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Setting the stage for bench-to-bedside movement of anti-HIV RNA inhibitors–gene therapy for AIDS in macaques

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1. ABSTRACT

Despite significant progress over the last two decades, treatment of HIV infection remains a tremendous challenge. Although antiretroviral therapy has proved quite effective in most HIV-infected patients, increasing recognition of toxicity and the emergence of multidrug resistant HIV strains has fueled the development of alternative therapeutic approaches. Introduction of genes to inhibit HIV replication into CD4+ T lymphocytes or hematopoietic stem cells represents a potentially attractive but still unproven strategy. Despite the availability of a diverse range of molecular strategies that are able to provide potent inhibition of HIV replication in the laboratory, translation of these *in vitro* successes to *in vivo* therapies has been difficult. Fundamental challenges facing AIDS gene therapy at the present time includes the need to increase the efficiency of gene transfer *in vivo*, to confer upon genetically-modified T cells the ability to have a selective growth advantage *in vivo*, and the development of additional techniques to decrease the probability of emergence of resistant viruses. As one of the leading animal models for AIDS and for hematopoietic stem cell gene therapy, nonhuman primates are ideally suited to help address many of these basic questions. This review will provide a general overview of RNA-based genetic strategies for inhibition of HIV and SIV replication, criteria to be considered in the selection of promising inhibitory strategies for *in vivo* use, and key questions that can be addressed in the macaque model.

2. RATIONALE FOR DEVELOPMENT OF GENETIC THERAPIES FOR AIDS

While the introduction of highly active antiretroviral therapy (HAART) has resulted in impressive suppression of circulating plasma viral RNA and decreases in mortality and morbidity in HIV-infected patients (1, 2), it is becoming increasingly clear that the long-term clinical benefit of these regimens is likely to be limited by several factors. These factors include patient compliance, the substantial cost of multidrug regimens, and the persistence of virus in lymphoid and non-lymphoid reservoirs. Even in patients who are able to maintain suppression of plasma viral RNA levels to undetectable levels for periods of up to 3 years, HIV persists in latently infected CD4+ T cells (3, 4), and replication rapidly rebounds in most patients following discontinuation of therapy (5, 6). There is now increasing recognition of a wide spectrum of toxicities associated with chronic administration of HAART, including diabetes, hyperlipidemia, and vascular disease (7, 8). Development of drug-resistant viruses is also an increasing concern. Transmission of resistant viruses, including multidrug resistant strains, has been well documented, and in some urban areas, may occur in up to 16% of patients with primary infection (9). A small but significant population of patients have developed multidrug resistant strains, and maintain levels of plasma viremia of 10^5 copies/ml or more, despite treatment with 6 or more drugs (10).

