transfection with a wild-type *DHFR-TS* fragment [9-kb Cla I fragment of pK300 (*16*)] and selection for thymidine prototrophy. The +/Δ1 strain of *L. major* was initially transfected with pX63TKNEO-TPASE to give rise to line TPASE1. TPASE1 was subsequently transfected with pX63PAC-Mos1 in the presence of both G418 and puromycin. Selection against DHFR-TS expression was accomplished by plating cells on semisolid M199 medium containing TdR (10 μg/ml) and MTX (100 μM) (*21*) but lacking G418 or puromycin.
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17. *Leishmania* DNAs were prepared by the LiCl miniprep method (*33*) and used in standard Southern blot analysis (*34*) with probes prepared by the random priming method (*35*).
18. Several of these colonies did show higher molecular weight bands; however, their mobility was the same as higher molecular weight bands arising from the input transfecting DNA, and we attribute this to the presence of partial digestion of monomer or multimeric pX63PAC-Mos1, which is expected to occur in both *Escherichia coli* and *Leishmania* (*16, 36*).
19. We obtained the sequences shown in Fig. 1C by inverse PCR, using different pairs of primers for each side of the transposon. The 5′ side primers (as defined by the transposase ORF within the *Mos1* element) were B440 (5′-GCCGAACTGCAAGCATTATTGG; sense) and B441 (5′-TGAAGCGTTGAAACCACCGTTC; antisense); the 3′ side primers were B442 (5′-TCCACAAATTGCCCGAGAGATG; sense) and B443 (5′-ATGTGATGGAGCGTTGTCATGG; antisense). First, genomic DNAs were separated on agarose gels after restriction digestion, and DNA fragments in the size range for the new *mariner*-hybridizing band were isolated. These were then digested with Sau 3A, self-ligated under dilute conditions, and then used in standard PCR reactions (*37*). Products differing in size from those arising from the parental plasmids were identified by gel electrophoresis, inserted into pGEM-T (Promega), and sequenced with ΔTaq 2.0 polymerase and the Taquenase kit (United States Biochemical). Sequences were deposited in GenBank under the accession numbers B07506 through B07511.
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24. RNAs were prepared with Tri-reagent (Sigma) (*38*), following the procedures of the manufacturer. cDNA was synthesized from ~1 μg of total RNA, with a random hexamer priming protocol (*39*). A nested RT-PCR protocol was used to amplify chimeric *Leishmania*-MosHYG mRNAs (*39*). For the first PCR, the anchor primer was B466 (5′-AACGCTATATAAGTATCAG), corresponding to nucleotides 1 through 19 of the mini-exon, and the *HYG*-specific primer was B467 (5′-AAAGCACGAGATTCTTCG). Primers for the second round of PCR were B145 (5′-ATCAGTTTCTGTACTTTA; nucleotides 15 through 32 of the mini-exon) and the *HYG*-specific primer B456 (5′-ATCAGAAACTTCTCGACAG). RT-PCR products were cloned into pGEM-T and sequenced. Sequences were deposited in GenBank under the accession numbers AA195776 through AA195783.
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41. We thank D. Hartl for the gift of *Mos1* DNA, S. Chatterjee and M. Lee for helpful discussions, and D. E. Dobson, L. A. Garraway, L. K. Garrity, A. Hubel, and L. R. O. Tosi for comments on the manuscript. Supported by NIH grant AI2964.

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HSV-TK Gene Transfer into Donor Lymphocytes for Control of Allogeneic Graft-Versus-Leukemia

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In allogeneic bone marrow transplantation (allo-BMT), donor lymphocytes play a central therapeutic role in both graft-versus-leukemia (GvL) and immune reconstitution. However, their use is limited by the risk of severe graft-versus-host disease (GvHD). Eight patients who relapsed or developed Epstein-Barr virus–induced lymphoma after T cell–depleted BMT were then treated with donor lymphocytes transduced with the herpes simplex virus thymidine kinase (HSV-TK) suicide gene. The transduced lymphocytes survived for up to 12 months, resulting in antitumor activity in five patients. Three patients developed GvHD, which could be effectively controlled by ganciclovir-induced elimination of the transduced cells. These data show that genetic manipulation of donor lymphocytes may increase the efficacy and safety of allo-BMT and expand its application to a larger number of patients.

Allogeneic bone marrow transplantation is the treatment of choice for many hematologic malignancies (*1*, *2*). It is now recognized that the "allogeneic immune advantage," in addition to the effectiveness of high-dose chemoradiotherapy, is responsible for the curative potential of allo-BMT (*1*, *2*). Although the nature of effector cells has not yet been fully elucidated, transplantation of allogeneic bone marrow produces superior results compared to autologous or syngeneic transplants (*3*). However, the therapeutic impact of the allogeneic advantage is limited by the risk of a potentially life-threatening complication, GvHD. Severe GvHD can be circumvented by the removal of T lymphocytes from the graft (*2*). However, T cell depletion increases the incidence of disease relapse, graft rejection, and reactivation of endogenous viral infections (*4*). Thus, the delayed administration of donor lymphocytes has recently been used for treating leukemic relapse after allo-BMT. Patients affected by recurrence of chronic myelogenous leukemia, acute leukemia, lymphoma, and multiple myeloma after BMT could achieve complete remission after the infusion of donor leukocytes, without requiring cytoreductive chemotherapy or radiotherapy (*5*). Other complications related to the severe immunosuppressive status of transplanted patients, such as Epstein-Barr virus–induced B lymphoproliferative disorders (EBV-BLPD) (*6*, *7*) or reactivation of CMV infection (*8*), also benefited. However, severe GvHD represents a frequent and potentially lethal complication of this delayed infusion of donor T cells (*9*). No specific treatment exists for established GvHD. We investigated the genetic manipulation of donor lymphocytes,

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which could enable their selective elimination and abrogation of GvHD, thereby making marrow transplantation more efficacious, safer, and available to a larger number of patients.

