Gene transfer in humans using a conditionally replicating lentiviral vector


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We report findings from a clinical evaluation of lentiviral vectors in a phase I open-label nonrandomized clinical trial for HIV. This trial evaluated the safety of a conditionally replicating HIV-1-derived vector expressing an antisense gene against the HIV envelope. Five subjects with chronic HIV infection who had failed to respond to at least two antiviral regimens were enrolled. A single i.v. infusion of gene-modified autologous CD4 T cells was well tolerated in all patients. Viral loads were stable, and one subject exhibited a sustained decrease in viral load. CD4 counts remained steady or increased in four subjects, and sustained gene transfer was observed. Self-limiting mobilization of the vector was observed in four of five patients. There is no evidence for insertional mutagenesis after 21–36 months of observation. Immune function improved in four subjects. Lentiviral vectors appear promising for gene transfer to humans.

In preclinical studies, a highly efficient T cell culture system and a lentiviral vector containing a long antisense sequence to HIV envelope were developed (1–3). In a prior phase I trial, autologous CD4 cells from HIV-infected subjects were expanded ex vivo by using anti-CD3/CD28 beads as artificial antigen presenting cells, and adoptive transfer of the activated CD4+ T cells was shown to result in dose-dependent increases in the steady-state CD4+ T cell counts by induction of resident CD4+ cell proliferation and a sustained decrease in CCR5 expression in vivo (4). Concurrently, an HIV-1-based lentiviral vector expressing a 937-base antisense gene against the HIV envelope, termed VRX496, was developed (Fig. L4). This vector retains the full LTRs of HIV, and, therefore, expression of the antisense is up-regulated upon wild type HIV infection of the cell. This LTR-dependent transcriptional up-regulation is in contrast to self-inactivating vectors, where the LTRs are modified by deletion of the U3 region, and transgene expression is driven from a heterologous internal promoter. An advantage of using a long antisense sequence is that it targets multiple sites of HIV genomic RNA, constraining the pathogenic virus’ ability to form resistance mutants without adversely affecting viral fitness (2).

In preclinical studies bringing these two technologies together, the rationale for the present clinical study was supported by the potent antiviral effects that were demonstrated in vitro, regardless of patient status or the tropism of the infecting virus (5).

After extensive preclinical safety tests (6, 7) approval by institutional review boards, the Food and Drug Administration (FDA), the FDA’s Biological Response Modifiers Advisory Committee (now called the Cellular, Tissue, and Gene Therapies Advisory Committee), and the National Institutes of Health Office of Biotechnology Activities, a phase I open label nonrandomized clinical trial, was initiated to investigate the safety and tolerability of autologous T cells modified with the VRX496 vector. In this study, 5 subjects with HIV infection that was resistant to at least two antiviral regimens and who had a viral load >5000 copies per ml and CD4+ T cells counts between 200–500 cells per mm3 were serially enrolled. Each subject was given a single infusion of ~1 × 10^10 gene modified autologous CD4+ T cells in a single dose. The primary endpoints for safety were incidence of adverse events, viral load, CD4+ count, and emergence of a replication-competent lentivirus (RCL) derived from the vector (i.e., distinct from wild-type (wt)-HIV). The primary feasibility endpoint was the ability to manufacture autologous lentiviral engineered CD4+ T cells from subjects with drug-resistant HIV infection. Secondary endpoints included measurements of vector persistence and immunological functional assessments.

