-type virus-infected animals 8632 and 8736 does it remain possible that GCV at the early time regimen had some limiting effect in postponing the onset of disease. On histological analysis, the severity of disease was reduced by drug treatment of the wild-type virus-infected animals (Table 1). This difference was not observed with the mutant virus.

Surprisingly, all of the TK-2 mutant virus-infected animals showed an accelerated disease progression (Fig. 1). The results of virus isolations, tumor cell cultivations, and DNA tests indicated that the mutant virus had a replication advantage, at least during the early phase of infection. In comparison to the wild-type virus, the TK-2 mutant virus DNA was detectable significantly earlier after infection in PBMCs. In addition, tumor cell lines could be grown from day-7 PBMC samples in the case of the mutant virus only (Table 1). Thus the suicide gene obviously provided an advantage for the mutant virus replication in the early phase of infection. Early studies had already shown that the *tk* gene was required for HSV replication under reduced serum concentrations, while it was dispensable under high serum concentrations in actively dividing cells (Jamieson et al., 1974). The infection of mice with *tk* deletion mutants of HSV leads to latent, nonpermissive infection of nondividing cells such as neurons. A reactivation of the mutant virus from latent infection was achieved only if the deletion had been repaired (Efstatthiou et al., 1989). This indicates that TK activity is a relevant factor for replication and secondarily for pathogenicity if there are suboptimal replication conditions such as low concentration of growth factors, reduced mitosis rate, or poor nucleotide availability.

In tissue culture, the TK-2 virus mutant had been shown to be much more sensitive to ACV or GCV treatment in comparison to wild-type virus when tested for limiting dilution titers. In this case, GCV induced up to 1000-fold repression of virus replication (Hiller et al., 2000). Similar inhibition factors were observed in the presence of GCV with recombinant viruses reisolated from TK-2-infected animals (Table 2). In the early phase of infection, however, the *tk*-expressing mutant replicated in cell culture more rapidly compared with wild-type virus (Fig. 4). Under low serum concentrations, the titer difference was even more pronounced. In the late phase of replication, both strains reached similar titers. The availability of nucleotides is a limiting factor for DNA synthesis. TK is a key enzyme in the pyrimidine salvage pathway of nucleotide biosynthesis. Thus HSV TK expression leads to increased intracellular levels of TTP, which facilitate viral DNA replication. The TK activity and the susceptibility to antiviral drugs were transferred from varicella zoster virus to HSV by the transfer of the *tk* gene (Bevilacqua et al., 1995). In comparison to HSV TK, the endogenous TK activity of herpesvirus saimiri and closely related rhadinoviruses seems to be much weaker and has been assigned to the genes *orf21* and *orf36* (Albrecht et al., 1992; Cannon et al., 1999; Cazaux et al., 1998; Hiller et al., 2000). Thus the insertion of the HSV *tk* gene into herpesvirus saimiri would result in an increased TK activity in infected cells, which would explain enhanced virus replication and, subsequently, enhanced pathogenicity.

In our experiments with monkeys, a single TK-expressing mutant was compared with wild-type virus to keep the animal number as small as possible. The enhanced replication and pathogenesis were likely to be due to TK expression. However, it cannot be formally excluded that secondary effects unrelated to TK expression might cause the different phenotype. This could be addressed by testing additional *tk* virus mutants and revertants for their pathogenic potential to formally prove that the phenotype is caused by the *tk* gene. Such additional experiments would be standard and feasible if smaller, less-developed animals such as mice could be used for this purpose. However, in this case the additional virus mutants would have to be tested for pathogenesis in primates. Such experiments have to meet high ethical standards, and the applications for primate experiments are carefully reviewed for their expected scientific benefit. We decided not to perform such additional tests, because the aim would be mainly confirmatory and the
scientific benefit would be questionable based on our detailed characterization of the viruses in culture before and after the animal experiment. We previously tested a series of independent but similar tk mutants concerning their TK protein expression, their lytic replication, and their GCV sensitivity in simian and human T cells, with similar results for different independent mutants (Hiller et al., 2000). Moreover, we have tested the replication of the reisolated viruses for their sensitivity to GCV and observed that it was fully preserved in the mutant while poor in the wild type (Table 2). Thus it is excluded that the tk mutant virus lost TK expression by escape mutation.