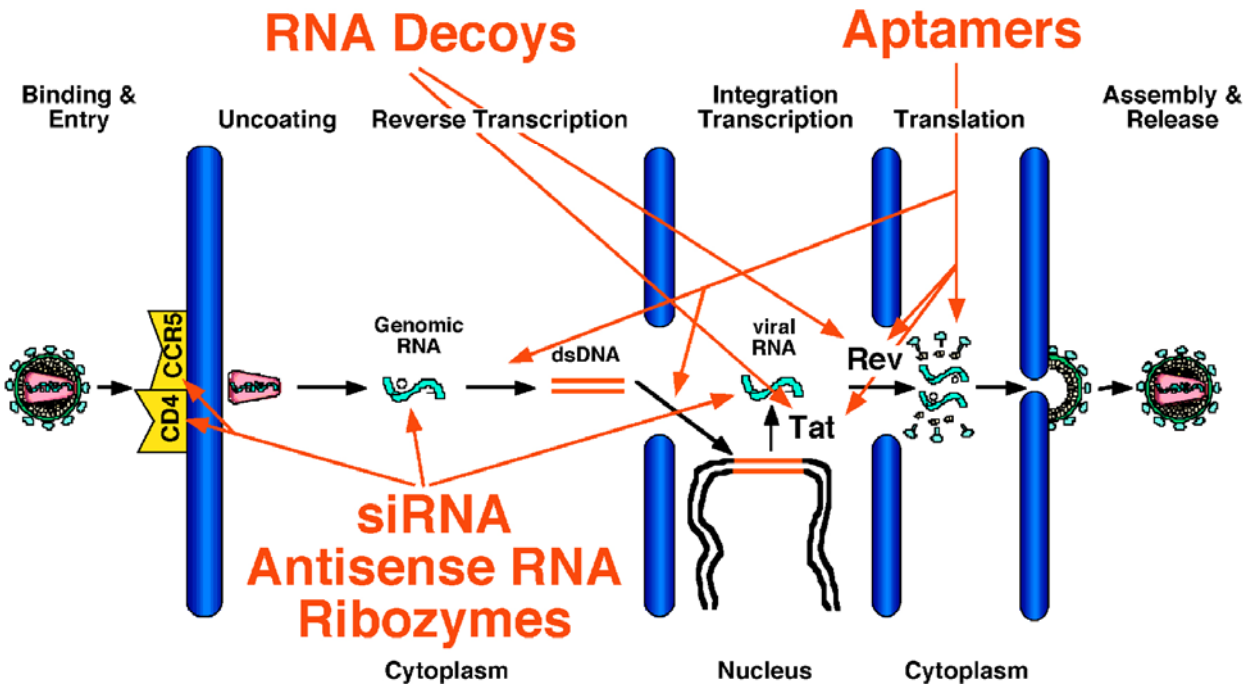

Figure 1. Potential sites of action of RNA-based inhibitors of HIV replication. ds DNA - double stranded DNA.

As a result of these factors, accumulating evidence suggests that a significant percentage of patients treated with HAART will not be able to maintain long-term suppression of HIV-1 replication below detectable levels (11, 12). Rates of virologic failure after 1 to 2 years of therapy range from 20% to 40% in treatment-naïve cohorts, and from 40% to 70% in treatment-experienced cohorts (12, 13). Although the continued development of new antiretroviral drugs may partially alleviate the problem of drug resistance and facilitate patient compliance, the diverse biologic and practical issues previously detailed suggest that long-term maintenance of virologic control will be an ongoing challenge in the treatment of HIV infection.

The continued need to develop alternative strategies to inhibit HIV replication in infected individuals has provided a strong impetus for research on genetic therapies for HIV disease. Over the past decade there has been considerable progress in the development of genes able to inhibit HIV replication, and a variety of potent inhibitors are now available. As compared with conventional antiretroviral therapy, the use of genetic therapies offers several advantages, including the ability to target points in the virus life cycle distinct from those inhibited by standard antiviral agents (and thus minimize the potential for the development of cross-resistant viruses) and the potential for life-long efficacy following a successful treatment. These strategies have been shown to inhibit HIV replication in culture by as much as 1000-fold or more in transformed cell lines, primary CD4+ T cells and either T cells or macrophages derived from transduced $CD34^+$ progenitor cells (14-16). However, the translation of these effective *in vitro* strategies to clinically efficacious treatments has remained problematic, in large part due to the inefficient techniques available to stably introduce these genes into the desired target cell populations *in vivo*. Studies in nonhuman primates infected with simian immunodeficiency virus (SIV) offer the opportunity to address basic questions related to gene transfer as well as to assess the efficacy of different genetic strategies in the leading animal model for the study of AIDS. The following review will provide an overview of different genetic strategies under study for treatment of AIDS and highlight areas of particular interest that can be addressed in nonhuman primate models.

3. GENETIC STRATEGIES TO INHIBIT HIV REPLICATION

In many respects, HIV infection is an ideal candidate to develop genetic therapies. The HIV genome is relatively small (approximately 9,800 base pairs) and encodes for 9 viral genes, which have been the subject of intensive study over the past 15 years. The relatively large body of information on these genes, their function in the viral life cycle, and interactions with cellular proteins has provided investigators with a number of different targets for gene-based therapies. Virtually all of the steps in the retroviral life cycle have been targeted by gene therapy strategies, ranging from host factors necessary for viral entry to viral factors necessary for replication and assembly (Figure 1), and the reader is referred to several reviews for a comprehensive survey of these approaches (14, 17-19). Although both protein and RNA inhibitory genes have the capability to target important steps in the retroviral life cycle, protein-based approaches have the potential

disadvantage of generating a cytotoxic T lymphocyte (CTL) response against a foreign protein eventually leading to the elimination of the genetically-modified cells, a finding that has been observed in at least one clinical trial (20) and in macaques (21). The following discussion will highlight those RNA-based approaches that have proved most effective in inhibiting viral replication, several of which have been studied in or are about to enter testing in clinical trials.

3.1. Overview of RNA-based approaches

Inhibition of HIV replication with RNA-based approaches relies on the specificity of Watson-Crick base pairing (antisense inhibitors, ribozymes or RNAi) or on the specific binding of HIV proteins to RNA secondary structures (aptamers, RNA decoys). RNA inhibitors avoid the potential disadvantage of a host CTL response against the therapeutic vector that protein-based inhibitors may encounter. RNA-based inhibitors have been designed to target either viral genes or host genes that are dispensable for normal cellular function but essential for viral replication. The targeting of host genes involved in viral replication significantly reduces the risk of generating viral escape mutants; however, these targets may also increase the potential for cellular toxicity. Although most RNA-based approaches to inhibit HIV replication interact with their targets with a high degree of specificity, the potential for off-target effects exists (22, 23). Inhibitors will have to be carefully screened to reduce potential side effects due to off-target interactions.