(HSV-TK) has been successfully transferred into various cell lines to confer ganciclovir sensitivity, and its efficacy has been demonstrated both in vitro and in vivo (*10–12*). However, an absolute prerequisite for the efficacy of this strategy is that all infused donor lymphocytes carry the "suicide" gene. For this purpose, we devised a simple strategy on the basis of retroviral vector–mediated gene transfer and expression in transduced cells of a cell surface marker not expressed on human lymphocytes, followed by positive immunoselection of the transduced cells (*13*). This strategy ensures that virtually 100% of the peripheral blood lymphocytes (PBLs) carry the suicide gene (*13*).

We used a retroviral vector (SFCMM-2) (Fig. 1A) (*13*, *14*) for the transfer and expression into human PBLs of two genes: The first encodes a truncated (nonfunctional) form of the human low-affinity receptor for nerve growth factor (ΔLNGFR) (*14*); the second encodes the HSV-TK–NEO fusion protein (Fig. 1A). ΔLNGFR is located on the cell surface and allows rapid in vitro selection of transduced cells by the use of magnetic immunobeads. In addition, a surface marker allows easy ex vivo detection and characterization of the transduced cells by fluorescence-activated cell sorting (FACS) analysis (*13*). The safety and efficacy of this vector were extensively tested in vitro and in vivo in small-animal models (*15*). In this pilot clinical study, the proportion of transduced donor lymphocytes after one round of gene transfer was in the 20 to 50% range. The proportion of transduced donor cells after one round of selection ranged between 95.0 and 99.6% (*16*).

Twelve patients who experienced severe complication after allo-BMT for a hematologic malignancy or immunodeficiency participated in this study. Here we report on the first eight patients for whom an adequate follow-up is available (Table 1). For the treatment of leukemic relapse, SFCMM-2–transduced donor lymphocytes were infused at increasing cell doses, beginning at 10^5 cells per kilogram of body weight to a total of 4×10^7 cells/kg. For the treatment of EBV-BLPD, transduced donor lymphocytes were infused at an initial dose of 1×10^6 cells/kg. The higher initial dose was necessitated by the rapid progression of this complication and was suggested by the previous clinical study (*6*). No toxicity or complication that could be related to the gene transfer procedure was observed in this study (*17*).

Extensive manipulation of the cells that undergo ex vivo gene transfer by retroviral vectors could potentially modify their immune repertoire and their activation status, thus affecting their in vivo survival and function. In all but one of the patients who received transduced donor lymphocytes, genetically modified cells could be repeatedly detected in the circulation, in marrow aspirates, and in tissue biopsies (*18*). The proportion of transduced donor cells among circulating lymphocytes ranged between the level of detection by polymerase chain reaction (PCR) (10^{-4}) and 13.4% (Fig. 1B). At various times of followup, some patients had only low frequencies of circulating transduced cells, below the level of detection in FACS analysis. In these cases, ex vivo–transduced donor cells could be selected by culture in the presence of G418 (a neomycin analog), and selected cells homogeneously expressed the cell surface marker (Fig. 1C). Additionally, the presence of transduced donor lymphocytes in PBLs, BM samples, and GvHD lesion sites could be confirmed by PCR analysis and followed over time (Fig. 1D). The persistence of transduced cells could still be detected for more than 12 months after the last infusion of transduced donor cells. Persistence of antigen-specific responses was also observed; in the patient treated for an EBV-induced lymphoma, we detect-

