Construction of a Minimal HIV-1 Variant that Selectively Replicates in Leukemic Derived T-Cell Lines: Towards a New Virotherapy Approach

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Abstract

T-cell acute lymphoblastic leukemia is a high-risk type of blood-cell cancer. We analyzed the possibility of developing virotherapy for T-cell acute lymphoblastic leukemia. Virotherapy is based on the exclusive replication of a virus in leukemic cells, leading to the selective removal of these malignant cells. We constructed a minimized derivative of HIV-1, a complex lentivirus encoding multiple accessory functions that are essential for virus replication in untransformed cells, but dispensable in leukemic T cells. This mini-HIV virus has five deletions (*vif*, *vpR*, *vpU*, *nef*, and *U3*) and replicated in the SupT1 cell line, but did not replicate in normal peripheral blood mononuclear cells. The stripped down mini-HIV variant was also able to efficiently remove leukemic cells from a mixed culture with untransformed control cells. In contrast to wild-type HIV-1, we did not observe bystander killing in mixed culture experiments with the mini-HIV variant. Furthermore, viral escape was not detected in long-term cultures. The mini-HIV variant that uses CD4 and CXCR4 for cell entry could potentially be used against CXCR4-expressing malignancies such as T-lymphoblastic leukemia/lymphoma, natural killer leukemia, and some myeloid leukemias. (Cancer Res 2005; 65(8): 3347-55)

Introduction

Virotherapy is based on the selective replication of viruses in specific target cells to efficiently remove these cells from the patient. This approach has been proposed as a novel therapeutic means against certain cancers and is currently being evaluated in clinical trials. Some success has been reported in the treatment of head and neck cancers using an engineered adenovirus (1–4), although doubts remain about the absolute restriction of virus replication in cancer cells (5). In an ideal setting, the viral infection exclusively targets malignant cells. At the start of therapy, a large number of target cells in the patient will enable fast spreading of the viral infection. The number of target cells will rapidly decline due to massive virus replication, resulting in a concurrent reduction of the virus population. Therapeutic virus variants may need to be optimized and fine tuned to increase their replication specificity or to modulate their cytopathogenicity. For instance, cytotoxic genes may be incorporated into the viral genome or virus spread may be improved by the inclusion of genes encoding fusogenic proteins (6). Experiments have thus far focused on virotherapy of solid tumors. None of the previously used therapeutic viruses are able to infect lymphoid cells, making these viral systems less useful for the targeting of leukemic cells.

In this study, we set out to construct viruses that are able to replicate in and to kill leukemic T cells. At the same time, these viruses should be harmless to untransformed T cells. As a starting point, we used the HIV-1 because it specifically targets cells of the lymphoid system (e.g., T cells and macrophages). HIV-1 infects cells by recognition of the CD4 receptor in combination with a coreceptor, which is either the chemokine receptor CCR5 or CXCR4 (reviewed in refs. 7, 8). HIV-1 can use other coreceptors to some extent *in vitro*, but these receptors do not seem to play a major role *in vivo*. For our strategy, we selected a CXCR4 using HIV-1 isolate because this marker is present on the surface of 53% of the human T-cell population as compared with only 2.5% for CCR5 (9). CXCR4 is also present on T-cell acute lymphoblastic leukemic cells (T-ALL; ref. 10). Interestingly, its SDF-1 chemokine ligand may in fact play a role in the circulation and homing of leukemic cells (11). The latter property may imply an important role for CXCR4 as a surface marker of leukemic cells. Therefore, a therapeutic virus that uses CD4 and CXCR4 could potentially target a significant fraction of T-ALL cases.

Although HIV-1 seems an interesting candidate due to its target cell specificity and possibly also due to its cell killing capacity, safety is an obvious issue for a therapeutic virus based on this pathogen. The therapeutic virus should not be able to infect and replicate in untransformed cells but should exclusively infect and kill leukemic T cells. To achieve this goal, we considered the long-standing observation that the HIV-1 accessory proteins vif, vpR, vpU, and nef are dispensable for *in vitro* replication in leukemic T-cell lines, yet essential for replication in untransformed peripheral blood mononuclear cells (PBMC). The vif protein is essential for replication in PBMC and T cells expressing APOBEC3G to prevent hypermutation of newly made HIV-DNA by this cellular cytidine deaminase (12, 13). Many leukemic T-cell lines lack this deaminase activity (12, 14–16), which makes vif a nonessential viral protein. Another, yet unidentified, cellular host restriction factor is inhibited by the vpU protein (17). Furthermore, vpU plays a role in particle release (18) and degradation of CD4 (19, 20). The vpR protein is incorporated into viral particles (21) and increases the duration of the G_2 phase in untransformed cells, which is optimal for HIV-1 long-terminal repeat (LTR) transcription (22). In addition, vpR is needed for nuclear import of the HIV-1 preintegration complex in quiescent cells (23). Both cell cycle arrest and nuclear import are not required for replication in T-cell lines. In fact, there is evidence of selection against a functional

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

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vpR protein in tissue culture studies of HIV-1 infection (22, 24). Nef down-regulates cell-surface receptors (including CD4), interferes with signal transduction pathways, enhances virion infectivity and viral production, induces apoptosis in bystander cells, but also protects infected cells against apoptosis (reviewed in refs. 25–28). Furthermore, it has been proposed that nef promotes infection of resting cells in a B-cell–dependent manner (29) and that nef is important for infection of quiescent cells (30–32). These nef functions augment viral replication in untransformed cells, but nef is usually much less important for replication in leukemic T-cell lines. All nef functions should be neutralized in our therapeutic virus. In particular, the dual role of nef in regulation of apoptosis needs to be abolished in this viral therapy. Preventing apoptosis in untransformed bystander cells is crucial for the safety of our approach, whereas increased apoptosis of infected leukemic cells would be beneficial for the cell killing capacity of the therapeutic virus. The combined literature data convinced us that it should be feasible to construct a T-cell leukemia–specific version of HIV-1.

