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RIBOZYMES IN GENE THERAPY OF HIV-1

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1. ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is the primary etiologic agent for Aquired Immune Deficiency Syndrome (AIDS). HIV-1 is a lentivirus, a separate genus of the Retroviridae, which are complex RNA viruses that integrate into the genome of host cells and replicate intracellularly. Ribozymes are catalytic RNA molecules with enzyme-like cleavage properties, that can be designed to target specific RNA sequences within the HIV-1 genome. In addition to the genomic RNA, several RNA intermediates, including splice variants, can be targeted by a single ribozyme. We and others have demonstrated the ability of ribozymes to suppress HIV-1 replication in a variety of cultured cells. Ribozyme gene therapy for HIV-1 infection is a therapeutic approach that offers several potential advantages over conventional therapies in that it can potentially impact on both viral load and restoration of the immune system. Ribozyme gene therapy may be used as an adjunct to chemotherapeutic drugs, effecting viral suppression, and facilitating immune restoration without problems of patient compliance. Currently, an anti-HIV-1 ribozyme is being tested in two separate Phase I Clinical Trials.

2. INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) and its associated disorders are caused by the human lentivirus, human immunodeficiency virus (HIV-1) (1-6). The primary target cells for HIV-1 infection *in vivo* are the $CD4^+$ T-lymphocytes. In AIDS there is a progressive and irreversible destruction of the immune system; with an inexorable decline in $CD4^+$ T-lymphocyte cell numbers over time, acting to severely compromise the immune system (1-6). This results in immune failure concurrent with multi-organism opportunistic infection. The disease course of AIDS consists of four stages - initial infection, acute syndrome, clinical latency, and eventually clinical disease (1-9) with viral replication found at all of these stages (9-11). This progression is despite a host immune response, with relatively high levels of antibodies to the HIV structural proteins env and p24 as well as HIV-specific cytotoxic T-lymphocytes (1-6). All studies described in this review were approved by the appropriate review committees, and the clinical investigations were performed in accordance with the guidelines proposed in the Declaration of Helsinki.

2.1. The infectious cycle of HIV-1

HIV-1 is the prototypic member of the lentivirus family of retroviruses. The HIV-1 genome (approximately 9.8 kb in size) contains the same three replicative genes *(gag, pol, env)* found in the genomes of all simple retroviruses. In addition, HIV-1 contains regulatory *(tat, rev, tev, vpr, nef*) and accessory *(vpu, vif)* genes (12, 13) (figure 1). HIV generally infects cells containing the CD4 receptor in combination with other co-receptors, such as the chemokine receptors CXCR-4 and CCR-5 (14-17). On infection, there is fusion of the viral and target cell membranes. Following viral uncoating, genomic RNA (in a complex with the viral proteins integrase, polymerase and

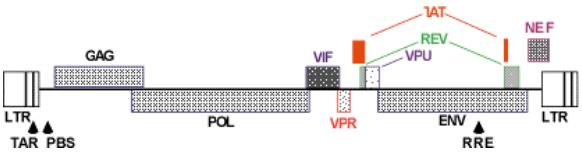

Figure 1. Genomic structure of HIV-1 showing regulatory and accessory genes.