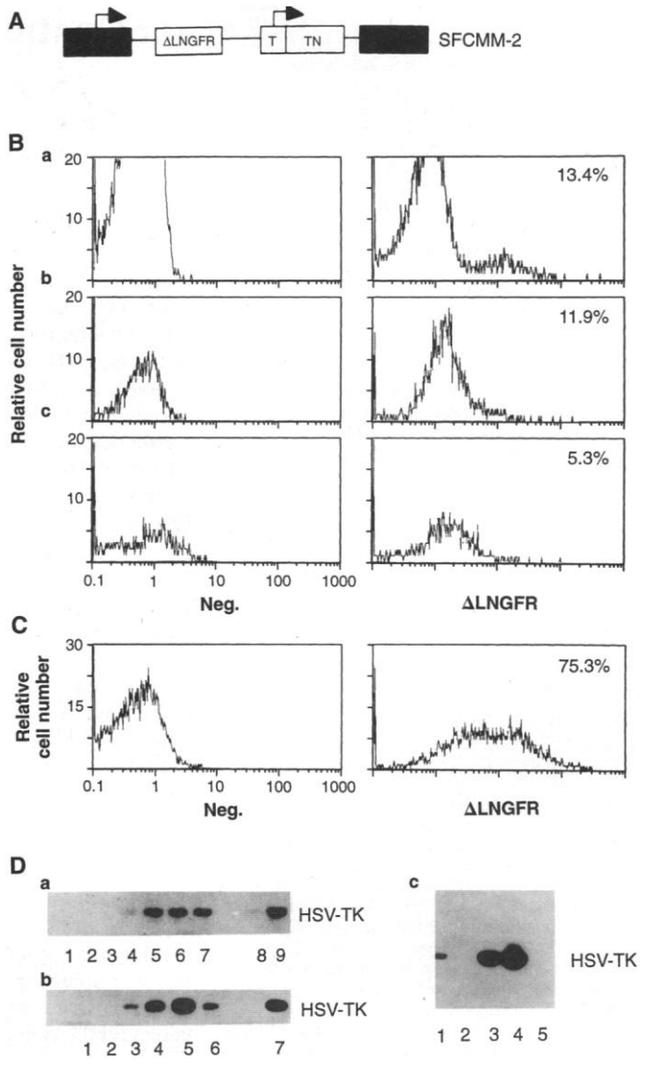

Fig. 1. Ex vivo detection of transduced donor lymphocytes. (**A**) Schematic map of integrated SFCMM-2 proviral genome, indicating the HSVTK internal promoter (T). Solid boxes denote long-terminal repeat sequences. ΔLNGFR, modified form of the low-affinity receptor for nerve growth factor; TN, fusion gene encoding a bifunctional protein carrying both HSV-TK activity and neomycin resistance. Arrows indicate transcription promoters. (**B**) FACS analysis for expression of ΔLNGFR on the cell surface and appropriate controls (Neg.) from peripheral blood (**a** and **b**) and bone marrow (**c**) samples of patient 1 (a) and 8 (b and c). The proportion of positive cells is indicated. (**C**) FACS analysis for expression of ΔLNGFR on lymphocytes obtained after G418 selection of PBLs from patient 2 and the appropriate control. The proportion of positive cells reported, 75.3%, is derived from comparison with the negative control and is underestimated because analysis of the sample revealed the presence of a cell population that homogeneously expressed the cell surface marker. On the *x* axis of (B) and (C), it is labeled as the logarithmic scale of green fluorescence. (**D**) (**a** and **c**) PCR analyses for the presence of the HSV-TK sequence on various samples from patient 8. (a) Lanes 3 to 6, peripheral blood samples obtained at monthly intervals beginning at day 3 from the second infusion (Fig. 3); lane 7, bone marrow sample obtained 23 days from infusion; lanes 1 and 2, negative controls; lanes 8 and 9, positive controls at two different levels of detection of positive cells (10^{-4} and 10^{-3}, respectively). (c) Lanes 1 and 3, two skin biopsy samples consistent for GvHD; lanes 2 and 5, negative controls; lane 4, positive control. (**b**) PCR analysis from patient 2. Lanes 3 to 6, peripheral blood samples obtained at monthly intervals beginning at day 4 from the second infusion (Fig. 4); lanes 1 and 2, negative controls; lane 7, positive control.

Table 1. Clinical characteristics of the eight patients described in the text. Disease status was assessed through examination of marrow aspirates and biopsy, cytogenetic examination, and molecular analysis. EBV-BLPD was determined by serologic data and histologic evaluation as indicated. Exclusion criteria for the infusion of transduced donor lymphocytes included the presence of aGvHD grade II or higher and CMV reactivation requiring ganciclovir treatment. This study was approved by the Institutional Ethical Committee (*14, 31*), and all patients gave informed consent. NHL, non–Hodgkin lymphoma; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; CmML, chronic myelomonocytic leukemia; EBV-BLPD, Epstein-Barr virus–induced B lymphoproliferative disorder; CR, complete remission; PR, partial response; NR, no response; NE, not evaluable.

Patient	Disease	Complication (weeks after BMT)	First infusion (weeks after complication)	No. of lymphocytes infused ($\times 10^6$ cells/kg)	Clinical outcome	GvHD
1	NHL	EBV-BLPD (+7)	2	1.5	CR	Acute
2	CML	CML relapse (+28)	11	38.6	PR	Acute
3	CML	CML relapse (+84)	192*	4.5	PR	No
4	AML	AML relapse (+45)	1	3.3	NR	No
5	AML	AML relapse (+14)	1	4.9	NR	No
6	CML	EBV-BLPD (+5)	1	0.5	NE	No
7	AML	AML relapse (+24)	2	11.1	CR	No
8	CmML	CmML relapse (+19)	4	20.5	CR	Chronic

*Patient 3 received idrossiurea and interferon-α before the infusion of transduced donor cells.