In this study, we constructed an HIV-1 variant with deletions of the accessory genes encoding vif, vpR, vpU, and nef. In addition, a large deletion in the U3 region of the LTR promoter was introduced. *In vitro* studies showed that this virus replicates efficiently in different T-cell lines, but this mini-HIV variant was unable to replicate in untransformed cells. Viral replication results in efficient killing of the infected T cells, and this candidate therapeutic virus was able to selectively remove leukemic T cells from a coculture with untransformed cells.

Materials and Methods

Cells lines. C33A cervix carcinoma cells (ATCC HTB31; ref. 33) were grown as a monolayer in Dulbecco's minimal essential medium supplemented with 10% (v/v) FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, 20 mmol/L glucose, and minimal essential medium nonessential amino acids at 37°C and 5% CO_2. These cells were transfected by the calcium phosphate method as described previously (34).

The human T lymphocyte cell lines SupT1 (ATCC CRL-1942; ref. 35), MT2, MT4, A3.01, C8166, CEMx174, and Jurkat (ATCC TIB-152) were cultured in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (v/v) FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C and in 5% CO_2. Transfections were carried out by electroporation as described (36) using a Bio-Rad Gene Pulser (Hercules, CA). PBMC from four healthy donors were mixed and grown under the same conditions as the T-cell lines with the addition of 100 units/mL human interleukin (IL)-2 after initial stimulation with phytohemagglutinin (5 μg/mL) for 2 days. Infections were done with C33A-produced HIV-1 stocks with the indicated amounts of virus.

CA-p24 levels. Culture supernatant was heat inactivated at 56°C for 30 minutes in the presence of 0.05% Empigen-BB (Calbiochem, La Jolla, CA). CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom, Berlin, Germany) as the capture antibody, alkaline phosphatase–conjugated anti-CA-p24 monoclonal antibody (EH12-AP), and the AMPAK amplification system (Dako Diagnostics Ltd., Glostrup, Denmark) as described previously (37, 38). Recombinant CA-p24 expressed in a baculovirus system was used as the reference standard.

NL4-3 virus stock preparation from half genomes and virus evolution. The HIV-1 molecular clone used in the initial replication experiments is the chimera pNL4-3, which contains the 5′ half of NY5 and the 3′ half of LAI sequences joined at a shared *Eco*RI site in the *vpR* gene (39). The different deletion mutants were described previously (40). Infectious virus was reconstructed from the two half genomes by *Eco*RI digestion and subsequent ligation. Virus stocks were generated by transfection of this ligation mixture into SupT1 cells and harvesting of the virus at peak infection as judged by the appearance of massive syncytia. The supernatants of the cell cultures were passed through a 0.22-μm filter and stored in small aliquots at −70°C. These virus stocks were quantitated by CA-p24 ELISA and used for infections. Virus evolution was initiated with virus inoculums corresponding to 5 ng of CA-p24 for the cell lines SupT1, MT2, and C8166 and 50 ng of CA-p24 for the cell lines MT4, A3.01, and Jurkat. Virus replication was monitored by syncytia formation when possible (SupT1, MT2, C8166, and Jurkat) and otherwise by CA-p24 measurement (MT4 and A3.01). Cell-free supernatant (0.5-400 μL) was transferred at the peak of infection to uninfected cells.

DNA-constructs. Full-length molecular HIV-1 clones were made in the pLAI background (41) with an additional *Cla*I restriction site. First, the subconstruct, pKS-AS, was made by ligating the 3,816-bp *Apa*I-*Sal*I fragment from pLAI into the polylinker of pBluescript II KS (Stratagene, La Jolla, CA). The *Cla*I site was introduced near the 5th codon of the integrase open reading frame (ATA to ATC at position 3,825 relative to the transcription start site of HIV-1) by the MutaGene *in vitro* mutagenesis kit (Bio-Rad) and the oligonucleotide RJ001 (5′ GGG CCT TAT CGA TTC CAT CTA 3′). This plasmid pKS-AS-001 was cut with *Apa*I and *Sal*I and the 3,816-nucleotide (nt) fragment with the *Cla*I site was inserted into pLAI to yield the infectious molecular HIV-1 clone pLAI-001.

The generation of pLAIΔ5 occurred in three steps. PCR with primers RJ001 and 6N (5′ CTT CCT GCC ATA GGA GAT GCC TAA G 3′) was done with the pDR2483 plasmid (40) as template to generate a 882-bp PCR fragment (encompassing a 618-bp deletion spanning the open reading frames of *vif* and *vpR*). This PCR fragment was cut with *Cla*I and *Eco*RI and cloned into pLAI-001 to yield pLAIΔ2 (*vif-vpR*). pLAIΔ3 was made by replacing the wild-type 2,722-bp *Eco*RI-*Bam*HI fragment in pLAIΔ2 by the corresponding fragment of pDR2487 (40) with a 117-nt deletion in the *vpU* open reading frame. A 7,833-bp *Xba*I-*Bam*HI fragment from pLAIΔ3 was used in a triple ligation with a 2,041-bp *Afl*II-*Xba*I vector fragment from pLAI-001 and a 642-bp *Bam*HI-*Afl*II fragment from pDR2486 (40) to make the pLAIΔ5 plasmid (*vif-vpR-vpU-nef*-U3).

All constructs were verified by restriction enzyme analysis and BigDye terminator (Applied Biosystems, Foster City, CA) sequencing with appropriate primers on an automatic sequencer (Applied Biosystems DNA sequencer 377). Plasmid DNA was isolated with Qiagen Plasmid isolation kits according to the protocol of the manufacturer (Qiagen, Chatsworth, CA).