3.2. Antisense inhibitors

In the simplest scenario, the RNA-based inhibitor is expressed as an antisense message against crucial targets in HIV, binds to the homologous HIV target sequences, and interferes with further HIV mRNA processing, transport, transcriptional initiation, or leads to HIV mRNA degradation. As shown in Figure 1, antisense targets have been developed to interfere with all intracellular phases of the HIV life cycle. Antisense-based strategies have been shown by multiple investigators to provide potent inhibition against HIV-1 replication, particularly when sequences longer than 600 bp are used for inhibition (24-27). In contrast to siRNA-based strategies, in which mutation of a single base-pair may abrogate inhibition (28, 29), the ability of antisense molecules to target multiple base pairs significantly reduces their susceptibility to evolution of escape mutations (25, 27), a particularly important characteristic given the rapid generation of variant HIV-1 strains. The molecular mechanisms that underlie antisense-mediated inhibition of expression of target genes remain incompletely understood. Previous studies of antisense-mediated regulation of gene expression in polyoma virus-infected cells documented nuclear retention of sense-antisense hybrid RNAs, associated with base modifications consistent with those mediated by double-stranded RNA-dependent adenosine deaminase activity (dsRAD) (30). Additionally, double-stranded (ds) RNA serves as an activator of protein kinase R, an interferon-induced serine-threonine protein kinase that mediates of the antiviral and antiproliferative actions of interferons by phosphorylating the alpha-subunit of the protein synthesis initiation factor 2 (eIF2alpha) and thereby inducing a general block in the initiation of protein synthesis. Activation of PKR may limit the potential of antisense gene therapy because of induction of apoptosis.

3.3. Ribozymes

Ribozymes are an extension of the antisense approach, but include a small catalytic RNA sequence that cleaves the phosphodiester backbone. Ribozymes were developed based on the findings of Zaug and Cech in the 1980's when they described RNA molecules in Tetrahymena that were capable of self-splicing activity (31). Other self-splicing RNA molecules have subsequently been described in certain plant and animal viroids, newt satellite DNA and fungal mitochondrial DNA. These ribozymes have critical secondary structures necessary for self-splicing function described as hammerhead, hairpin and axe-head ribozymes. Importantly, for gene therapy applications, sequence-specific complementary targeting domains can surround the catalytic domain, with its secondary structures, to cleave the target RNA molecule. Many groups have demonstrated the feasibility of using ribozymes against HIV (32-35). Because ribozymes cleave target mRNA, this strategy may protect cells from infection if the ribozyme cleaves the incoming HIV genomic RNA prior to reverse transcription. The advantage of ribozyme inhibitor is that they catalyze an enzymatic reaction, so the level of expression necessary for viral inhibition is expected to be lower. A disadvantage of ribozymes is the relatively short complementary targeting domain is susceptible to the generation of viral resistance.

3.4. RNA Interference

RNA interference (RNAi) is a highly conserved mechanism for post-transcriptional gene silencing originally discovered in plants and lower animal species. These short interfering RNA (siRNA) of 21-23 nucleotides with 2 or 3 bps of 3' overhang are generated by pairing of short transcripts, by processing from the inverted repeats in microRNA (miRNA), or by polymerization from RNA templates. The presence of double-stranded RNA triggers the sequence-specific degradation of the homologous gene mRNA in the RNA-induced silencing complex (RISC). Small inhibitory RNAs (siRNA) targeting conserved functions in HIV (Tat, Rev or the primer binding site) (36, 37) or host genes associated with HIV replication (the chemokine coreceptor CCR5) (38, 39) have been described by several groups in cell lines, primary CD4+ T cells and even in CD4+ T cells derived from transduced CD34+ cells (36, 37, 39-41). However, the strong inhibition mediated by siRNA inhibitors is associated with potent selection pressure on viral replication, leading to the evolution of resistant virus. With the short sequence homology used by siRNAs, a single-base pair mutation in the target sequence can result in abrogation of inhibition (42, 43). Indeed, *in vitro* evolution of resistance to siRNAs has already been observed for both poliovirus and HIV-1 (28, 29).