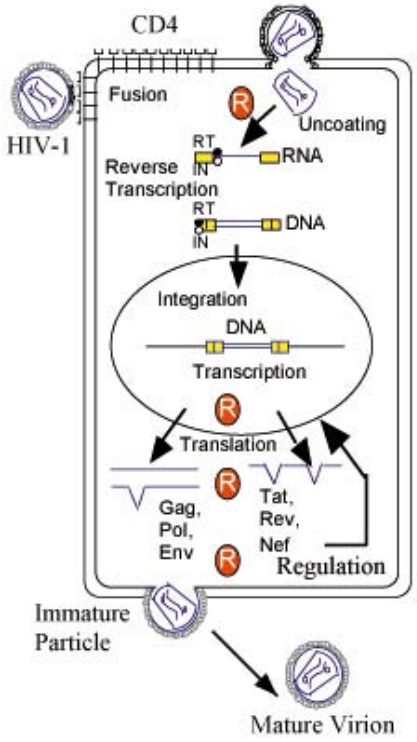

Figure 2. Infectious cycle of HIV-1 and potential sites of ribozyme action (R) against HIV-1 RNA. HIV-1 binds cell surface receptors, fuses with the host membrane and enters the cell. Following uncoating of the viral core, viral RNA is converted to DNA by reverse transcriptase in the cytoplasm, and transported to the nucleus where integrase mediates integration into the host genome. Transcription and translation of provirus gives rise to mature HIV-1 viral particles which are shed from the infected cell.

reverse transcriptase) enters the host cell cytoplasm (12-15). Viral reverse transcriptase and polymerase enzymes convert the genomic RNA to cDNA, and a dsDNA copy is transported to the cell nucleus and then integrated into the host genome by the action of the viral integrase protein. This proviral DNA is then a stable component of the host cell genome and it may remain dormant or become actively transcribed to genomic or subgenomic viral RNA yielding mature virions which are released by budding (12, 13). A $CD4^+$ T-lymphocyte productively infected with HIV-1 has a half-life of approximately two days (9-11). The HIV infectious cycle is shown in figure 2.

2.2. Gene therapy approaches to HIV-1

AIDS pathogenesis is a relatively complicated series of events with pathologic sequelae as well as drug-induced side effects (18-25). Several different gene-based approaches (26) have been described for the potential inhibition of HIV-1 replication. These include the expression of i) intracellular antibodies to viral proteins (27, 28); ii) antisense RNA to inhibit the reverse transcription, processing and translation of HIV-1 RNA (29) ; iii) mutant HIV structural or regulatory genes with dominant repressor activity such as *rev* M10 (30, 31) or *tat* (32-34), iv) RNA decoys such as RRE (35) and TAR (36) to inhibit HIV-1 transcription and processing; and v) ribozymes to catalytically cleave and thus inactivate HIV-1 RNA species (37-43).

Ribozymes have the potential to act at several stages in the HIV infectious-cycle. These are i) the initial entry of genomic viral RNA into the target cell, ii) the transcription of genomic and subgenomic RNA molecules, iii) prior to and during translation of mRNA to viral proteins, and iv) prior to encapsidation of the genomic RNA. The cleavage of HIV RNA by ribozymes at any of these stages (indicated in figure 2) can lead to a decrease in intracellular viral replication.

2.3. Designing and testing ribozymes

Ribozymes are attractive potential therapeutic agents due to the specificity of binding and cleavage, potential for turnover and lack of immunogenicity. Here we summarise the factors which need to be considered in the design and testing of ribozymes, with particular emphasis on anti-HIV ribozymes.

2.3.1. Choice of the RNA target site

Target site selection is generally based on three criteria; i) biological significance of the target RNA, ii) the presence of an appropriate target triplet sequence and iii) accessibility of this sequence to ribozyme action (39, 42). The biological significance of a target RNA must be assessed for each target gene. For example, we have shown that the *psi* packaging region, a biologically significant site in all retroviruses, is susceptible to ribozyme-mediated inhibition. For hammerhead ribozyme cleavage, the target site is generally GUX, although in certain cases NUX may be used (where N represents any nucleotide and X represents A, C or U) (44). Ribozyme recognition triplets may be identified within the target RNA, and accessibility assessed using RNA secondary structure analysis algorithms, or by empirical experiments such as chemical modification mapping (45). However, these methods provide only an approximation as to whether or not the target site is accessible since they do not take into account RNA tertiary structures or potential RNA-protein interactions, but they are still useful as a first step in the design of ribozymes. An example of an anti-HIV ribozyme is shown in figure 3.