Results

Patient Characteristics. Baseline characteristics for the five subjects with chronic HIV infection who participated in the study are shown in Table 1. The first subject was infused in July 2003, and the fifth subject was infused in September 2004. All subjects had drug-resistant HIV infection, having received therapy with a mean 7.6 (range 4–10) different antiretroviral agents. Enrolled patients must have been on their current therapy for at least 3 months and agree to continue their therapy for at least 6 months. Four subjects elected to remain on their present failing therapy, and one subject was intolerant of antiretroviral therapy. Fig. 5, which is published as supporting information on the PNAS web site, provides the date of infusion for each patient and the dates of the initiation of their current anti retroviral regimens. Peripheral blood mononuclear cells were obtained by apheresis, the CD4+ T cells were enriched by negative selection, and the cells were transduced with the VRX496 lentiviral vector at a multiplicity of infection (MOI) of 5 as described in Supporting Methods, which is published as supporting information on the PNAS web site. All subjects were infused with the target dose of ~10 billion cells, and it is noteworthy that the CD4+ cells were efficiently transduced, as the average vector copy number per cell for the 5 infused products was 2.1 (range 1.0 to 4.1). This scale-up cell manufacturing process was performed under GMP-compliant conditions, confirming our preclinical data (3, 5), and substantiating the promise of lentiviral vectors as generally more efficient in transduction than other viral vectors in a number of...
There was no evidence of ongoing HIV replication as measured by before and after cell-expansion proviral copy numbers or the generation of a vector-derived RCL determined by biological RCL assay (Methods) in any of the cell products (Table 1), nor in any of the clinical vector lots to date (7). The infusions were well tolerated, and no subjects experienced serious adverse events that were judged as possibly, likely, or definitely related to the VRX496 cells.

**Primary Trial Endpoint.** Subjects were monitored at 1, 2, 3, 7, 14, and 21 days, 6 weeks and at 3, 6, and 9 months for viral load, CD4 count, emergence of any potential RCL, and for immunological parameters. Importantly, no subject has clinical or laboratory evidence of vector-derived RCL in vivo, which was evaluated by monitoring for VSV-G DNA in peripheral blood mononuclear cells (PBMCs), VSV-G RNA in patient plasma, generation of antibody against the VSV-G protein by ELISA (Table 2, which is published as supporting information on the PNAS web site), and a biological RCL assay performed on patient PBMCs 6 months after dosing. Mean viral load at screening ranged from 19,970 to 188,500 copies per ml, with a median viral load of 68,224. The change from baseline in viral load log values in all
patients is shown in Fig. 1B. Viral load changes greater than 0.5 log were noted as meaningful because they are outside of the variation of the viral load assay. Transient increases in viral load was observed in patients shortly after dosing presumably because of cytokine release; this increase has previously been observed (9). Subject 1 entered the study with a mean baseline viral load of 188,500 (5.27 logs) copies per ml, and, at the 6 and 12 month points, his viral load declined to 12,700 copies per ml and 36,300 copies per ml, which correlates to a drop of 1.17 and 0.71 logs, respectively. At the 6-month time point, subject 5 had a 0.75 log drop in viral load, and his viral load returned to baseline levels at the 9-month and 1-year time points.

The case history of subject 2 exemplifies our patients, for whom chronic HIV-1 infection was resistant to available antiretroviral agents (Fig. 1C). He is a 41 year old who has been HIV positive for at least 15 years. Medical history is significant for hyperlipidemia (related to antiretrovirals), peripheral neuropathy bilaterally in hands and feet (related to antiretrovirals), lymphadenopathy, and buccal wasting related to HIV lipodystrophy. Prior HIV therapies include zidovudine (1989), zalcitabine (1994–1995), sanquinavir (1994–1997), stavudine (1996–1999), lamivudine (1996–1999), indinavir (1996), ritonavir (1997–1999), amprenavir (1997–1999), and didanosine (1998–1999). His regimen at the time of enrollment included efavirenz (April 1998–), abacavir (November 2001–), didanosine (1998–1999), stavudine (1996–1999), lamivudine (1996–1999), and tenofovir (March 2003–).

Subject 2 was given a single infusion of VRX496 CD4+ T cells on October 13, 2003, when he had a mean baseline viral load of 54,100 copies per ml (4.64 logs). At 6 and 12 months, his viral load declined to 8,627 and 1,063, a drop of 0.8 and 1.7 logs, 54,100 copies per ml (4.64 logs). At 6 and 12 months, his viral load declined to 8,627 and 1,063, a drop of 0.8 and 1.7 logs, respectively (Fig. 1C). With the exception of subjects 3 and 4, no study participants changed antiretroviral therapy during the 1 year after infusion of VRX496 CD4+ cells. Subjects 1 and 2 changed regimens between years 1 and 2. Fig. 5 provides treatment histories for all patients.