3.5. Aptamers

Aptamers are synthetic single-stranded nucleic acids that can be designed to serve as ligands for both protein and nucleic acid targets (44). Aptamers have typically been isolated using a process termed SELEX (systematic evolution of ligands by exponential

enrichment). In this system, random sequences of 40-60 nucleotides are flanked by PCR primers, one of which contains the T7 promoter. These sequences are transcribed into RNA and bound to specific targets (ie, HIV-1 reverse transcriptase (RT)). The bound RNA molecules are first purified with the target protein and then amplified using RT-PCR. After several rounds of transcription, binding and amplification, this process selects for RNA molecules with specific binding to the target of interest. The SELEX approach has been used by several groups to generate aptamers specific for HIV proteins, including RT, Tat and envelope (45-49). X-ray crystallographic studies of an RT-specific aptamer aptamers have revealed a secondary structure termed a pseudoknot (50) with extensive contact with the template-primer-binding cleft of RT, even though this aptamer lacks primary sequence homology.

Aptamers have several key features: 1. High affinity binding—Kds for ligands are often <1 nM; 2. High specificity—in many cases aptamers have been designed to distinguish proteins that differ in only a few amino acids; 3. Ability to inhibit multiple distinct sites in the retroviral life cycle; 4. Relative resistance of aptamer-mediated inhibition to the effects of single point mutations and the evolution of resistant virus variants, because aptamers generally have multiple sites of contact with their targets.

3.6. RNA Decoys

Several HIV proteins bind specifically to RNA secondary structure and regulate virus-specific functions. The Rev protein facilities nuclear export of unspliced and singly spliced HIV mRNA by binding to the Rev-responsive element (RRE), and shuttling the RRE-containing viral RNA transcripts to the cytoplasm. Multiply-spliced viral mRNAs, such as those encoding for the regulatory proteins Tat and Rev, lack the RRE and are transported to the cytoplasm by a Rev-independent pathway. The HIV Tat protein activates HIV expression by binding to the stem-loop bulge of the transactivating region (TAR) in the HIV LTR, interacting with cellular factors, and stimulating early transcriptional elongation. Additionally, the extracellular release of Tat can activate HIV expression in neighboring cells. Whereas antisense or ribozyme inhibitors can block expression of HIV within the same cell, the RNA decoys may block the activity of extracellular Tat protein. As these functions are essential for HIV viral replication, expression of RNA decoys that can bind to and sequester these proteins will inhibit viral replication. One disadvantage of RNA decoys is that the RNA decoys generally require high levels of expression to compete with the natural targets. Using multiple decoy repeats has been shown to provide better inhibition (51, 52) but also increases the potential for vector instability.

4. VECTOR DELIVERY SYSTEMS

Many viral delivery systems are being studied for potential gene therapy applications. Adenoviral and adenoviral-associated viral (AAV) vectors have demonstrated efficient gene transfer into many target tissues; however, these vectors are not appropriate for tissues with high proliferative capacity, such as hematopoietic stem cells or T cells, because these vectors do not efficiently integrate into the host cell genome. Oncoretroviral vectors, such as Moloney murine leukemia virus-based vectors, and more recently lentiviral vectors, have proved to be the most appropriate vector system for targeting hematopoietic stem cells and primary T cells for human gene therapy. Advantages of retroviral vectors for delivery of RNA-based inhibitory approaches include the fact that the vector stably integrates into the target cell genome, is replicated as the host cell proliferates, and, importantly, can direct expression of the RNA inhibitor to either the nucleus or the cytoplasm via the use of different promoters. Oncoretroviral vectors have several advantages, including years of experience in design and testing, well-established packaging cell lines validated for production of clinical-grade vectors and high levels of expression. Lentiviral vectors have distinct advantages such as the ability to transduce nondividing or quiescent cells, the ability to be efficiently pseudotyped with several different envelopes, to be concentrated to relatively high titer, and self inactivating (SIN) LTRs. Lentiviral vector transcripts that are expressed from an intact LTR will be upregulated in response to Tat but also have the potential for being packaged by wild-type virions and transferred to nontransduced cells. In contrast, expression of transgenes delivered by SIN lentiviral vectors is controlled by inserted promoters, and these transcripts will not be efficiently packaged by wild-type virions. The use of HIV-based lentiviral vectors for AIDS gene therapy can potentially provide additional inhibition of viral replication through competition for regulatory proteins, for packaging into virions, and for RT in heterodimeric virions. Competition of HIV-1 lentiviral vector transcripts for packaging of wild-type HIV-1 RNA has been demonstrated by several groups (24, 53, 54). Additionally, HIV-induced insertional mutagenesis has been reported only rarely (55), in contrast to the well-documented ability of oncoretroviruses to transform cells (56). This difference may be due to the preferential integration of HIV-1 within the coding regions of genes versus the preferential integration of oncoretroviruses in the upstream transcriptional control elements of genes (57) or to the intrinsic activity of the different promoters/enhancer elements in transactivating proximal genes. One disadvantage of using HIV-based lentiviral vectors to transduce inhibitors of HIV-1 replication is that the expression of the inhibitor sequences during production of the therapeutic vector can lower the vector titer. A final criterion that applies to both oncoretroviral and lentiviral vectors is vector stability– because of the propensity of retroviruses for recombination, inhibitory sequences that contain multiple repeats (e.g. of RNA decoys) are often subject to recombination and deletions following retroviral integration.