ed transduced cells that exhibited EBV-specific activity for the first time after administration of donor cells. The frequency of EBV-specific cells was ~1:1000, as compared to the frequency of 1:1300 that was detected in the donor PBLs before and after ex vivo vector transduction. This level was achieved after administration of a small dose of donor cells (Fig. 2 and Table 1). After administration of ganciclovir to treat GvHD, we could still detect this specific reactivity in the peripheral blood of the patient at the reduced frequency of 1:3250. No linear correlation was observed between the number of infused transduced donor lymphocytes and persistence or detection. Rather, the intensity of antigen response and proliferation of donor cells seemed to affect levels of detection. In particular, patient 1 reached the level of 13.4% of positivity after a total injection of only 10^6 cells/kg to treat EBV-BLPD. Patient 8, who achieved similar levels of positivity (11.9%), did so after receiving an order of magnitude higher infusion of cells to treat chronic myelomonocytic leukemia (CmML) relapse. Other patients who received even higher cell doses did not reach similar levels of positivity. As expected, viral antigens were able to induce a more rapid and intense specific proliferation of transduced cells than leukemic cells (*19*).

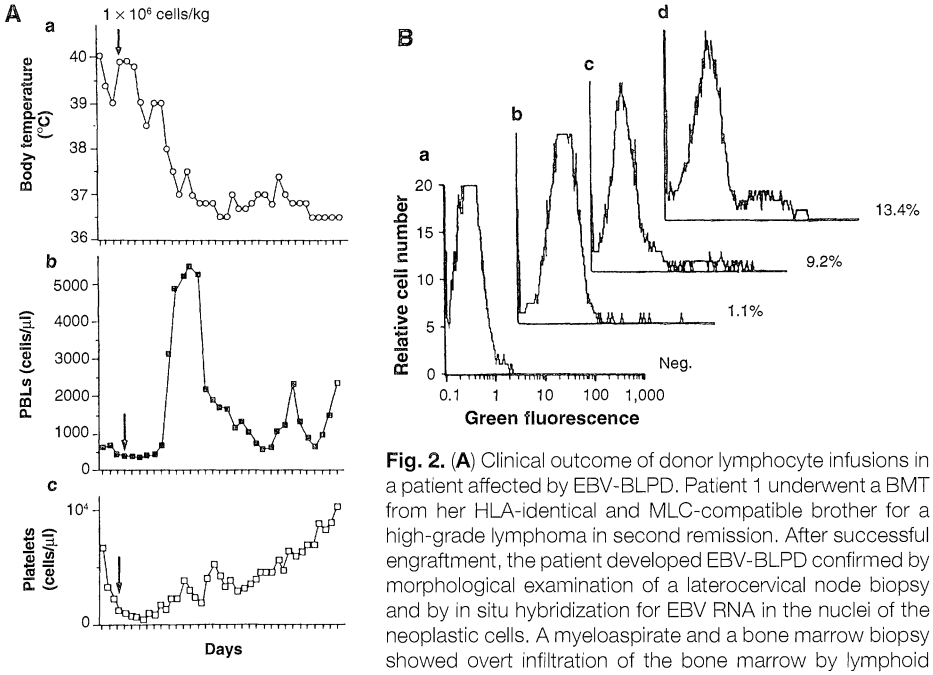

Fig. 2. (**A**) Clinical outcome of donor lymphocyte infusions in a patient affected by EBV-BLPD. Patient 1 underwent a BMT from her HLA-identical and MLC-compatible brother for a high-grade lymphoma in second remission. After successful engraftment, the patient developed EBV-BLPD confirmed by morphological examination of a laterocervical node biopsy and by in situ hybridization for EBV RNA in the nuclei of the neoplastic cells. A myeloaspirate and a bone marrow biopsy showed overt infiltration of the bone marrow by lymphoid paratrabecolar nodules causing marrow failure. The patient received a total dose of 1.5×10^6 cells/kg (*6*). In the 2 weeks after administration of donor cells (arrow), all clinical symptoms associated with EBV-induced B cell proliferation regressed as shown by body temperature decrease (**a**). At the time of regression of clinical symptoms, a sharp increase in PBL counts was observed (**b**). Circulating transduced donor lymphocytes were almost exclusively $CD3^+CD8^+$ lymphocytes (>90% of total mononuclear cells from day +10 to day +15), with high proliferation index. Meanwhile, a hematological reconstitution occurred, shown by an increase in the number of circulating platelets (**c**). During this time, marked donor cells increased progressively in the patient's peripheral blood up to 13.4% of total mononuclear cells (**B**). (**a**) Negative control; (**b** to **d**) three blood samples obtained at day 3, 10, and 20 after infusion. The proportion of positive cells is indicated.

The in vivo function of genetically modified donor lymphocytes was revealed by antitumor responses, immune reconstitution, and alloreactivity (*20*). In five out of the eight patients in this series GvL was detected, with three complete responses (Table 1). Three patients developed GvHD. The patients who achieved full remission included patient 1, treated for an aggressive EBV-induced lymphoma, and patient 8, treated for leukemic relapse (Figs. 2 and 3). These results were obtained in the absence of any chemotherapeutic agent. A third patient (patient 7, Table 1) achieved full remission after administration of genetically modified donor lymphocytes and chemotherapy. Patient 3 relapsed twice after two subsequent allogeneic BMTs for CML and could be maintained in CML chronic phase for over 5 years. During this period, all donor cells disappeared from the marrow and the circulation. After the long interval in chronic phase, the CML progressed to accelerated phase and was treated by the infusion of transduced donor lymphocytes (total dose, 4.5×10^6 cells/kg). This was followed by conversion to chronic phase and by the reappearance of donor chimerism, as monitored by the progressive conversion of the blood type from B to the donor type A (*21*). In this patient, the increase of the donor blood type erythrocytes closely followed the appearance of circulating transduced donor lymphocytes.