2.3.2. Testing ribozymes by *in vitro* cleavage

Following selection of potential target sites, the corresponding ribozyme sequences can be synthesised and tested in *in vitro* cleavage reactions using standard conditions (50mM Tris HCl, pH 7.5, 10mM $MgCl_2$, at 37^oC). Efficiency of cleavage can be assessed by production of the expected size cleavage products on

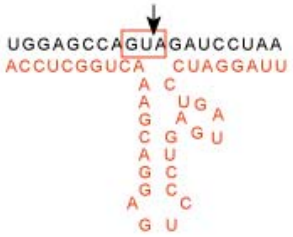

Figure 3. Target sequence within *tat* together with hammerhead ribozyme. Site of cleavage is indicated by an arrow.

polyacrylamide gel electrophoresis (46-48). A correlation between *in vitro* cleavage efficiency and *in vivo* ribozyme activity has been observed for some ribozymes (39). However, this is not always the case and *in vitro* cleavage efficacy does not always correlate with *in vivo* activity (41).

2.3.3. Testing ribozymes in cell culture

Ribozymes can be tested in a cell culture system by incorporating the ribozyme sequence as a DNA copy into an expression vector. We and others have used relatively simple plasmid-based expression vectors in which the ribozyme is incorporated downstream of a mammalian promoter, as well as more complicated vectors based on the genomes of retroviruses or other viruses (48). In order to improve transcription efficiency and potentially increase the stability and sub-cellular targeting of ribozymes within cells, the ribozymes can be constructed as chimeras with other genes including tRNA, U1 and U6 snRNA. We have extensively used constructs where the ribozyme sequence is incorporated within the 3'-untranslated region of the neomycin resistance gene. This resulted in high level expression of the neo/ribozyme transcript. It also allowed positive selection with G418 of cells expressing the construct (39, 42, 48) .

Ribozymes have been shown to modulate the expression of a number of different genes in various cell culture systems. However, the rapid degradation of ribozyme cleavage products has made direct detection of the RNA cleavage products extremely difficult. Ribozymes have been shown to inhibit expression of genes encoding enzymes, structural genes and genes encoding cytokines, as well as inhibition of viral replication (49, 50**).** Ribozymes targeted to various sites within HIV are able to inhibit HIV-1 replication in cultured cells (38, 39, 41-43, 51-55). Ribozyme-mediated inhibition of HIV replication can be assayed by ELISA monitoring of viral p24 protein production.

2.4. Ribozyme-mediated inhibition of a murine retrovirus

Ribozymes as potential anti-retroviral agents were initially tested in a Moloney murine leukemia virus (MoMLV) model system. We demonstrated that the *psi* packaging site, which is essential for packaging of viral genomic RNA, can be effectively targeted, resulting in inhibition of MoMLV replication with the efficacy of substrate cleavage *in vitro* by *psi* packaging site-targeted ribozymes correlating well with inhibition of MoMLV replication *in vivo* in a cell culture system (39). This work was extended to show a high level of ribozyme specificity when targeting the *psi* packaging site. Ribozymes could be designed to specifically target the MoMLV *psi* packaging site whilst not affecting the MoMLV variant sequence within the retroviral vector expressing the ribozyme itself. As there is minimal variation between the two sequences, these results indicated a high degree of specificity of ribozymes (56).

3 ANTI-HIV-1 RIBOZYMES

3.1. The potential application of ribozymes to the treatment of AIDS

AIDS is a disease with a viral etiology and a marked immune component; the virus slowly destroys the cells of the immune system leading to immunodeficiency (1-10). An effective treatment needs to address two facets of the disease; viral load and immune function. To date, therapies for the treatment of HIV-1 infection have focused on the reduction of viral load using drugs that interfere with replication of the virus with only a modest effect on the restoration of T-cell counts. Triple combination therapy (TCT), generally involving two reverse transcriptase inhibitors and one protease inhibitor, is the most recent and relatively successful development in the management of HIV infection (18-20). Whilst TCT has shown great promise, often reducing to undetectable the level of HIV-1 in the blood, side effects are now being noticed including lipodystrophy, hyperlipidemia, and protease-related diabetes (21, 22). In addition, strains of HIV are emerging which are resistant to both reverse transcriptase and protease inhibitors (22, 23). New therapies which address the other aspect of HIV/AIDS disease management, namely immune restoration, need to be considered (24, 25).