At study entry mean CD4+ T cell counts ranged from 220 to 316 cells per μl (mean, 274). At the 1 year time point, CD4+ T cell counts were elevated relative to baseline in four of five study participants (Fig. 1D).

Persistence of the lentiviral engineered cells was assessed by quantitative PCR of peripheral blood mononuclear cells (Fig. 2A). There was prolonged engraftment as measured by detection of the unique sequence tag in most, with 2 of 5 subjects having detectable VRX496 CD4+ T cells at one year and later with frequencies of 0.04% and 0.023% of total PBMCs. The mean half-life of modified cells in circulation was 23.5 (±7.7) days, with a range from 19 to 37 days during the first 6 months after infusion. However, the apparent decay kinetics are complex and, presumably, represent the death of infused cells, migration to tissues and secondary lymphoid organs, and the accumulation of clonal progeny consequent to cellular division (10, 11). Evidence to support the latter is suggested by the observation that subjects 4 and 2 had detectable frequencies at 1 and 2 years respectively, after being below the limit of assay quantification at 9 months (0.02%). One may speculate that the 6 month drops in viral load observed in subjects 1 and 5 may have been sustained had the modified cells persisted, thus providing rationale for multiple infusions in follow-on trials.

A major concern for the use of a VSV-G pseudotyped HIV vector is the formation of a vector-derived RCL, particularly with VSV-G envelope sequences, because such RCLs would have a broader tropism than HIV. To address this safety issue, we used sensitive molecular assays for detection of VSV-G DNA and RNA in the final T cell product and in peripheral blood, respectively, at 3, 7, 21, and 90 days after infusion. VSV is an RNA virus and natural infection is possible although rare and typically limited to persons who work with livestock. Detection of VSV-G DNA would be a strong indicator that a recombination event has occurred, which may represent an RCL. Because the vector genome does not contain VSV-G sequences, persistent expression of VSV-G nucleic acid, in particular DNA, would indicate a recombination event between the vector and helper during vector production, indicating the possibility of a vector-derived RCL. Had this situation occurred, it would have triggered a full biological RCL assay as confirmatory testing. In addition to testing for VSV-G sequences, a biological amplification assay was used to test for vector-derived RCL in both the final cellular product and in patient PBMCs isolated by apheresis 6 months after infusion. No VSV-G envelope sequences were detected in any final product or in any subject (Table 2). In addition, no subject developed antibodies to VSV-G that could be detected in plasma as measured by ELISA.

Another potential safety concern associated with the use of lentiviral vectors is vector mobilization, also commonly referred to as conditional replication (12). Mobilization is possible for vectors that retain their full LTRs when packaging proteins are provided in trans such as during HIV infection. Although vector mobilization in vivo to nontarget tissues may have adverse safety consequences, mobilization of the vector payload into uninfected CD4+ cells could amplify the antiviral effects (12, 13). VRX496

Table 1. Baseline subject characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<td>M</td>
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<td>Caucasian</td>
<td>African American</td>
<td>African American</td>
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<td><strong>Baseline viral load</strong></td>
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<td>46,150</td>
<td>32,400</td>
<td>19,970</td>
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<tr>
<td><strong>Baseline CD4 count per mm³</strong></td>
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<td>316</td>
<td>273</td>
<td>308</td>
<td>220</td>
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<tr>
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<td>15</td>
<td>15</td>
<td>15</td>
<td>10</td>
<td>9</td>
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<tr>
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<td>5 NRTI, 4 PI</td>
<td>6 NRTI, 1 PI</td>
<td>2 NRTI, 1 NNRTI, 1 PI</td>
<td>5 NRTI, 1 NNRTI, 1 PI</td>
</tr>
<tr>
<td><strong>No. of VRX496 CD4 cells infused</strong></td>
<td>1 × 10⁶</td>
<td>1 × 10⁶</td>
<td>None</td>
<td>1 × 10⁶</td>
<td>1 × 10⁶</td>
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<tr>
<td><strong>Average vector copy no. per cell</strong></td>
<td>95</td>
<td>100</td>
<td>80</td>
<td>98</td>
<td>94</td>
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<tr>
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<td>&lt;50</td>
<td>&lt;50</td>
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</tr>
</tbody>
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NRTI, nucleoside reverse-transcriptase inhibitor; NNRTI, nonnucleoside reverse-transcriptase inhibitor; PI, protease inhibitor.