Additional studies will be necessary to directly compare the effectiveness of oncoretroviral and lentiviral vectors *in vivo*, a point that reinforces the importance of nonhuman primate studies. However, the use of HIV-1-based vectors in nonhuman primates is complicated by the fact that macaques express an endogenous inhibitor, TRIM-5 alpha, that provides a relative block to the nuclear import of the preintegration complex (58). While this effect can

be in part overcome by increasing the multiplicity of infection, this species-specific block to efficient lentiviral transduction suggests SIV-based vectors may prove to be a more appropriate choice for lentiviral-mediated transduction in the macaque model.

5. CRITERIA FOR SELECTION OF AN ANTI-HIV INHIBITOR

With all the possible targets for inhibition and the different mechanisms of inhibition, some selection criteria must be employed to narrow the pool of RNA inhibitors to those with the highest probability for *in vivo* success. The first criterion must be the absence of toxicity including immunogenicity. As previously discussed, RNA inhibitors have the potential to target multiple phases of the HIV life cycle while avoiding induction of CTL responses to transduced cells. Although the interaction of RNA-based inhibitors with viral or host targets is generally highly specific, and thus reduces the potential for toxicity, off-target base pairing (as with siRNA, antisense or ribozymes) and cellular interactions with accumulated target proteins bound to RNA inhibitors (as with decoys or aptamers) have the potential to interfere with normal cellular functions. Double-stranded RNA hybrids that activate the IFN pathway could shut down host-cell protein synthesis, induce apoptosis, and potentially inhibit neighboring cells through a bystander effect. Additionally, the transduction protocol itself could negatively affect normal cellular proliferation or differentiation due to the *ex vivo* transduction conditions (stimulation conditions, cytokine treatment), or toxicity associated with additives (serum, polybrene or protamine sulfate), or vector particles (VSV-G-pseudotyped vectors).

The second criterion for the selection of an inhibitor is the stage of the viral lifecycle at which inhibition occurs. If inhibition of viral replication occurs prior to viral integration, infection of the transduced cell may be blocked. Inhibition of the expression of viral proteins may reduce the spread of the infection and potentially prevent presentation of viral antigens and subsequent CTL-mediated cytolysis. If inhibition of viral function occurs after viral packaging or in subsequent rounds of infection, as for some RT inhibitors, one would not necessarily expect to find a selective advantage of transduced cells. The choice of inhibitor should protect the transduced cell as early in the viral life cycle as possible to minimize the latent pool of infected cells and to maximize the potential selective advantage of the transduced cell to proliferate *in vivo*.

The next criterion for selecting the inhibitor is the potency of viral inhibition. A wide range of efficacy in the ability of RNA-based approaches to inhibit HIV replication has been reported, ranging from 50% inhibition to over 10,000-fold inhibition (36, 39, 59). Interpretation of these differing degrees of efficacy is complicated by the fact that the experimental conditions used, such as the cells studied (transformed cells versus primary CD4+ T cells) and culture conditions (especially multiplicity of infection) can significantly impact the observed efficacy. The molecular mechanisms that affect the potency of RNA inhibitors are incompletely understood but may include differences in intracellular compartmentalization, inhibitor stability, levels of expression, and the stoichiometry of inhibitor/target interactions necessary to achieve inhibition. Which *in vitro* conditions are likely to be predictive of *in vivo* efficacy are unknown. Relatively few studies have directly compared the efficacy of different RNA inhibitors using the same culture conditions. Although more comparative and *in vivo* studies are needed, clearly a clinically efficacious gene therapy vector should provide strong inhibition of viral replication in transduced cells.

A final consideration in the selection of a gene therapy vector would be the inclusion of a combination of several inhibitors targeting different points in the viral life cycle and using different mechanisms of inhibition. As observed with HAART, a multi-pronged gene therapy approach may avoid the development of resistant virus and may increase the potency of inhibition compared to a single inhibitor. These types of studies are currently underway (60).