Traditional anti-viral drugs for infectious disease are small molecules. However, recent advances in molecular biology have made gene-based therapies a possibility. As noted above, ribozymes are enzymatic molecules that can cut specific sequences in HIV and destroy the virus. Ribozymes cut HIV at several stages of its life cycle and are active against strains that are resistant to conventional anti-viral therapy. Ribozyme gene therapy could be used as an adjunctive or stand-alone therapy, and is potentially cost-competitive with other anti-viral therapies.

3.2. Design and testing of anti-HIV-1 ribozymes

Due to the considerable sequence variation among HIV-1 isolates, its rapid mutation rate in response to treatment with anti-retroviral agents such as reverse transcriptase inhibitors and protease inhibitors, it is important to choose ribozyme target sites critical for viral replication and highly conserved in sequence between clades. We selected several potential target sites, and have focused on two well-conserved target sites in our studies, one within the *psi* region (39) and the other within the 5' region of the first coding exon of *tat* (42). These two sites are highly conserved between HIV-1 clades and subsequently, ribozymes targeted to both these sites, and especially the *tat* site (figure 3), were shown to inhibit a wide range of HIV-1 isolates (39, 42, 51, 57).

3.2.1. Anti-HIV-1 ribozymes in cell culture

Hammerhead ribozymes targeting different sites within the genome of HIV-1 have been shown to effectively inhibit HIV-1 replication in cultured cells. These include ribozymes targeting other sequences within the *tat* gene (54), the HIV-1 5'-leader sequence (55), *env* (58) and *gag* (59). Hairpin ribozyme constructs targeted to *tat* and *env* and expressed from a $tRNA^{Val}$ transcriptional cassette within various expression vectors have also been shown to confer resistance to several HIV-1 isolates (52, 60-62).

We have used constructs in which the ribozyme is cloned into the 3'-untranslated region of the *neo* gene, with expression driven by the SV40 promoter in a eukaryotic expression vector, or a MoMLV-based retroviral vector. Ribozymes targeted to both the HIV *tat* gene (42, 51, 57). and the *psi* packaging site of HIV-1 (39), were able to protect T-lymphocyte cell lines from HIV-1 pathogenesis by delaying HIV-1 replication and reducing absolute virus levels. In the SupT 1 T-cell line, replication of HIV-1 was inhibited by 70-95% for the laboratory adapted isolates SF2 and IIIB and by 2-4 logs for primary clinical isolates (39, 42, 51, 57). In a second T-lymphocyte cell culture system, CEM T4 cells were transduced with amphotropic retrovirus containing the same neo/ribozyme expression cassettes (51, 57).

The most effective ribozyme construct in both assay systems was the one directed to a site in the first coding exon of *tat* (39, 42, 51, 57). This construct was designated RRz2 and formed the basis for our later pre-clinical work. The efficacy of RRz2 in the T-lymphocyte cell line studies was confirmed using primary cells, both total peripheral blood mononuclear cells (PBMC) and $CD4^+$-enriched T-lymphocytes (PBL). PBMC were obtained from random, normal, blood donor packs using Ficoll/Hypaque separation and $CD4^+$-enriched by depletion of $CD8^+$ T-lymphocytes using $CD8^+$ MicroCELLector flasks (Applied Immune Systems/RPR Gencell). Both total PBMC and $CD4^+$-enriched PBL were then transduced with the vector, LNL6 and the RRz2 retrovirus, selected with an appropriate donor-specific dose of G418 and then challenged with laboratory strains of HIV. RRz2 was shown to be effective in this assay giving inhibition of 70-90% when compared to the LNL6 control (42). Other ribozyme-containing constructs were less effective in this assay (57). In addition, RRz2 offered protection against a range of clinical HIV-1 isolates, including AZT- and nevirapine-resistant strains (57).