In our studies, we used the promoter of the housekeeping gene phosphoglyceroyl kinase-1 (pgk-1) for transgene expression (Hiller et al., 2000). This promoter has been reliable in human T cells using either herpesvirus saimiri or retrovirus vectors (Cooper et al., 2000; Follenzi et al., 2000; Hiller et al., 2000). In another study, the human cytomegalovirus (HCMV) immediate-early enhancer had been applied for driving transgene expression from a herpesvirus saimiri vector (Stevenson et al., 2000a). In that case, pluripotent murine embryonal stem cells were transduced. During differentiation of these cells, a differential transgene expression was encountered. These observations are in line with a previous series of reports on mosaic-like expression from the HCMV enhancer in transgenic animals (Baskar et al., 1996a,b; Furth et al., 1991; Koedood et al., 1995). In contrast, the pgk-1 promoter has also been used frequently and reliably in transgenic applications with ubiquitous transgene expression. In human T cells transduced with herpesvirus saimiri vectors, this promoter has yielded strong and long-term transgene expression (Hiller et al., 2000, and unpublished data).

In contrast to the nonpermissive episomal persistence of herpesvirus saimiri genomes in human T lymphocytes, the leukemogenesis induced by herpesvirus saimiri in neotropical monkeys is characterized by massive virus replication and strong viral gene expression. T cells from infected New World monkeys are semipermissive: they are transformed and produce virus particles at the same time. Moreover, herpesvirus saimiri also replicates under systemic infection conditions in other cell types, presumably in a broad spectrum of cells, including epithelia and connective tissue, and possibly also in endothelial cells. Replication of herpesvirus saimiri in cell culture is well known for epithelial kidney cells such as Vero and OMK (Daniel et al., 1976b) and for the pancreatic carcinoma cell line Panc-1 (Simmer et al., 1991; Stevenson et al., 2000b). A lung carcinoma cell line was also shown to support inducible virus production (Hall et al., 2000). Moreover, various primary culture conditions from canine lung or human fetal tissues have yielded infectious virus (Daniel et al., 1976a; Melendez et al., 1975). Although only few virus genes such as stpC and tip are expressed in nonpermissive human T cells, a broad variety of virus genes is transcribed during lytic replication, including the endogenous tk gene of herpesvirus saimiri (Fickenscher et al., 1996; Hiller et al., 2000). In vitro, wild-type and tk recombinant virus-infected neotropical T-cell lines were highly sensitive to GCV, obviously due to the endogenous TK function of herpesvirus saimiri. In contrast, wild-type virus replication was poorly susceptible to GCV treatment in culture. This again supports our hypothesis that rapid virus replication is the reason why the leukemogenesis in common marmosets did not react to antiviral treatment. It is conceivable that further virus gene products with homology to cellular proteins and with possible growth-stimulatory function could support leukemogenesis in the susceptible monkeys. However, deletion mutagenesis has shown that most of these accessory genes are not essential for transformation and leukemogenesis. This has been published, for example, for the viral interleukin-17 and for the viral superantigen homolog (Duboise et al., 1998b; Knappe et al., 1997, 1998a,b). However, it remains possible that such accessory genes influence the phenotype as described for the viral homolog to superantigens (Duboise et al., 1998b). Generally, most of the accessory genes are presumed to play an important functional role during the apathogenic persistence in the natural host of the virus, the squirrel monkey (Saimiri sciureus).

Although the results of this study were unexpected, they do not argue against herpesvirus saimiri as a vector for human T cells for several reasons. First, herpesvirus saimiri-transformed human T cells are not permissive and do not produce virus particles, as described in several studies (Biesinger et al., 1992; Fickenscher et al., 1996, 1997; Knappe et al., 2000). Similarly, TK-2-transduced human T cells were not permissive to the virus (Hiller et al., 2000). Second, the TK/GCV prodruk activation system is the prototype strategy. In the context of herpesvirus vectors, alternative suicide genes should be generally considered, such as the enzyme cytochrome P450 2B1, which activates the cytostatic drug cyclophosphamide in the periphery (Chen et al., 1996). Third, it is a general aim of vector development to preclude virus replication in vivo by generating packaging cell systems. Viral deletion variants for essential genes will only replicate in trans-complementing packaging cell lines expressing the deleted function. On this basis, a pathogenetic role of TK expression would be excluded. However, for all replication-competent DNA vector systems, possibly even including adenvirus vectors, HSV tk as a transgene should be carefully reconsidered.