6. TARGET CELL POPULATIONS FOR GENE THERAPY

The two dominant cell populations considered for genetic modification for treatment of HIV disease are $CD4^+$ T cells and hematopoietic stem cells (HSC). Most gene therapy clinical trials in HIV-infected individuals have focused on adoptive transfer of genetically modified $CD4^+$ T cells (18, 61-65). The use of $CD4^+$ T lymphocytes for adoptive transfer experiments in HIV-infected individuals has a number of advantages, including the development of efficient techniques for retroviral transduction of T cells, their relative ease of procurement, as well as an established safety and feasibility track record of adoptive T cell therapy in humans (66). Initial clinical trials of genetically modified $CD4^+$ T cells in HIV-infected patients demonstrated the ability of the transdominant RevM10 protein to prolong the survival of $CD4^+$ T cells compared with a control vector (64, 65). However, overall levels of gene marking were relatively low, the persistence of genetically modified cells relatively short (~6 months after retroviral gene transfer) and no evident clinical benefit was observed. Subsequent trials of genetically modified T cells containing a chimeric HIV-specific T cell receptor (CD4zeta) in HIV-infected subjects have employed improved techniques for the transduction and expansion of T cells, resulting in levels of genetically modified cells that exceeded 1% to 3% 8 weeks after infusion (61, 63). Although some subjects in these studies exhibited transient decreases in HIV load in some viral reservoirs (e.g. rectal tissue-associated RNA), no sustained virological, immunological or clinical benefit was observed. Taken together, these studies highlight the potential utility of adoptive T cell transfer studies to evaluate the *in vivo* efficacy of anti-HIV genes. However, they also highlight one of its limitations, the relatively limited persistence of genetically-modified T cells after *ex vivo* expansion and reinfusion.

The potential use of genetically modified hematopoietic stem cells has its own set of distinctive

advantages and disadvantages. Introduction of genes into hematopoietic stem cells that would render their progeny resistant to HIV infection is a particularly attractive strategy, especially since the cells infected by HIV-1 are largely (if not exclusively) of hematopoietic origin (67). The cardinal features of hematopoietic stem cells, self-renewal (potentially resulting in a life-long supply of genetically modified cells) and pluripotency (leading to production of multiple lineages of cells containing inhibitory genes) represent distinct advantages of this approach. Finally, in light of the tremendous proliferation of progeny from hematopoietic stem cells (68) and the increased turnover of $CD4^+$ T cells in HIV-infected individuals (69), transduction of even a small fraction of hematopoietic stem cells with a gene that confers a survival advantage on genetically-modified cells should lead to an *in vivo* expansion of a significant population of cells resistant to HIV infection.

Despite these potential advantages, there are multiple barriers to the development of stem cell gene therapy for AIDS. One of the fundamental challenges of stem cell gene therapy for any disease has been the difficulty in introducing genes into hematopoietic stem cells. Initial trials using murine leukemia viruses (MLV) vectors in nonhuman primates achieved only low levels of gene marking of hematopoietic cells. However, modifications to retroviral transduction protocols and the *in vivo* administration of hematopoietic growth factors have recently resulted in significant improvements in the efficiency of gene transfer to human and nonhuman primate hematopoietic stem cells. These modifications include: 1) the use of hematopoietic growth factor combinations such as Tpo, Flt3 ligand (Flt3L) and stem cell factor (SCF) that act on primitive stem cells to enhance retroviral transduction *in vitro* (70, 71); 2) the use of fibronectin or a recombinant fibronectin fragment (CH-296) as an *in vitro* matrix to enhance retroviral transduction and help maintain the pluripotency of transduced cells (72); and 3) the *in vivo* administration of hematopoietic growth factors such as SCF and Flt3L to increase the susceptibility of $CD34^+$ cells to transduction by oncoretroviral vectors (73, 74). In nonhuman primates, these modifications have resulted in the ability to obtain levels of gene marking that can transiently exceed 50% of peripheral blood leukocytes (PBL) at periods 2 to 4 weeks post-transplant with steady-state levels out to over 1 year in most animals of 5 to 20% of all cells (74-77). Results in human stem cell gene therapy trials using murine oncoretroviral vectors have generally been less dramatic but have demonstrated rates of gene transfer in PBL of up to 5% (78). In human infants with severe combined immunodeficiency-X1 (SCID-X1), reinfusion of autologous $CD34^+$ cells transduced with an oncoretroviral vector encoding the oncoretroviral vector encoding the gamma c cytokine receptor chain resulted in high levels of genetically modified lymphocytes (between 30% and 100%) 155 days post-transplant, associated with restoration of T and B cell function to normal levels (79). This high level of genetically modified lymphocytes reflects in part an *in vivo* survival advantage of lymphocytes carrying the transgene, similar to what may occur for genetically-modified $CD4^+$ T cells in HIV-infected subjects. Together, these recent results suggest that modified transduction protocols coupled with improvements in retroviral vectors can achieve sufficiently high levels of genetically-modified hematopoietic stem cells to treat many human diseases, including HIV infection.