*Value determined at the time of cell harvest.


is an example of a conditionally replicating HIV vector that replicates only in the presence of wild type HIV, and it was
selected for the initial clinical evaluation because it displayed less efficient mobilization in vitro than other vectors [(3) and Biological Response Modifiers Advisory Committee Meeting, October 26, 2001, www.fda.gov/ohrms/dockets/ac/01/briefing/ 3794b3.htm], thereby maximizing safety as much as possible while retaining the wt-LTR for HIV-specific gene expression. To
determine vector mobilization in vivo, we tested for the presence of VRX496 genomic RNA in the plasma by quantitative RT-PCR assaying for the specific primer to a non-protein-encoding gene fragment of the GFP (Gtag), which is used to distinguish VRX496 from wt-HIV in the patient (Fig. 1A). Gtag RNA was
detected in all subjects after infusion (Fig. 2B). Vector mobili-

zation in plasma was transient as it was not evident after day 60. Subject 4 had the highest magnitude of mobilization, which may be related to the observation that he had the highest average vector copy per cell (4.0) in his infused CD4+ T cell product (Table 1).

To monitor for clonal outgrowth as a consequence of potential insertional mutagenesis, the T cell receptor repertoire was assessed in all subjects by using a PCR-based spectratyping assay. There were no changes toward increased skewing or oligoclonality and, importantly, no evidence of clonal outgrowth at 21–36 months after infusion. An example of the distribution of the T cell receptor V-β gene usage is shown in Fig. 6, which is published as supporting information on the PNAS web site. Insertion-site analysis was also extensively evaluated as described next.

Integration-Site Analysis. Detailed analysis of vector integration sites was performed because of concerns for insertional mutagenesis that have been raised by trials using oncoretroviral vectors (14). The sites of integration of the antisense env vector were examined in transduced T cells harvested before infusion into patients. Cells from all five of the subjects were analyzed, yielding a total of 192 unique integration site sequences. Integration sites were mapped onto the human genome sequence (Fig. 3A) and the distribution compared with human genomic features and previously determined HIV integration sites (15–18). Studies have shown that integration by HIV or HIV-based vectors was favored in gene-rich regions, and this trend was also seen for the antisense env vector (Fig. 3A; P = 2 × 10−10 for comparison with random placement). Similarly, integration by the antisense env vector was favored in features associated with gene dense regions such as G/C-rich sequences and Alu elements (P = 6.5 × 10−8 and P < 0.0001, respectively, for comparison with random). Integration by VRX496 was strongly favored in transcription units, with 74% of sites within these sequences (P < 1.2 × 10−14 for comparison with random), as seen in previous HIV data sets. A similar trend was seen for integration sites from each patient analyzed individually. Comparison with transcriptional profiling data revealed that genes hosting integration events by VRX496 were biased toward relatively high-level expression (P = 0.004; Mann–Whitney test). A detailed genomic and statistical analysis of the VRX496 integration events is included in Appendix, which is published as supporting information on the PNAS web site.

Of note, integration in gene-dense regions was more strongly favored in the antisense env vector data than in previously studied HIV vector or virus data sets (Fig. 3B; P = 2.9 × 10−5 for comparison with pooled HIV integration data). The reason for the stronger favoring of gene-rich regions by VRX496 is uncertain; one possibility is vector independent: that the optimally stimulated primary T cells used in this study contain higher levels of cellular factors promoting integration in gene-rich regions. Taken together the data so far demonstrate the vector displays integration similar to wild type HIV.