When the RRz2 and LNL6 vectors were used to transduce PBL from HIV-1 infected patients. Cell viability was significantly higher in the RRz2-transduced HIV-1 infected PBL during a two week culture period. No difference in viability between RRz2- and LNL6-transduced PBL was observed in cultures from non-infected donors (57). This result was the first direct evidence that a ribozyme can impact on the survival of HIV-1 infected, patient-derived PBL in culture (57) and implies that the ribozyme-expressing cells may have a growth viability advantage within HIV-1-infected individuals. This hypothesis is presently being tested in Phase I Clinical Trials by our group (see below).

3.2.2. Specificity of anti-HIV ribozymes

To validate the specificity of ribozyme action against HIV-1, we compared Rz2 with another ribozyme, Rz1, which targets the 5' splicing region of the *tat* gene. This ribozyme was designed to target GUCN, in which N is a G in HIV-1 IIIB and an A in HIV-1 SF2. The data from *in vitro* and *in vivo* studies with Rz1 showed that this ribozyme could protect cells only against those HIV isolates whose genomic sequence was cleavable *in vitro*. This study demonstrated the importance of the first base pair distal to the NUX within helix I of the hammerhead structure for both *in vitro* and *in vivo* ribozyme activities (51). The Rz2 ribozyme, in contrast to Rz1, is able to inhibit replication of both SF2 and IIIB strains of HIV-1, due to sequence conservation at the Rz2 target site (42, 51, 57).

A multiple-passage assay similar to those used to detect the emergence mutant strains resistant to reverese-transcriptase inhibitors, was employed to analyse HIV-1 sequence variation and viral replication dynamics in ribozyme-expressing cells. These studies demonstrated that expression of RRz2 ribozyme in transduced human T-lymphoctes did not provide selective conditions for the emergence of HIV-resistant mutations over five sequential viral passages, and resulted in the rapid disappearance of certain quasi-species of HIV-1 (57). The rapid disappearance of this "less fit" quasi-species in the RRz2-though not the LNL6-transduced cells argues for a RRz2 selective pressure. These data further support the potential clinical use of the Rz2 anti-HIV ribozyme.

3.3. Towards an anti-HIV ribozyme gene therapeutic

The two major challenges for gene therapy applications are i) the efficient delivery of therapeutic sequences into relevant cell populations and ii) the subsequent expression of the therapeutic gene within patients. Retroviral vectors remain the preferred delivery system as they are relatively efficient, have a good track record for safety, integrate into host genomes in a stable fashion and have previously shown persistent levels of expression (63, 64). To date, over 300 gene therapy clinical trials have been approved worldwide, and the majority use retroviral vectors to carry marker or therapeutic genes into target cell populations (65, 66). Most of clinical trials (including our own) utilise retroviral vectors based on MoMLV. However, the percentage of cells infected by these retroviruses are limited by culture conditions and, more importantly, MoMLV-based vectors require cell division for stable integration. Recently, gibbon ape leukemia virus (GaLV) (67) or vesicular stomatitis virus (VSV) G-glycoprotein pseudo-typed retroviral vectors (68) have been demonstrated to provide more efficient infection of human hematopoietic cells. Other relatively recent advances have involved the reduction of retroviral packaging cell culture temperature to 32^oC, using concentrated virus (69) and the use of a recombinant fibronectin fragment (CH296) (70). These modifications