Fluorescence-activated cell sorting analysis. Flow cytometry was done with R-phycoerythrin–conjugated mouse monoclonal anti-human CD4 (clone MT310, Dako) and FITC-conjugated mouse monoclonal anti-human CD8 (clone DK25, Dako). The cells from 1-mL culture were collected (4 minutes at 4,000 rpm, Eppendorf centrifuge) and incubated with a mixture of both monoclonal antibodies in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FCS) for 30 minutes at room temperature and washed with 800-μL FACS buffer. The cells were subsequently collected (4 minutes at 4,000 rpm, Eppendorf centrifuge) and resuspended in 20 μL of 4% paraformaldehyde. After a 5-minute incubation at room temperature, 750 μL of FACS buffer were added and the suspension analyzed on a FACScalibur flow cytometer and CellQuest Pro software (BD Biosciences, San Jose, CA). The machine was set for a 30-second collection time. Cell populations were defined based on forward/sideward scattering and isotype controls were used to set markers. For the mixed SupT1 and PBMC culture experiments, gates for PBMC ($CD4^+CD8^-$ and $CD4^-CD8^+$) and SupT1 ($CD4^+$ and $CD8^+$) were set using a separate control culture. Twenty-four consecutive T-ALL samples of children under 18 years were immunophenotyped at diagnosis. In addition, CD4-FITC/CCR5-PE/CD3-PerCP/CD8-APC and CD4-FITC/CXCR4-PE/CD3-PerCP/CD8-APC combinations were used.

Virus rescue assay. To test for low level replication in PBMC, we used a virus rescue assay. At day 13 post infection of PBMC, the cells from 1-mL culture were collected (4 minutes at 4,000 rpm, Eppendorf centrifuge), washed once with 1-mL PBS, and resuspended into medium. A dilution series (1, 10×, 100×, 1,000×, 10,000×) was made and each dilution subsequently mixed with SupT1 cells. The cell cultures were regularly inspected for virus replication by CA-p24 ELISA on the culture supernatant and visual inspection for syncytia formation.

Mixed culture SupT1/PBMC infection. Freshly isolated PBMC were stimulated for 2 days with phytohemagglutinin (5 μg/mL), washed twice with medium, and mixed with SupT1 cells. The cell mixture was analyzed by FACS staining for CD4 and CD8 as described above. The culture was divided into equal 10-mL samples, containing ~1 million PBMC and 2 million SupT1 cells, which were infected with different virus variants (input 40 ng CA-p24). Daily samples were taken for CA-p24 ELISA and anti-CD4/CD8 FACS analysis.

Results

Construction and testing of HIV-1 deletion variants. For an initial survey of the effect of removing multiple accessory functions from the HIV genome, we used a set of deletion mutants that were previously constructed as 5′ and 3′ half genomes (Fig. 1). These constructs are based on the CXCR4 using NL4-3 molecular clone (40). Substantial deletions were introduced to remove as much as possible the individual open reading frames without affecting overlapping frames and/or critical sequence elements. The mutations were designed such that a stop codon was present immediately after the deletion (40). Because of the substantial deletions, it seems impossible that such mini-HIV-1 variants could restore the genetic information, especially because there are no homologous genes or sequence elements known to be present in the human genome. The 5′ genome half contained either a deletion of the *vif* gene, the *vpR* gene, or both. The 3′ genome half contained either a deletion of the *vpU* and *nef* genes or the *nef* gene and the upstream, nonessential part of the U3 domain of the viral LTR promoter sequences. The *nef* deletion removes the 5′ part of this gene that is positioned upstream of the LTR promoter. The U3 deletion removes the 3′ part of the overlapping *nef* gene.

The different 5′ and 3′ HIV-1 genome halves were combined by *Eco*RI digestion and ligation as described previously (40). The resulting DNA mixture was used to transfect the leukemic T-cell -line SupT1. The supernatant was harvested at the peak of infection as witnessed by massive syncytia and used as virus stocks. In total, eight HIV-1 deletion variants were made, including four single deletions (*vif*, *vpR*, *vpU*, and *nef*), one double mutant (*vif-vpR*), one triple mutant (*vpR-nef*-U3), and two quadruple mutants (*vif-vpR-vpU-nef* or $\Delta 4^{A}$ and *vif-vpR-nef*-U3 or $\Delta 4^{B}$). The virus stocks were used to infect the leukemic T-cell line SupT1 and PBMC from healthy volunteers as untransformed control cells. Virus replication was monitored over time by measuring CA-p24 protein production in the culture medium (Fig. 2). The differences between the two cell systems are profound. Deletion of up to four accessory genes has little effect on HIV-1 replication in the transformed SupT1 cells (Fig. 2, *left*). Similar results were obtained in the T-cell lines A3.01 and CEMx174, and the human T-cell lymphotrophic virus-I–transformed T-cell lines MT2, MT4, and C8166 (see Supplementary figures). In contrast, only the individual removal of the *vpU* and *nef* genes was compatible with HIV replication in PBMC (Fig. 2, *right*). The other single deletion mutants (*vif* and *vpR*) and all combined deletions abrogated virus replication. These results indicate that it is feasible to remove at least four viral gene segments without grossly affecting virus replication in the context of a leukemic cell, but these mini-HIV variants seem completely unable to replicate in untransformed PBMC.