Immune Assessments. To characterize immune function, we analyzed the response of T cells to HIV antigens by ELISPOT. The frequency of T cells secreting IFN-γ was assessed at baseline, 3 and 6 months after infusion by stimulation with a panel of overlapping 15-mer peptides that corresponds to the entire coding region of HIV-1 env and gag. The frequency of T cells responding to env was elevated in three of five subjects at 3 and 6 months after infusion when compared with baseline (Fig. 4). In addition, the responses to env after infusion were higher in magnitude in three patients at 3 months (P < 0.05) and 6 months (P < 0.001) than a series of matched control patients with chronic HIV-1. In contrast, the T cells from the subjects generally did not have augmented responses at 3–6 months after therapy after gag stimulation compared with matched controls.
before any subject changed antiretroviral therapy. Finally, serum protocol, and the immune response changes were documented from HIV controls (treatment responses were elevated in comparison with samples responses at 3 and 6 months after infusion (Fig. 7). Low responses at baseline, and three of five subjects had increased response to diphtheria toxin was measured. Subjects had absent or confidence interval for the control subjects is plotted.

Fig. 4. Immunologic assessment of HIV-1 env-specific effector cells secreting IFN-γ. Blood samples were obtained at baseline and at 3 and 6 months after gene-transfer therapy. PBMCs were isolated from study subjects and HIV-1-positive control subjects (n = 25) by a standard Ficoll separation technique. IFN-γ production after HIV-1 env in vitro stimulation of PBMCs was assessed for an env antigen-specific response by a standard ELISPOT. The mean ± 95% confidence interval for the control subjects is plotted. *, subject 1 did not have a 6-month sample available for analysis.

(P > 0.5; Fig. 7A, which is published as supporting information on the PNAS web site).

To characterize CD4 memory responses during the protocol, the response to diphtheria toxin was measured. Subjects had absent or low responses at baseline, and three of five subjects had increased responses at 3 and 6 months after infusion (Fig. 7B). The after-treatment responses were elevated in comparison with samples from HIV controls (P < 0.03). No subject was vaccinated during the protocol, and the immune response changes were documented before any subject changed antiretroviral therapy. Finally, serum from patients was analyzed for neutralizing antibodies to laboratory HIV strains and to autologous viral isolates (19). No subjects developed neutralizing antibodies to env.

By ELISPOT analysis, there was little improvement of the immune response of patient 2 to the HIV gag or env that could possibly explain the mechanism for the drop in viral load. Therefore, additional immune analysis was performed by using intracellular cytokine staining (ICS) (see Supporting Methods) after stimulation of patients’ PBMCs with overlapping peptides from Gag, Pol, Env, and Nef corresponding to the HIV-1 clade B consensus sequence. The CEF (CMV, EBV, and Flu) peptide was used to measure the cytokine response to other, non-HIV, pathogens. There was a measurable improvement in the anti-Pol and Nef responses as early as 1 year after dosing (Fig. 7C) and significant improvement in general immune function at year 2 after dosing. However, it cannot be known whether this improvement is a result of the study treatment alone, or results from the change in medication the patient was taking between years 1 and 2. During this time, while continuing on kaletra and zidane, the patient went off sustiva and switched from lamivudine and tenoforiv to Truvada, which is a combination of tenoforiv and tricabine. However, it should be noted that, to our knowledge, antiretroviral drugs, if efficient in controlling HIV replication, are not able to restore the immune response to HIV or other pathogens in patients who are failing highly active antiretroviral therapy (HAART).