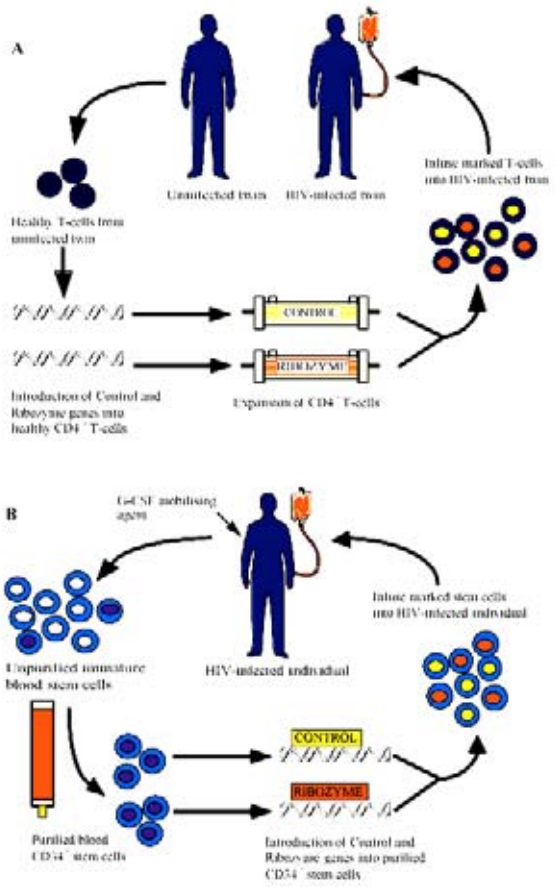

Figure 4. Schematic representation of the Phase I clinical trial procedures. A: $CD4^+$ T-lymphocyte twin study. Healthy T-cells from the HIV-1-negative twin are transduced with control and ribozyme vectors and infused into the HIV-1-positive identical twin. B: Autologous $CD34^+$ blood stem cell study. Mobilised peripheral blood $CD34^+$ stem cells harvested from HIV-1-positive individuals are transduced with control and ribozyme vectors and reinfused into the same individual. The $CD34^+$ blood stem cells engraft in the bone marrow and contribute to all the hematopoietic lineages including T-cells and macrophages.

act to increase the effective multiplicity of infection, and increase amphotropic viral transduction efficiency (71, 72).

Adeno-associated virus (AAV), is another viral system being developed as a gene delivery vector that has been shown to be capable of efficient insertion of anti-HIV-1 genes into hematopoietic cells (73). AAV vectors have been used to insert an HIV-1 antisense construct into human T-cells, resulting in a significant reduction of HIV levels following challenge with purified HIV-1 (74). AAV-based vectors have several potential advantages over retrovirus-based vectors that are relevant for HIV-1 gene therapy (75). They appear to have no potential for homologous recombination, and high transduction in hematopoietic cells, including progenitor cells is possible. In addition, AAV is non-pathogenic, replication-incompetent and is able to transduce non-dividing cells. However, for clinical application there is still a major limitation on the size of exogenous sequence that can be inserted, and there is the potential for adenovirus contamination. It is possible that integrated sequences will excise or not remain stable long term. For example, the expression of a truncated rat nerve growth factor receptor reporter gene delivered to hematopoietic cells using an AAV vector was observed to decline by 50-90% over a 2 month period (76).

HIV-1 based lentiviral vectors represent another approach for the introduction of therapeutic genes (77, 78). They have a number of potential advantages over existing vectors. Such vectors are expected to have the ability to infect non-dividing cells and they offer the possibility for *tat*-inducible and therefore HIV-1 infection-specific expression (65, 79).

As to the appropriate target cells for an anti-HIV-1 ribozyme gene therapeutic, hematopoietic $CD34^+$ stem cells would appear to be the ideal choice since they have the potential to give rise to several generations of protected cells in multiple lineages. Another approach to the treatment of HIV-infected individuals is the infusion of gene manipulated $CD4^+$ T-lymphocytes. The battle between the immune system and HIV-1 in an infected individual is balanced, and any approach which weakens either virus replication or gives the immune system a slight but maintained advantage may be sufficient to tip that balance (10, 11). Therefore, if ribozyme constructs can protect $CD4^+$ T-cells from HIV-1 infection (and its sequelae) in patients, the decline in the number of $CD4^+$ T-cells could be halted or even reversed, providing potential clinical benefit. Concerns regarding a $CD4^+$-based therapy, include i) the effect on susceptibility of the $CD4^+$ cells to HIV infection due to *ex vivo* activation during culture, ii) the role played by non-T-cell populations such as the macrophage and iii) possible down-regulation of therapeutic gene expression from the proviral LTR in the absence of T-cell activation (80). The use of both mature $CD4^+$ cells and $CD34^+$ stem cells for the delivery of anti-HIV-1 ribozymes is described below.