Long-term culturing of the mini-HIV-1 variants. The genetic stability of the mini-HIV-1 constructs was tested in long-term infection experiments. With a focus on the Δ4 viruses, the deletion mutants were passaged repeatedly on a variety of T-cell lines for up to 162 days. We analyzed 11 independent cultures of distinct virus-host cell combinations (see Supplementary data). Virus evolution could optimize replication of the mini-HIV-1 variants and this was apparent in some cultures. However, none of the evolved viruses restored their ability to replicate in PBMC. The integrity of the introduced gene deletions was checked by sequence analysis of large PCR fragments that overlap the deletions, and no repair was apparent in any of the 11 cultures tested. Obviously, there should be other changes within the viral genome that are responsible for the observed replication changes, but we did not detect any consistent mutations in the viral gene segments that were analyzed.

Construction of the Δ5 virus; replication studies and cell killing capacity. Based on the previous results, we wanted to combine all five deletions in the LAI molecular clone (Δ5; *vif-vpR-vpU-nef*-U3). For cloning purposes, we first made a Δ3 construct (*vif-vpR-vpU*) that was included in the subsequent analyses. The availability of these plasmid infectious clones allowed us to test distinct phases of the virus replication cycle. The production of

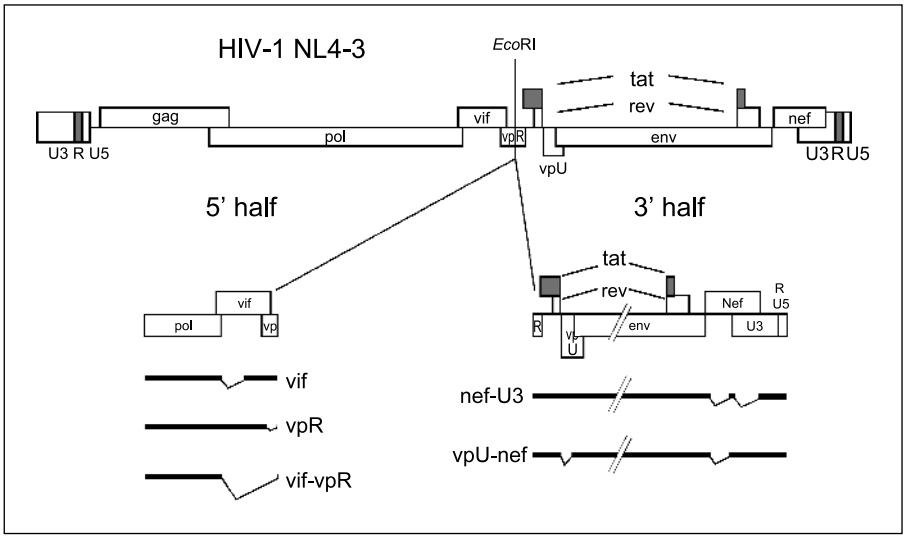

Figure 1. Construction of the mini-HIV-1 variants. Schematic overview of the HIV-1 deletion variants that were made by *in vitro* recombination of 5′ and 3′ fragments of HIV-1 NL4-3 at the *Eco*RI site as described previously (40). The location of the deletions in the different constructs is indicated.

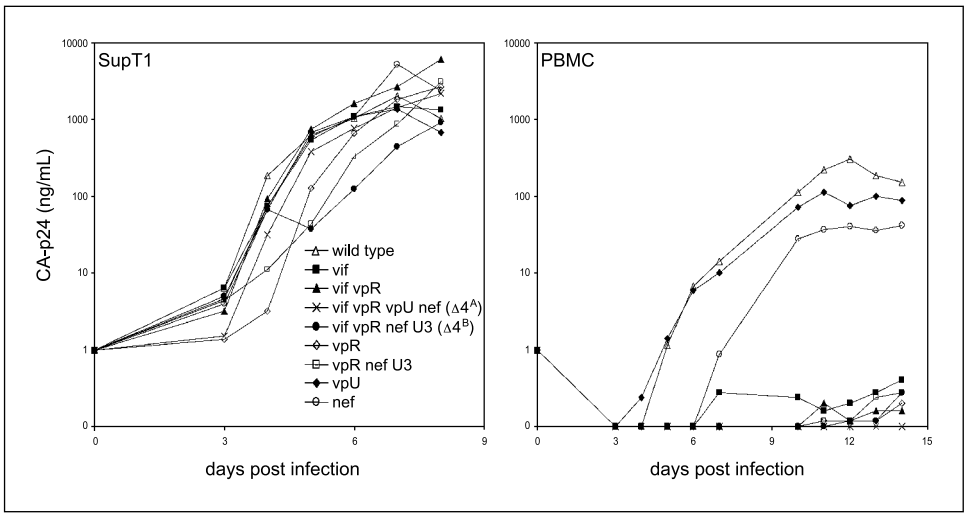

Figure 2. Replication of the mini-HIV-1 viruses in the SupT1 T-cell line and PBMC. Virus stocks of the HIV-1 deletion variants were produced in transfected SupT1 cells and were used to infect SupT1 cells (*left*) and PBMC (*right*). Virus replication was monitored by frequent measuring of CA-p24 in the supernatant after an initial virus administration corresponding to 25 ng CA-p24 in a 5-mL culture. Similar results were obtained in three independent infections (with different PBMC pools).

virus particles was tested by transfection of these plasmids in C33A cells that lack the CD4 receptor to prevent multiple rounds of infection. We measured no significant reduction in the amount of virus produced by the Δ3 and Δ5 mutants compared with the wild-type LAI (Fig. 3). These results confirm that none of the deleted genes or sequence elements play an important role in viral gene expression (transcription, splicing, and translation) and assembly of new virions.