Discussion

Our results demonstrate the expected high efficiency of gene transfer in vitro at the clinical scale, and the observed long term persistence of gene-modified T cells in vivo in these subjects with late stage HIV infection is encouraging and indicates that the VSV-G pseudotyped vector may not have substantial intrinsic immunogenicity when given in a single cellular dose, as has been observed with most forms of gene transfer therapy. To date our studies have not uncovered evidence of insertional mutagenesis, with observation of 21–36 months. However, given that the
latency period was 3 years for adverse events to become clinically evident in the case of stem cell gene transfer of common γ-chain by using an oncoretroviral vector (14), safety will not be fully established until longer follow-up is completed and more subjects have been treated. In accordance with the long-term follow-up guidelines provided by the FDA, the patients enrolled in this trial will be followed annually for 15 years after infusion.

An increase in the cellular responses to HIV was observed in four of five patients, and three of those experienced a concomitant improvement in their T cell memory responses as well. The magnitude of the responses to env were higher than we have observed in studies of patients with chronic HIV-1 infection who have been vaccinated with HIV genes (20). Finally, we observed a robust antiviral effect in one subject who had been refractory to conventional antiviral therapy. The mechanism of the delayed antiviral effect remains unclear and may be related to immune enhancement and/or to vector mobilization (12), consistent with the transient vector mobilization that we have observed. The antiviral response observed in patient 2 cannot yet be definitively linked to vector expression in the T cells instead of an effect from the T cell infection alone, however, several studies involving T cell infusions in HIV patients have been performed, and sustained control of viral load has not been observed. One can speculate that vector mobilization resulting in greater antisense pressure against HIV leads to the generation of HIV variants in patients with reduced pathogenicity, which, in combination with the immune reconstituting effects of adoptive cellular therapy, could lead to enhanced control of viral replication. This situation would be reminiscent of the subset of individuals with advanced multidrug-resistant HIV that develop an immunologic profile comparable to that of long-term nonprogressors, where it has been suggested that functional immunity can be reconstituted as a result of a de facto attenuated vaccine process that occurs consequent to the generation of drug resistant HIV with reduced fitness (21).

The retention of the LTRs in VRX496 provides the potential for the vector to be packaged by using wt-HIV proteins and, thus, mobilized to CD4-bearing cells. For most applications, SIN vectors are thought to be safer because they cannot mobilize because of a deletion in the U3 region of their LTRs. However, in the setting of HIV, mobilization may be beneficial (12), resulting from production of immunizing virus like particles, or a combination of both mechanisms. Mathematical modeling evaluating the hypothesis of vector spread to T cells indicates that the antiviral effects of conditionally replicating vectors are complex and depend critically on the efficiency of mobilization (13). Although VRX496 contains an anti-HIV gene and, thus, should inhibit production of HIV proteins capable of packaging the vector, at lower copy numbers, silencing or variegation may result in productive infection and mobilization (22).

This trial provides intriguing data in a pilot study evaluating a gene therapy vector and viral replication in the setting of HIV. In particular, conditionally replicating lentiviral vectors may have promise in a number of chronic viral infections. Two follow-on studies are presently underway to further evaluate the potential of this vector when given in multiple doses (www.clinicaltrials.gov). Taken together the results support the clinical promise of this efficient T cell culture system and gene transfer using lentiviral vector technology.

Methods
Clinical Protocol. Details of the protocol have been published (23). The final protocol was later modified to remove dose escalation. To be eligible for the study, subjects must have failed at least two HAART regimens as a result of drug resistance or be intolerant to antiretrovirals, with viral load of >5,000 copies per ml and CD4 counts between 150 and 500. In addition, subjects must have had a Karnofsky performance score of at least 80 and no signs of opportunistic infections. Subjects were monitored after infusion at 1, 2, 3, 7, 14, and 21 days, 6 weeks, and at 3, 6, and 9 months for viral load (Amplicor assay; Roche, Indianapolis, IN), CD4 count, emergence of potential RCLs, and for immunological parameters. Adverse events were defined in part by observation of a sustained 0.5-log increase in viral load, or 30% decrease in CD4 count within 3 weeks after dosing. Additional information is provided in Supporting Methods, which is published as supporting information on the PNAS web site.

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