Thus, the procedures associated with ex vivo gene transfer of the marking and thera-

peutic genes by the SFCMM-2 vector preserved the in vivo potential of allogeneic PBLs and their antigen-specific reactivity. Additionally, this observation was further confirmed in a small series of patients in which genetically modified and unmodified donor PBLs were used. In a patient with moderate skin GvHD, it was possible to determine by immunostaining and PCR that both cell populations positive and negative for the cell surface marker ΔLNGFR were contributing to the lymphocyte infiltrate present in the lesion (*22*).

Two patients in this series developed acute GvHD and were treated with ganciclovir (*23*) (Table 2). Patient 2 relapsed 6 months after allo-BMT and was treated by increasing doses of transduced donor cells. Marked cells could be detected in the peripheral blood by FACS analysis and PCR. After the third infusion of transduced donor lymphocytes (total of 1×10^6 cells/kg), values of the patient's bilirubin and liver function enzymes rapidly increased (Fig. 4). A liver biopsy revealed acute GvHD, and the patient was treated with ganciclovir (10 mg/kg per day). All transduced donor lymphocytes and all GvHD clinical and biochemical signs then disappeared (Fig. 4 and Table 2), in the absence of any local or systemic toxicity. Elimination of transduced donor cells preceded normalization of liver enzymes with similarly sharp kinetics (Fig. 4 and Table 2). Indeed, after 1 day of treatment, genetically modified cells decreased below the level of PCR detection (10^{-4}) and remained below this level until a second infusion of transduced cells was administered 21 days later. Because no immunosuppressive drug was administered to this patient, the complete abrogation of the severe liver GvHD could be attributed exclusively to the expression of the TK transgene. Four weeks after the infusion of donor lymphocytes, patient 1 progressively developed signs of acute GvHD, confirmed by a skin biopsy. The intravenous administration of two doses of ganciclovir (10 mg/kg) resulted in progressive rapid reduction from 13.4% to below PCR detection (Table 2). The treatment was followed by the disappearance of clinical signs of skin GvHD. About 3 weeks after infusions of HSV-TK–transduced cells, patient 8 presented chronic GvHD involving skin, oral mucosa, and lungs. Immunohistochemical analysis and PCR revealed the presence of genetically modified cells in the biopsy. This patient was then treated with ganciclovir (10 mg/kg per day) for 7 days, resulting in improvement of clinical conditions and a decrease of GvHD signs. The correlated reduction in the proportion of circulating transduced donor cells to 2.8% was achieved in the first 24 hours (Table 2). Complete elimination of genetically modified donor lymphocytes could not be obtained despite extended treatment.

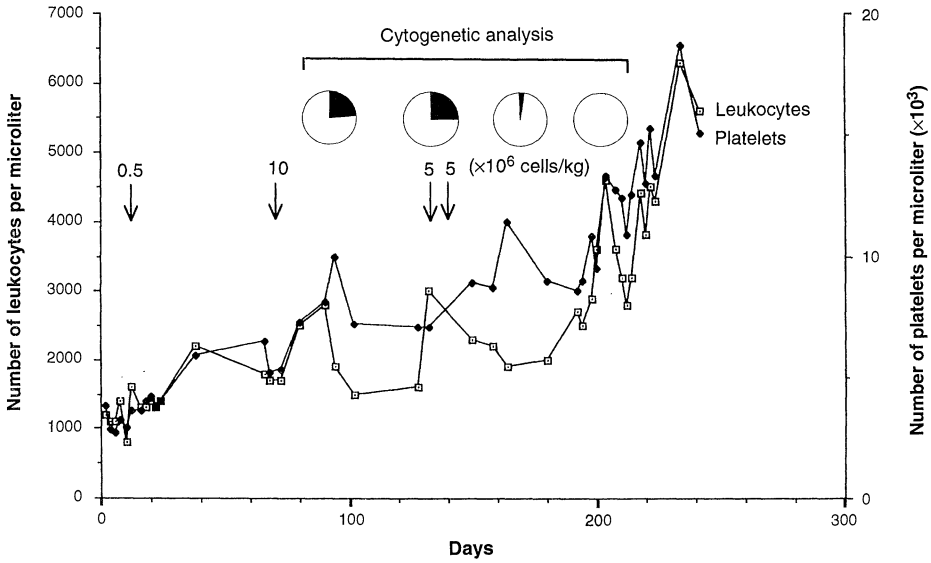

Fig. 3. Clinical outcome in a patient affected by chronic myelomonocytic leukemia (CmML) relapse treated by the infusion of donor lymphocytes. Patient 8 underwent BMT from her HLA-identical MLC-compatible brother but relapsed 3 months after transplant, as revealed by cytogenetic analysis, followed by a decrease in peripheral blood counts and marked morphologic myelodisplastic bone marrow. The patient received a total of 2×10^7 cells/kg donor lymphocytes. Marked cells could be easily detected by FACS analysis (Fig. 1B) and PCR (Fig. 1D) in the peripheral blood (reaching a peak at 11.9% of circulating mononuclear cells) and in bone marrow (reaching a peak at 5.3%). Infusions (arrows) were followed by a hematological reconstitution, as shown by the increase in the number of circulating (leukocytes and platelets. Bone marrow cytogenetic analysis revealed a progressive decrease in the number of host hematopoietic cells (shaded area), and 100% donor engraftment (unshaded area) was observed after four infusions.