We next compared the replication capacity of the Δ3 and Δ5 variants and wild-type HIV-1 on SupT1 cells (Fig. 4, *left*). The Δ3 mutant replicated efficiently, albeit somewhat reduced compared with wild-type HIV-1. A profound delay in replication was scored for the Δ5 virus, but all cells were eventually infected as witnessed by the appearance of massive syncytia. FACS analysis was used to determine the number of live cells in the infected cultures and an uninfected control SupT1 culture. The cell killing capacity was determined by dividing the number of cells in the infected culture by the number of cells in the control culture (Fig. 4, *right*). These results indicate that both the Δ3 and Δ5 viruses are competent to initiate a spreading infection that leads to the killing of all leukemic T cells. The cell killing capacity seemed to correlate with the replication capacity of these viruses.

The Δ3 and Δ5 viruses were also tested for their ability to replicate in PBMC cultures (Fig. 5, *left*). No spreading infection was scored for the Δ3 and Δ5 viruses, but the former variant produced a transient virus peak at day 3 and a slight increase in the CA-p24 level at the end of the replication experiment (days 11 and 12). Nevertheless, feeding these cultures with fresh PBMC did not result in detectable virus spread. We also used a more sensitive test to detect the presence of infectious virus. The PBMC harvested at day 13 were washed and subsequently used to infect SupT1 cells, which acts as a very sensitive indicator cell line for the presence of replication-competent HIV-1. Indeed, infectious virus could be recovered from the Δ3 culture, but only with 1,000-fold more input material as compared with the wild-type control (results not shown). Perhaps more important, no infectious Δ5 virus could be recovered, thus confirming the total replication block of this virus in untransformed PBMC.

The PBMC cultures were also analyzed for virus-induced cell killing (Fig. 5, *right*). PBMC contain mainly $CD4^+CD8^-$ and $CD4^-CD8^+$ cells that can be distinguished by FACS analysis. Only the former cells can be infected by HIV-1, and we therefore determined the ratio of the two cell types during virus replication. The results show efficient killing of the $CD4^+$ cells by the wild-type virus at day 3, coinciding with the peak in CA-p24 production. However, no decrease in the $CD4^+/CD8^+$ cell ratio is apparent for the Δ3 and Δ5 viruses, confirming their inability to cause a spreading PBMC infection.

Mini-HIV-1 virus replicates in a variety of leukemic T-cell lines. The results obtained thus far are summarized in Table 1. The Δ5 mini-HIV-1 virus is able to replicate in and to kill the leukemic SupT1 cells, but is not able to initiate a spreading infection in PBMC, and has consequently no effect on the $CD4^+$ cell population. The Δ3 virus has the advantage of being a more potent replicating virus in SupT1 cells, but it has the disadvantage that a very low level of replication in PBMC cannot be ruled out. Thus, the Δ5 backbone seems the preferred choice for the future development of a safe therapeutic virus.

Obviously, we aim for a therapeutic virus able to kill a variety of leukemic cells. We therefore analyzed replication of the Δ3 and Δ5 virus in several transformed T-cell lines (Fig. 6). Delayed replication was observed in all T-cell lines for the deletion mutants compared with the wild-type control. The Δ5 virus showed the most severe delay in Jurkat cells, but the wild-type control does not replicate vigorously in these cells either. As in the SupT1 experiments, all cultures were inspected for cell death

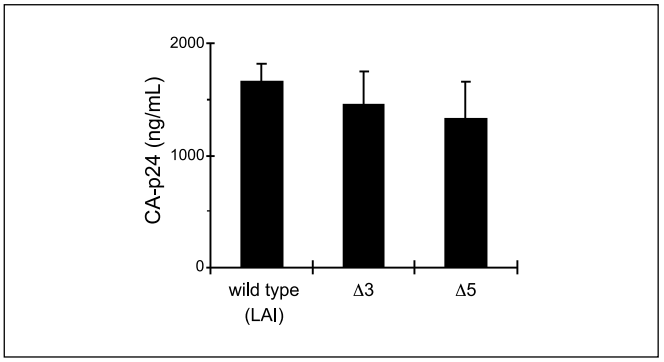

Figure 3. Virus production capacity of the mini-HIV-1 variants. Virus production was measured by CA-p24 ELISA on transfection of the nonsusceptible C33A cell line. *Columns,* average CA-p24 values of three independent transfections; *bars,* SD.

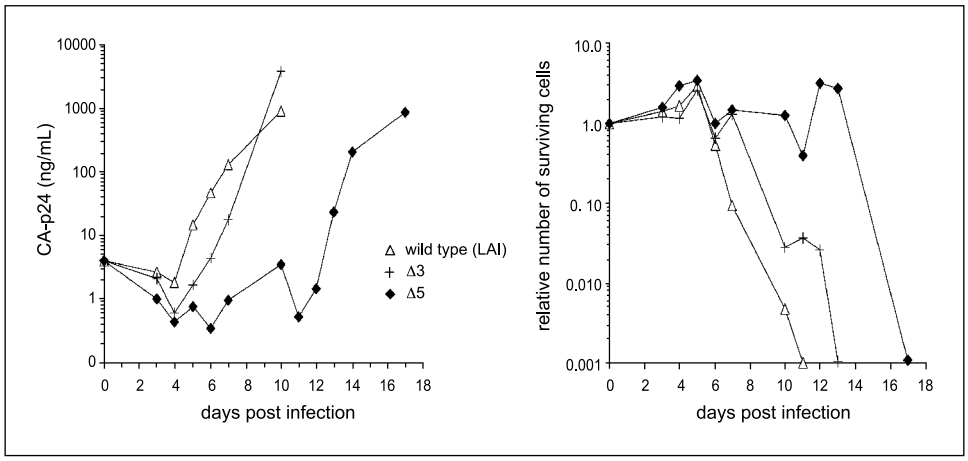

Figure 4. Replication and cell killing by the Δ5 virus in SupT1 cells. Virus replication of LAI (△), LAI-Δ3 (+), and LAI-Δ5 (♦) was determined by frequent measuring of the supernatant CA-p24 concentration after an initial virus administration corresponding to 20 ng CA-p24 in a 5-mL SupT1 culture. The cell killing capacity of these viruses was determined by calculating the number of cells compared with an uninfected culture. The (relative) cell number of each culture was determined by a 30-second time limit FACS analysis.

and complete cell death was obtained with both mini-HIV variants in the C8166, MT2, and MT4 cultures (results not shown). No complete cell killing was apparent in the Jurkat culture, but the same result was obtained for the wild-type virus. The kinetics of cell killing by the three viruses was directly related to their replication capacity in these cell lines.