3.4. Current Phase I clinical trials of an anti-HIV ribozyme

The question as to whether ribozymes can impact on AIDS disease course, and the two surrogate markers of advancing disease, viral load and $CD4^+$ T-cell counts, is about to be assessed. Clinical trials are currently being conducted by several groups including our own, to move towards addressing these questions. Our group has initiated two independent Phase I clinical trials to test the safety and feasibility of an anti-HIV-1 ribozyme gene therapeutic approach. The secondary aims of the studies are to assess the ability to detect ribozyme-containing cells in the bloodstream. Both clinical trials utilise the LNL6 vector and the recombinant RRz2. The two trials use different target cell populations: $CD4^+$ PBL or $CD34^+$ stem cells.

The first Phase I clinical trial involves identical twins, discordant for infection with HIV (figure 4A**)**. Healthy $CD4^+$ PBL from the uninfected twin were transduced with

either LNL6 or RRz2 and both cell populations cultured and expanded *ex vivo* (71) before infusion into the bloodstream of the corresponding HIV-positive twin. The patients were then monitored for signs of any adverse events, and assessed for $CD4^+$ lymphocyte counts, HIV-1 viral load, and presence of RRz2 and LNL6 vector in peripheral blood lymphocytes.

The second Phase I clinical trial involved the removal, transduction and infusion of $CD34^+$ blood stem cells within HIV-positive individuals (figure 4B). As in the $CD4^+$-based trial, two populations of marked cells were infused (LNL6 and RRz2-transduced cells). The rationale for this trial is that these transduced $CD34^+$ stem cells will home and reconstitute in the bone marrow compartment, with subsequent proliferation and differentiation to give rise to a variety of hematopoietic lineages including $CD4^+$ T-lymphocytes and macrophages. The patients were monitored for signs of any adverse events, and assessed for $CD4^+$ lymphocyte counts, HIV-1 viral load, and presence of RRz2 and LNL6 vector in bone marrow, and purified peripheral blood lymphocytes, monocytes and granulocytes.

In each of the trials, separate populations of cells were transduced with either retroviral vector containing the ribozyme (RRz2) or the vector alone (LNL6), the latter as an internal control. Approximately equal numbers of the two transduced cell types were then introduced into the recipient patients. This allowed monitoring of the survival of anti-HIV-1 ribozyme-expressing cells. Cell survival is monitored by simultaneously detecting ribozyme and control vector DNA sequences in peripheral blood using a semi-quantitative PCR procedure (71). In order to examine the ability of the CD34+ stem cells to repopulate multiple cell lineages, PCR is performed on bone marrow, and purified cell populations.

4. CONCLUSION

AIDS is an enormous therapeutic challenge due to the complexity of HIV pathogenesis, the multiple levels of control of proviral expression and the ability of the virus to rapidly mutate. Ribozymes offer a possible new paradigm: a gene therapy-based anti-HIV approach. Their potential advantages include specificity, multiple turnover, stable expression, and lack of immunogenicity. Studies to date, indicate that ribozymes are effective in suppressing HIV-1 replication in cell culture systems. Testing of the ability of a ribozyme gene therapapeutic to impact on AIDS are currently being tested clinically - initially in our own and other Phase I safety studies. The next step is for these studies to move to testing in Phase II clinical trials.

5. ACKNOWLEDGMENTS

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