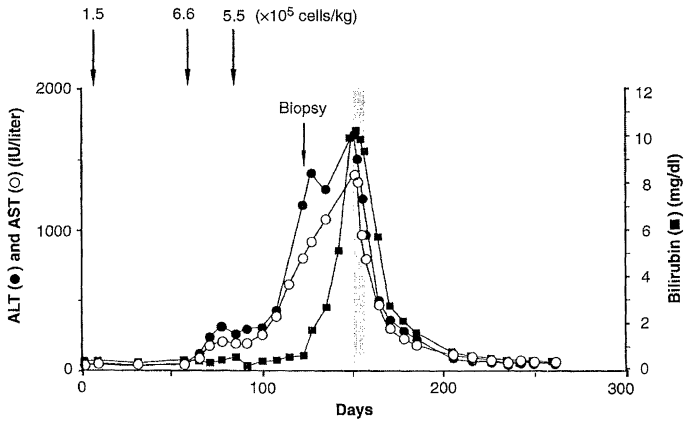

Fig. 4. Values of bilirubin and liver function enzymes in patient 2, who developed acute GvHD after the infusion of transduced donor lymphocytes. Long arrows, infusions of transduced donor cells; short arrow, timing of the liver biopsy performed to detect acute GvHD (biopsy). The gray area indicates the period of the four infusions of ganciclovir (10 mg/kg per day).

Although fluctuations in the numbers of circulating transduced lymphocytes were observed at various times of followup, reduction of circulating transduced donor lymphocytes comparable to that described in these three patients was never observed in any of the patients in the study in the absence of ganciclovir treatment. This is best compared to the lack of any spontaneous remissions of a grade II or higher of GvHD in the 258 patients treated with donor lymphocytes in the EBMT (European Bone Marrow Transplantation) international collaborative study (*24*).

The two treated patients who had achieved full remission before ganciclovir administration remained in full remission after elimination of donor lymphocytes by ganciclovir. Thus, the transfer of the HSV-TK gene by the SFCMM-2 vector may provide a tool for in vivo modulation of alloreactivity and effective and specific treatment of acute GvHD in the absence of any immunosuppressive drug.

As anticipated, no local or systemic toxicity was observed that could be related to the gene transfer procedure or to ganciclovir administration. No significant modifications of the total counts of leukocytes, PBLs, natural killer cells, or other T cell subsets could be

Table 2. Effect of ganciclovir treatment on elimination of genetically modified donor lymphocytes and on GvHD.

Patient	GvHD (grade)	Proportion of ΔLNGFR + PBLs (%)		Clinical outcome
		Preganciclovir	24 hours after ganciclovir	
1	Acute skin (II/III)	13.4	$<10^{-4}$	CR*
2	Acute liver (III)	2.0	$<10^{-4}$	CR†
8	Chronic (extended) lung, skin, G.I.	11.9	2.8	PR‡

*Complete remission was revealed by clinical observation of complete regression of all skin GvHD signs. †Complete remission was revealed by the disappearance of all physical and biochemical signs of liver GvHD (Fig. 4 and text). ‡Partial remission of the severe obstructive bronchiolitis was revealed by physical evaluation and spirometric analysis. Amelioration of skin GvHD, but not of oral mucosa, was also observed. In oral mucosa, persistence of chronic GvHD and of genetically modified cells was confirmed histologically and by PCR, respectively. In patient 1 and 2, given the complete elimination of clinical signs of aGvHD, a biopsy immediately after ganciclovir treatment was considered inappropriate and was not performed. However, followup biopsies performed later as part of the patients' monitoring were consistently negative for the presence of the transgene (PCR) and for GvHD.

detected in patients who had received ganciclovir treatment for GvHD. This confirms that lymphocytes do not produce the in vivo bystander effect observed in other cell types (*10*, *12*). The only unwanted effect observed in this study was a specific immune response to HSV-TK–NEO in one patient who had received genetically modified cells late after transplant, after return to immunocompetence (*25*). The immune response was expressed only to the HSV-TK–NEO fusion protein. No immunity to the cell surface marker ΔLNGFR was detected in any patient (*25*).