Mini-HIV-1 can selectively remove leukemic T cells from a mixed culture. In the real scenario of viral-mediated treatment of a leukemic patient, the blood will contain a mixture of leukemic and untransformed cells, and the viral therapeutic agent should selectively replicate and kill the leukemic target cells. To mimic this situation in our *in vitro* culture system, we made cocultures of the SupT1 cell line and PBMC. These cells can easily be distinguished by FACS analysis using the CD4 and CD8 surface markers. SupT1 cells represent double-positive intermediate T cells ($CD4^+CD8^+$), whereas PBMC contain a mixture of $CD4^+CD8^-$ and $CD4^-CD8^+$ populations (Fig. 7, *left*). The starting mixed cell culture was split into four samples and were mock infected or infected with an equal amount of the wild-type, Δ3, or Δ5 virus (Fig. 7, *right*). The cell composition was followed over time, showing the more rapid proliferation of leukemic SupT1 cells versus PBMC in the uninfected control (mock, *top*). The wild-type LAI virus replicates efficiently in this coculture system, which results in the removal of both the SupT1 cells and the $CD4^+CD8^-$ fraction of PBMC around days 6 to 8. In agreement with the replication curves in Fig. 4, the removal of SupT1 cells is somewhat delayed for the Δ3 virus and significantly delayed for the Δ5 variant (day 12), but these viruses did not affect the $CD4^+CD8^-$ PBMC fraction. These results indicate that it is possible to selectively remove leukemic T-cells from a PBMC mixture by the use of a mini-HIV-1 variant that selectively lost the ability to replicate in untransformed cells.

T-cell acute lymphoblastic leukemic cells express the CD4 and CXCR4 receptors. We have optimized a CXCR4 using HIV-1 variant for the selective replication in leukemic T cells. An initial survey of 24 consecutive T-ALL clinical samples was done to score for the presence of the viral CD4 receptor and the CXCR4/CCR5 coreceptors. The T-ALL leukemic cells were subdivided into immature ($CD1^-$, surface $CD3^-$), intermediate ($CD1^+$, Sm $CD3^{-/+}$), and mature ($CD1^-$, $CD3^+$) T cells. None of the leukemic T cells express CCR5, but a significant fraction of the cells express the CXCR4 marker ranging from an average of 35% positive cells (mature T-ALL), 50% (immature T-ALL), to 80% positive cells (intermediate T-ALL). The expression of CD4 follows approximately the same pattern: with 10%, 20%, and 40% positive cells in the respective subsets. These results indicate that the initial choice of a CD4-CXCR4 using HIV-1 strain as therapeutic virus seems appropriate. The SupT1 T-cell line can be classified as an intermediate T-ALL ($CD4^+$, $CXCR4^+$, and $CCR5^-$), which may be the optimal leukemic target subset for further development of HIV-based virotherapy.

Discussion

The use of nonreplicating viral vectors in gene therapy approaches has become standard because of their efficiency to

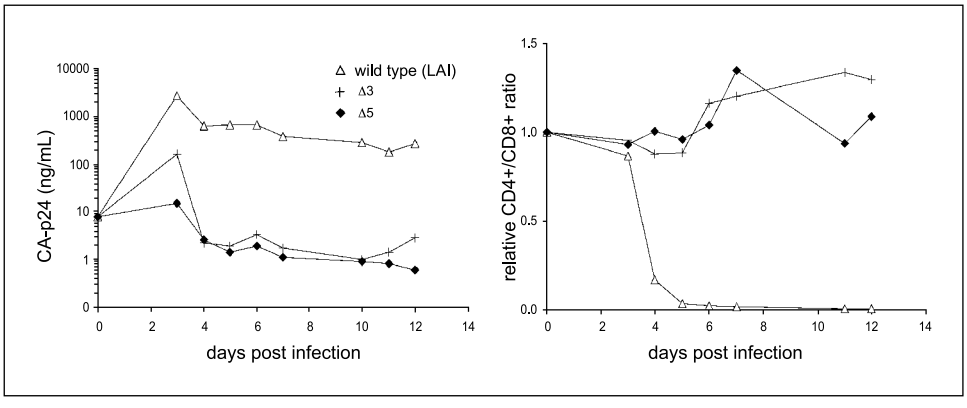

Figure 5. No replication and cell killing by the Δ5 virus in PBMC. Virus replication of LAI (△), LAI-Δ3 (+), and LAI-Δ5 (♦) was determined by frequent measuring of the supernatant CA-p24 concentration after an initial virus administration corresponding to 40 ng CA-p24 in a 5-mL PBMC culture. The CD4+ and CD8+ cell populations in the infections and a control without virus were quantified by a 30-second time limit FACS analysis and the CD4/CD8 ratio was calculated. CD4/CD8 ratio in the infections normalized for the control uninfected PBMC culture.