However, the considerable enthusiasm for the initial clinical success of the SCID-X1 gene therapy trials has been significantly tempered by the development of leukemia in 3 of 10 subjects enrolled in this protocol (80). While the mechanisms responsible for the development of malignancy remain under debate, it is possible that this event is related to the insertion of the retroviral vector encoding the gamma c chain near the LMO2 proto-oncogene or other cellular oncogenes (80). Although the development of malignancy in these cases may be due to factors unique to this setting (e.g. expression of the gamma chain), these events have nonetheless raised important questions about the safety of other stem cell gene transfer protocols. Thus, new clinical studies of stem cell gene therapy for HIV disease are not likely to be initiated for several years, especially given the uncertain benefit of this approach.

Although the inefficient transduction of HSC has represented the major barrier to the development of HSC gene therapy for AIDS, there are other barriers as well. Initial reports that $CD34^+$ bone marrow cells are susceptible to HIV infection (81) or infected *in vivo* (82), have not been confirmed by most investigators (83-85). However, there is clear documentation of abnormal hematopoiesis in HIV-infected people (reviewed in (86) and SIV-infected macaques (87), a factor that might interfere with differentiation of genetically modified hematopoietic stem cells. An additional potential barrier to stem cell gene therapy for AIDS is the loss of thymic function, either due to age or the effects of HIV infection. Although recent evidence suggests that there is some residual thymic function in a subset of HIV-infected people (88), there is reason to believe that thymopoiesis of genetically modified T cell progenitors may be abnormal in HIV-infected individuals and might limit the extent of immune reconstitution provided by stem cell gene therapy (89).

Results from a clinical trial examining the level of gene marking in HIV-infected people who received infusions of autologous $CD34^+$ cells transduced with an MLV vector encoding a *rev*-responsive element (RRE) decoy dramatically reinforce the challenges of human clinical trials of stem cell gene therapy for AIDS (90). Following reinfusion of transduced $CD34^+$ cells into HIV-infected subjects without conditioning, detection of RRE occurred only the first day after infusion, and even then only at very low levels (0.01 to 0.001%). A subsequent trial involving transduction of mobilized peripheral blood stem cells with an oncoretroviral vector expressing an HIV-1-specific ribozyme demonstrated more prolonged gene marking of peripheral blood lymphocytes out to 1 year or more, although levels were still quite low – on average 0.001 to 0.01% (91). Lymphocytes containing the ribozyme did appear to have a survival benefit over those

containing a control vector, but again no clear virologic or immunologic benefit was observed. Due in part to the limitations inherent in human clinical trials, it is uncertain which of a multitude of factors (lack of conditioning, inefficient transduction of HSC using oncoretroviral vectors, lack of cytokine mobilization, HIV-induced abnormalities in bone marrow, etc.) are likely to account for this extremely low and transient level of gene marking *in vivo*.

7. NONHUMAN PRIMATE MODELS FOR THE STUDY OF AIDS

The multiple challenges delineated above provide a strong rationale for preclinical analysis of gene therapy for AIDS in a nonhuman primate model. Infection of macaques with SIVmac represents the leading animal model for the study of AIDS (92). SIVmac is one of a number of lentiviruses that have been identified to infect nonhuman primates. Although these viruses do not appear to cause disease in their natural hosts, cross-species transmission to nonadapted hosts can result in AIDS, as observed when SIV from sooty mangabeys (SIVsm) was accidentally transmitted to Asian macaques, resulting in the SIVmac model (93). SIVmac and HIV share similar (though not identical) genetic organization (differing in the *vpx* and *vpu* genes), and are approximately 50% homologous based on nucleotide sequences (94). Both viruses are tropic for CD4+ T lymphocytes and macrophages and use CCR5 as a predominant coreceptor. The clinical course of SIVmac in rhesus macaques mirrors that of HIV infection in many respects, resulting in an initial burst of viral replication, followed by a partial control of viremia by host immune responses, followed by a more protracted course of progressive CD4+ T lymphocyte depletion, accompanied by increased susceptibility to opportunistic infections and malignancy (93). Although the outcome of SIVmac infection is variable and depends on the specific strain used, in general, macaques develop chronic levels of viremia of about 10^5 to 10^6 copies/ml (on average about 10-fold higher than most HIV-infected people) and progress to death in 1-2 years (in contrast to an average of 10 years in untreated HIV-infected people).