This study confirms the therapeutic potential of donor T cells in the context of allo-BMT (*5*, *6*). Because it is not yet possible to differentiate between GvL and GvHD effector cells and to predict which patients will develop the more severe grade of GvHD after an unmodified allo-BMT (*26*), immunosuppressive prophylaxis remains an absolute requirement. This immunosuppression and the more intense regimens associated with GvHD that arise despite prophylaxis significantly limit the benefit of allo-BMT, eliminating or reducing the allogeneic advantage (*27*). No specific treatment yet exists for established GvHD, and immunosuppressive regimens carry significant side effects and limited efficacy (*28*). In this context, the transfer of a suicide gene for selective and specific elimination of effector cells of GvHD provides a new tool to combine the benefits of allo-PBLs with the possibility of eliminating GvHD without toxic effects. However, the extensive manipulation of the cells that undergo ex vivo gene transfer could potentially modify the clinical outcome produced by transduced donor PBLs as compared to unmodified counterparts, resulting in abrogation or reduction of the allogeneic advantage, or exacerbation of GvHD. Therefore, the long-term survival and immunocompetence of transduced PBLs observed in this study is crucial not only for this model but also for all other applications involving ex vivo gene transfer into polyclonal lymphocytes (*29*). Because the mechanisms underlying GvHD, GvL, and antiviral responses are likely to be different and to involve different effector cells, the preservation of an intact immune repertoire as well as all functional properties in transduced PBLs is of crucial relevance. We propose that optimal effects achieved were attributable to the rapid positive selection system for transduced cells allowed by the cell surface marker (*13*). Ganciclovir-mediated elimination of HSV-TK–transduced cells was efficacious in the presence of acute GvHD. In only one case of chronic GvHD was ganciclovir treatment not fully effective. Transduced cells isolated ex vivo from this patient showed unmodified sensitivity to ganciclovir, suggesting that in vivo resistance could indeed be due to the activation state of the genetically modified lymphocytes. This is supported by the well-known cell replication–dependent activity of HSV-TK (*30*). If confirmed in extended clinical studies, these results will expand the number of candidate patients benefiting from T lymphocyte–depleted allo-BMT, by allowing the use of less-compatible marrow donors.

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16. Peripheral blood mononuclear cells (PBMCs) were collected from the BMT donor by leukapheresis and separated from the red cells and neutrophils by Ficoll-Hypaque density-gradient centrifugation. The PBMCs were then washed, counted, and cultured at ~2×10^6 cells per 2-ml well in 24-well tissue culture plates containing RPMI 1640 with 5% human serum in the presence of recombinant human interleukin-2 (IL-2) (Euro-Cetus Italy S.r.l., Milan, Italy). For gene transfer, donor lymphocytes were cocultured with the irradiated packaging cell line for more than 48 hours in the presence of Polybrene (8 μg/ml), beginning the first day of polyclonal stimulation with phytohemagglutinin (2 μg/ml). No modification of culture conditions was introduced until the end of the procedure (*13*, *14*). After gene transfer, transduced cells were selected for the expression of the cell surface marker ΔLNGFR by means of specific immunobeads. Cells were incubated with the murine monoclonal antibody 20.4 to human ΔLNGFR (American Type Culture Collection). After 30 min cells were washed and incubated with immunomagnetic beads coupled to a rat antibody to mouse immunoglobulin G1. After 45 min, positive cells were separated by use of magnetic immunobeads and incubated overnight at 37°C to remove antibody-coated beads. After one round of selection, the proportion of transduced cells was assessed by cytofluorimetric analysis (*13*). Up to this point of the study, a second round of selection was never required to achieve the indicated levels of purity. Cells were then cryopreserved for future infusions. In vitro ganciclovir sensitivity of HSV-TK–transduced lymphocytes (*15*), as well as acyclovir resistance (*10*), have been described previously. Before clinical use, cells were required to meet the specifications already in use in other clinical human gene therapy experiments. In particular, cells were tested for replication-competent retroviruses, IL-2–independent growth, and for adventitious infectious agents. Cells were infused by intravenous injection in saline with 4% human serum albumin at the time of diagnosis of complication (Table 1) and subsequently at monthly intervals (*14*). In some patients (Figs. 2 to 4) the schedule was adjusted in accord with clinical status and nature of the complication.
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18. Small amounts of peripheral blood in preservative-free heparin were obtained immediately before and after each lymphocyte infusion, weekly for the first 3 months after infusion, and monthly for the remainder of the first year and were used for the following immunological and gene marking studies: (i) cytofluorimetric analysis for the frequency of cells expressing the cell surface marker gene and for the ex vivo characterization of transduced cells; (ii) confirmation of the presence of transduced donor lymphocytes by PCR; (iii) G418 selection for detection and expansion of *neo*-transduced cells; (iv) ex vivo analysis of the persistence of ganciclovir sensitivity; and (v) ex vivo detection of immunity against transduced donor lymphocytes, by standard mixed lymphocyte culture and cytotoxic assays (*25*). Any diagnostic biopsy, including biopsies performed to detect GvHD, were used for the following studies: (i) immunohistochemical assays for the expression of the cell surface marker in infiltrating lymphocytes; (ii) analysis for vector gene presence by PCR; and (iii) immunophenotype of infiltrating lymphocytes. Serum, plasma, cells, and biopsies collected at specific time points were frozen for future analysis. After the administration of ganciclovir, peripheral blood samples were obtained daily for the first week and weekly thereafter. The in vivo selective elimination of transduced donor lymphocytes was monitored by means of the same immunological and gene marking criteria reported above.
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20. Patients were monitored weekly for the first month and