Table 1. Virus replication and cell killing capacity

	SupT1		PBMC		
	Replication	Cell killing	Replication	CD4+ killing	SupT1 rescue
LAI	++	++	++	++	+
LAI-Δ3	++	++	−	−	+
LAI-Δ5	+	++	−	−	−

transfer genetic information into target cells. In the search for new anticancer treatments, replication-competent viruses are being investigated as infectious and cytotoxic agents that should specifically target malignant cells. These oncolytic viruses are based on adenovirus (1), herpesvirus (42), vesicular stomatitis virus (43), poliovirus (44), and reovirus (45, 46). In this report, we describe the development of a leukemia-specific HIV-1 variant for use as a therapeutic virus against T-ALL.

HIV-1 was chosen as the starting material for our studies because of its restricted host cell range, which overlaps the cell types transformed in T-ALL, and its ability to kill infected cells, either directly by the virus or indirectly by the immune system. As an *in vitro* T-ALL model for virotherapy, we used the SupT1 cell line that is derived from an 8-year-old T-ALL patient (35). Screening for T-ALL markers confirmed that SupT1 is representative for an intermediate T-ALL, although it lacks the terminal deoxynucleotidyl transferase marker. To convert HIV-1 into a safe therapeutic virus, its ability to replicate in untransformed lymphocytes needs to be abolished firmly. To this end, we constructed minimal HIV-1 variants with up to five deletions (*vif*, *vpR*, *vpU*, *nef*, and *U3*). Such mini-HIV-1 variants have the desired phenotype: they replicate efficiently in SupT1 cells, yet are completely replication impaired in PBMC. No replication in long-term PBMC cultures was apparent for the Δ5 virus, even when the susceptible SupT1 cells were added as sensitive indicator cells. Furthermore, SupT1 cells could be selectively infected and killed in a mixed culture of leukemic and untransformed cells (SupT1 and PBMC). This Δ5 virus maintains only the five essential HIV-1 genes (*gag*, *pol*, *env*, *tat*, and *rev*), and its RNA genome size is reduced from 9,306 to 8,081 nt. We note that the

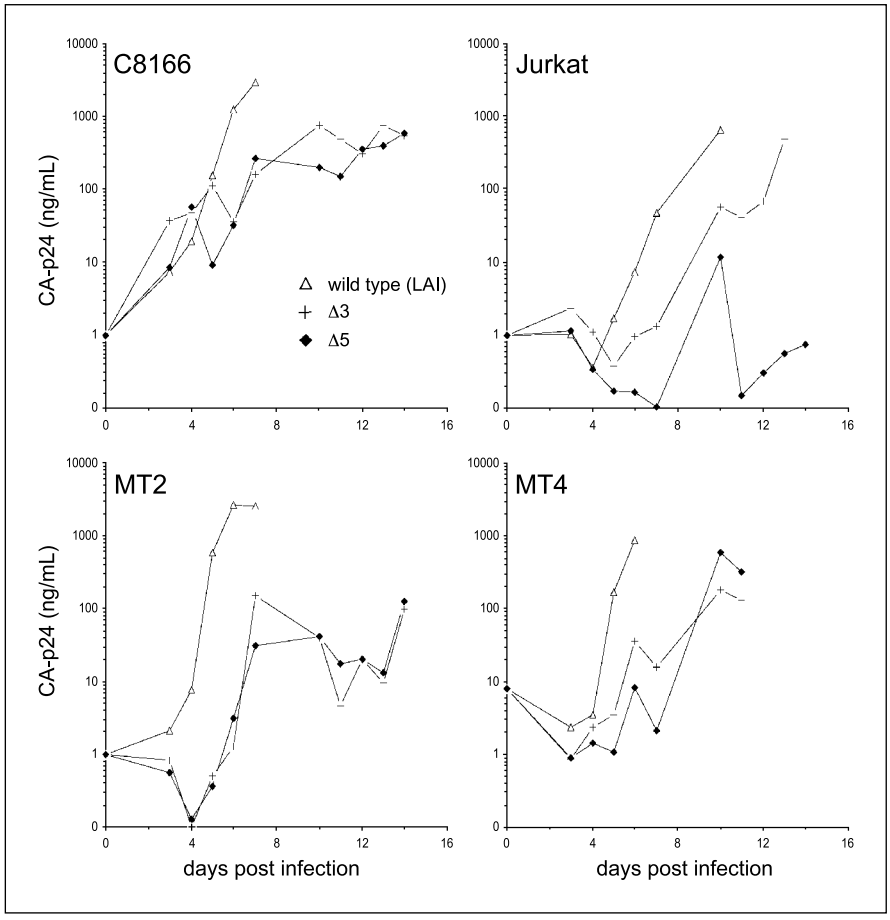

Figure 6. Replication of the mini-HIV-1 constructs in other T-cell lines. HIV-1 infections in the indicated T-cell lines were initiated with virus corresponding to 1 ng CA-p24. See Fig. 4 for details.