**MATERIALS AND METHODS**

**Virus and T-cell cultures**

Permissive OMK cells, primary lymphocytes, and transformed T cells were cultivated according to published protocols (Fickenscher and Fleckenstein, 1998;
Hiller et al., 2000). OMK cells (CRL-1556; American Type Culture Collection, Manassas, VA; Daniel et al., 1976b) were split once a week by a factor of 2, and the medium was exchanged on day 4 (Dulbecco’s modified Eagle's medium with 10% heat-inactivated FBS, 350 μg/ml glutamine, and 100 μg/ml gentamicin). Virus titrations were performed by limiting dilution in microwell plates (24- or 48-well plates). The titer was determined as the respective dilution step in which at least 50% of the infected parallel cultures (four to six each) developed specific cytopathic changes followed by lysis or complete (TCID_{50}).

Monkeys T cells were cultivated in absence of interleukin-2 in complete medium containing 45% RPMI 1640, 45% Panserin 401 (PAN, Aidenbach, Germany), 10% FBS, 350 μg/ml glutamine, and 100 μg/ml gentamicin. The wild-type virus C488 (Biesinger et al., 1990; Desrosiers and Falk, 1982) and the TK-expression mutant TK-2 (Hiller et al., 2000) have been described before in detail. TK-2 carries the HSV tk reading frame under the control of promoter and termination signals of the murine phosphoglycerol kinase gene. This expression cassette is inserted between orf75 and the terminal repetitions of herpesvirus saimiri genome (Hiller et al., 2000).

**Animals**

Common marmosets (Callithrix jacchus) were purpose-bred in the colony of the German Primate Center (Göttingen, Germany). The experiment was performed based on permissions according to the national animal protection and gene technology safety regulations. To limit the necessary number of animals, we compared the previously characterized virus mutant (TK-2) with wild-type virus under various drug treatment conditions. The infection conditions followed our protocol used before for cottontop marmosets (Knappe et al., 1998a,b). The animals were intravenously injected with either the wild-type or the mutant virus with 10^{6} TCID_{50} in 1 ml. The drugs GCV (Cymeven; Syntex, Grenzach-Wyhlen, Germany) and ACV (Zovirax; Glaxo Wellcome, Hamburg, Germany) were applied subcutaneously at daily doses of 30 mg/kg (ACV) or 15 mg/kg (GCV). The dosage was based on clinical protocols and on previous studies in macaques (Böttiger and Öberg, 1996). As soon as severe symptoms were realized, the animals were killed and subjected to necropsy.

**Analysis of blood and necropsy samples**

Leukocytes from Callithrix jacchus blood samples were sedimented and treated for 5 min with ACK buffer (0.15 M NH_{4}Cl, 1 mM KHCO_{3}, 0.1 mM EDTA, pH 7.3). The PBMCs were washed in saline and either stored as frozen cells for PCR or were used for cocultivation with OMK cells for virus isolation. Moreover, PBMC samples were taken into culture without interleukin-2 to isolate leukemia cell lines. Lysates of PBMC samples or of leukemia cell lines were subjected to routine PCR using the following sets of primers. The tk gene of HSV-1 was monitored with HF344 5'-AAT-GGG-GTC-TCG-GTG-GGG-TAT-C-3' and HF370 5'-ATT-ACG-ACC-AAT-GGC-CC-3'. Virus DNA of wild-type and mutant strains was detected with the orf75-specific primers HF586 5'-TGG-CTG-CTA-AGC-GGC-ATG-G-3' and HF597 5'-AGC-ACG-TTG-CCC-GAG-ATT-G-3'.

**ACKNOWLEDGMENTS**

This study was funded in parts by grants to H.F. from the Bayerische Forschungsstiftung (Munich, Germany), the Deutsche Forschungsgemeinschaft (Bonn, Germany), and the Wilhelm-Sander-Stiftung (Neu- stadt, Germany) and by a joint grant to H.F. and S.S. from the German-Israeli-Foundation (Jerusalem, Israel). We are grateful to Disa Böttiger (Stockholm, Sweden) and Andrew Davison (Glasgow, Scotland) for helpful suggestions. The authors thank Gerhard Hunsmann, Franz-Josef Kaup, Christiane Stahl-Hennig (Göttingen, Germany), and Bernhard Fleckenstein (Erlangen, Germany) for continuous support.

**REFERENCES**