monthly thereafter for detailed clinical history, complete physical examination (including skin examination for the presence of GvHD), and general laboratory evaluation [including complete blood count and differential, urinalysis, blood urea nitrogen creatinine, bilirubin, aspartate transaminase (AST), alanine transferase (ALT), alkaline phosphatase, Na, K, Cl, albumin, total protein, glucose, and radiographic evaluation] as indicated. Disease status was assessed through examination of marrow aspirates and biopsy, cytogenetic analysis, and molecular analysis.
21. C. Bonini and S. Rossini, data not shown.
22. M. Ponzoni and L. Ruggieri, data not shown.
23. If grade II GvHD or higher occurred, patients who had previously received transduced donor lymphocytes were treated with ganciclovir (10 mg/kg per day) for 7 days or less if all the GvHD signs and symptoms regressed. Patients who had previously received infusions of both transduced and untransduced donor lymphocytes were initially treated with ganciclovir at 10 mg/kg per day, to down-regulate GvHD.
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TECHNICAL COMMENTS

Dealing with Database Explosion: A Cautionary Note

Carol J. Bult *et al.* (*1*) report the first entire archea genome sequence of *Methanococcus jannaschii* (Mja). Because the initial gene assignments were conservative (*1*, *2*), we anticipated that much interesting biological information would be missing. We searched the database for additional open reading frames (ORFs), and found 15 ORFs: four within intergenic regions (M1 through M4, Table 1); five overlapping with previously identified ORFs (*1*, *2*) but that read off in a different frame (M5 through M9, Table 1); and six that are extended or truncated as a result of potential frameshifts (M10 through M15, Table 2).

Table 1. New ORFs in *M. jannaschii* (Mja) identified on the basis of similarity. ORFs were identified after purging out protein coding regions reported for the organism (*1*) and searched using BLASTX against the combined SwissProt+PIR+Genbank translations database through the NCBI Network BLAST server using a score cutoff of 60, as described previously (*6*). Corresponding matching protein, matching species–*Methanococcus vannielii* (Mva), *Bacillus subtilis* (Bsu), *Haemophilus influenzae* (Hin)—5′ start position, + or − strand, length of the ORF in amino acids (AA), 5′ to 3′ flanking ORFs, and the Poisson probability estimates are provided for each ORF. Other details available at www.golgi.harvard.edu/bhatia/neworfs/mja/table1.html

ORF	Matching protein	Matching species	Start 5′	Length (AA)	Flanking ORFs		*p*
					5′	3′	
M1	30S Ribosomal protein S14	Mva	415652+	55	469	470	10^{-19}
M2	Yqgp protein	Bsu	540515+	190	610	611	10^{-9}
M3	Amido phosphoribosyl transferase	Mja	1301085−	362	1352	1351	10^{-5}
M4	Unknown	Mja	1230530−	255	1283	282	10^{-18}
M5	Asparagine synthetase	Bsu	994621+	318	1055	1056	10^{-26}
M6	Modification methylase	Mja	1153501−	81	1208	1206	10^{-12}
M7	Modification methylase HINCII	Hin	1277783+	286	1327	1328	10^{-35}
M8	Helicase	Bsu	1548365−	182	1573	1572	10^{-6}
M9	Unknown	Mja	1329000+	58	1380	1381	10^{-12}

Table 2. Identification of potential frameshift(s) by similarity. Highly significant BLAST matches, of similar genes in alternative coding frames, were classified as frameshifts, manually assembled, and confirmed. Effect of the frameshift (extension or truncation) and length of the ORF as a result of the frameshift are also provided. M10 through M14 have suffered a single frameshift event, while M15 has apparently undergone a second frameshift. Other details available at www.golgi.harvard.edu/bhatia/neworfs/mja/table2.html

ORF	Matching protein	Matching species	Start 5′	Length (AA)	Frameshift		*p*
					Effect	Length (AA)	
M10	Restriction modification enzyme subunit M1	Mja	128577−	359	extension	583	10^{-93}
M11	Transposase	Mja	276289+	91	truncation	38	10^{-43}
M12	Polyferredoxin	Mja	457630−	410	extension	567	10^{-40}
M13	Unknown	Mja	14344−	72	truncation	16	10^{-35}
M14	Unknown	Mja	202169+	177	truncation	50	10^{-8}
M15	Unknown	Mja	809431+	32	extension	131	10^{-20}

Although the potential frameshifts we describe might be bona fide, it cannot be ruled out that they represent actual sequencing artifacts. Erroneous sequences in public databases are a substantial problem and have been estimated to be in the range of 0.37 to 2.9 errors per 1000 nucleotides (*3*), making data interpretation sometimes difficult. This is especially true, for example, in studies that utilize protein and DNA sequence information to estimate evolutionary distances (*4*). It is not known how the error rate in this study (*1*) compares with error rates in the database, but a previous study suggests that error rates generally vary between 1 in 5000 to 1 in 10,000 nucleotides (*5*).

The issue of sequencing artifacts is important and is expected to be a continuing problem in the future, considering the heightened surge of genome sequencing projects from model organisms, as well as from the human genome sequencing initiative.

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