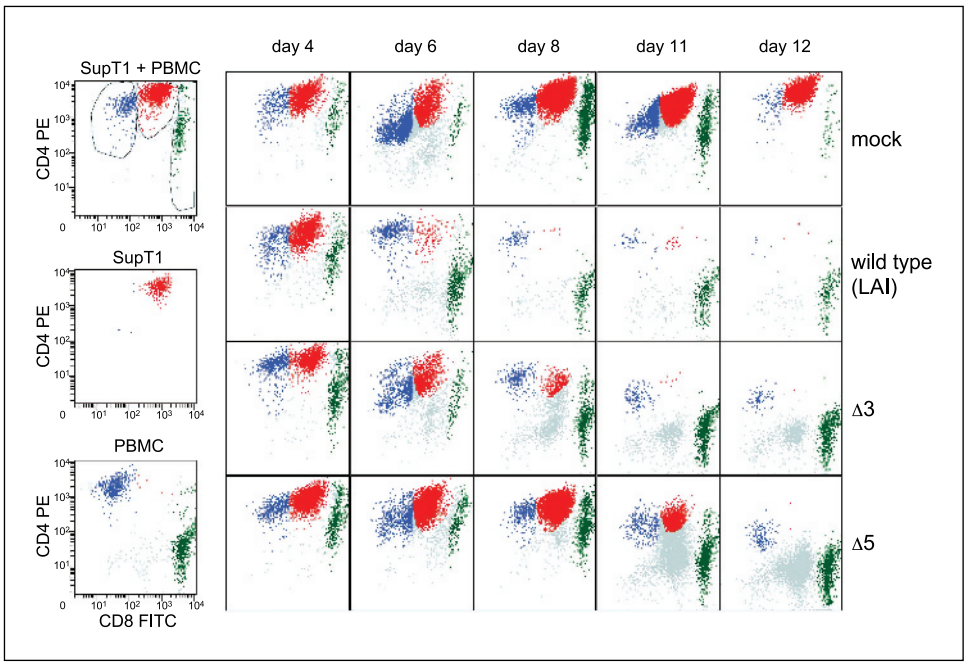

Figure 7. Selective killing of leukemic cells in SupT1/PBMC cocultures by mini-HIV-1 variants. Infections were started with virus corresponding to 40 ng CA-p24 or no virus (mock infections). *Left,* FACS dot plot of the initial mixture (*top*) with controls for separate PBMC and SupT1 cultures (*bottom*). The gates for CD4+ PBMC (*blue*), SupT1 (*red*), and CD8+PBMC (*green*) are indicated. *Right,* plots for the different infections were followed longitudinally.

construction of such a mini-HIV variant, stripped of all nonessential gene segments, was previously proposed by Temin (47) to convert HIV-1 into a "simple" retrovirus that may be useful as a safe live-attenuated virus for vaccination purposes.

Several serious safety features need to be addressed before one can propose the use of attenuated HIV-1 viruses for the treatment of leukemia or vaccination purposes. First, the high mutation and recombination rate of HIV-1 that allows the generation of escape variants over time. This is a common theme in antiviral therapy, which leads to the appearance of drug-resistant mutants. Furthermore, we have previously shown that the Δ3 HIV-1 vaccine candidate with deletions in the U3 region and *nef* and *vpR* genes is able to partially restore replication in prolonged cultures (48). We did not observe restoration of replication capacity with the various mini-HIV-1 variants in primary cells, even in so-called forced evolution experiments. Obviously, the large number of deletions in the Δ5 virus reduces the change of obtaining viral escape variants. In addition, some functions may be easier to compensate for; e.g. vpR has a "transcriptional" component (49) which may be overcome by improvement or rearrangement of transcription factor binding sites in the LTR promoter (48). However, the loss of specific interactions with host cell factors such as that between vif and cellular protein APOBEC3G (12, 50–52) may be much more difficult to replace by other viral components.

A second safety issue is the capacity of HIV-1 to induce apoptosis in uninfected bystander cells. This activity has been linked to the viral nef, vpR, Tat, gp120, and gp41 proteins (28, 53–57), but several of these functions are deleted in the Δ5 virus. We observed no bystander effect in the PBMC/SupT1 coculture experiment; the SupT cells were efficiently killed by the virus, but no effect on PBMC was scored.

A third safety concern of the use of replicating retroviruses like HIV-1 is the possibility of insertional mutagenesis because the virus integrates at a random position in the host cell chromosomes. In fact, a recent gene therapy trial using a retroviral vector caused two cases of integration-induced leukemia, despite the overall success of this therapy (58). There are two reasons why this issue may not be troublesome for the proposed virotherapy. We use the genetic backbone of HIV-1, and infection by this lentivirus has not been linked to integration-induced oncogenesis, despite massive virus replication in many HIV-infected individuals. Furthermore, our strategy is supposed to restrict the establishment of integrated HIV provirus in primary cells. Most notably, removal of the vpR function will block nuclear import of the infecting viral particle, and the absence of vif may result in APOBEC3G-mediated degradation of newly made viral cDNA. It is possible that removal of the accessory genes leads to alternation of the cell killing ability. The cell killing capacity of the mini-HIV variants could be enhanced by insertion of genes that trigger apoptosis (e.g. *TRAIL* to induce the death pathway, cytolytic genes for perforin or granzyme). Alternatively, one could elicit or enhance the immune response to virus-infected cancer cells by insertion of genes (e.g., for cytokines such as IL-2 and tumor necrosis factor α or chemokines such as IL-10 or SDF-1) that are able to recruit and activate immune cells.

To further improve the safety of a therapeutic HIV-1 virus, one could use the conditionally replicating HIV-1 variant that we recently constructed (48, 59–63). We propose to transplant the Δ5 deletions in this HIV-rtTA virus. In the therapeutic setting, this will allow us to turn off virus replication after successful removal of the leukemic cells. This will effectively block ongoing replication and evolution, and thus the generation of pathogenic escape variants.

We show that a significant number of T-ALL cases express the CD4 and CXCR4 surface markers that are required for mini-HIV-1 virotherapy. Because CXCR4 is involved in the control of migration of T cells and stem cells, virotherapy is of special interest for the eradication of residual cells (64–66). Jak kinase activation is the frequent cause of uncontrolled cell proliferation in malignancies. The proposed mini-HIV-1–based therapy might

lead to apoptosis of T-lymphoblasts via interference with Jak kinase activity because Jak kinase activation lies downstream of the CXCR4-LFA-1 pathway (67). The proposed strategy might also be applicable in patients suffering from natural killer leukemia as CXCR4 is expressed on natural killer T-cells (64). Application of virotherapy in cytokine-induced CXCR4 expression of myelomonocytic leukemia and B-cell leukemia might be an additional interesting field of research (11), although the reduction in CXCR4 expression by IL-2 therapy (64) might hamper this approach.

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