The significant genetic diversity between HIV and SIV prevents efficacy testing of many HIV-specific genetic therapies in macaques. However, the development of chimeric simian/human immunodeficiency viruses (SHIVs) based on the SIVmac backbone and containing HIV-1 sequences such as RT or envelope with the embedded *tat* and *rev* genes allows testing of genetic therapies directed at these HIV-specific targets in macaques. Finally, the close phylogenetic relationship of macaques with humans and their longstanding use as a preclinical model for bone marrow transplantation and stem cell gene therapy make the macaque model an ideal preclinical model for stem cell gene therapy for AIDS. Especially in light of the finding of malignancies associated with retroviral vectors in the SCID-X1 gene therapy trials, studies of stem cell gene therapy for AIDS in nonhuman primates are thus likely to play an even more important role, both to help analyze the potential benefit of genetic-modification of stem cells and to provide additional data on the potential risks of integrating retroviral vectors.

8. GENE THERAPY STUDIES FOR AIDS IN NONHUMAN PRIMATES

As noted above, the inability of most HIV-1-specific genetic inhibitors to inhibit SIV replication has represented a significant barrier to testing of genetic therapies for AIDS in nonhuman primates. To date, there has been one published study of CD4+ T cells transduced with a RNA-based inhibitor of HIV replication in the rhesus macaque model. In this study, CD4+-enriched T cells from 3 uninfected macaques were transduced with a retroviral vector containing antisense inhibitors to the *tat* and *rev* genes. The macaques received 3-4 infusions of transduced autologous CD4+ T cells (total 1.2 - 1.6 x 109 cells) (95). Animals were then infected with SIVmac239 one week after the last infusion. The short-term *in vivo* marking of circulating lymphocytes was 2.4-9.6% and long-term marking of CD4+ cells ranged from 0.2-1.2%. As compared with three untreated controls, the two animals with the highest levels of genetically-modified cells had lower levels of plasma antigenemia and virus-infected cells. In animals that received the antisense *tat/rev*-transduced cells, CD4+ counts were similar to normal animals; whereas in control animals, the CD4+ numbers declined significantly (95). These studies did not observe *in vivo* selection for vector-containing cells. The authors speculate that the reduction in acute-phase SIV replication by this relatively small number of transduced cells may have been due to the activation of the transduced cells resulting from the *ex vivo* stimulation or to increased homing of transduced cells to sites of SIV replication (i.e. the lymph nodes). Although these findings are encouraging, the limited number of animals and the lack of a control vector make determining the significance of these data difficult, especially in light of the fact there is considerable animal-to-animal variation in the course of SIV infection. Two subsequent trials of reinfusion of autologous lymphocytes transduced with a retroviral vector expressing macaque interferon-beta showed lower levels of gene marking and no clear benefit (96, 97)

9. QUESTIONS TO BE ADDRESSED IN THE MACAQUE MODEL

The potential for gene therapy of AIDS has yet to be realized. Currently, there are more questions than answers. The definitive proof-of-principle experiments or clinical trials have not yet been done. *In vitro* experiments allow for the initial evaluation of viral inhibition; however, the most important questions for a gene therapy strategy can only be answered in an *in vivo* system. *In vitro* studies are conducted under highly idealized conditions generally employing populations of cells that are >90% genetically-modified and challenged with virus under conditions that are of uncertain relevance for *in vivo* replication. Additionally, *in vitro* experiments cannot adequately

Table 1. Key questions related to gene therapy for AIDS to be addressed in nonhuman primates

What is the best vector for delivery of RNA inhibitors to lymphocytes and hematopoietic stem cells?
Which specific inhibitors will offer the best *in vivo* protection and confer a survival benefit on genetically-modified cells?
What level of gene marking will be necessary to provide a therapeutic benefit? In what cell population?
How can the frequency of genetically-modified cells obtained *in vivo* be improved?
How frequently will the virus evolve resistance to specific RNA inhibitors?
Will candidate gene therapy vectors cause toxicity, such as leukemia?

address issues such as immunogenicity or whether genetically-modified cells will have a survival benefit *in vivo*. Because of the advantages of nonhuman primates as a model for AIDS and for gene therapy, the macaque model should be very valuable in addressing some of these important questions in gene therapy for AIDS (Table 1).

The most basic questions for gene therapy of AIDS relate to the vector– which vector, which inhibitor, or combinations of inhibitory genes – and many of these issues may initially be addressed *in vitro*. But ultimately, *in vivo* experiments or clinical trials must be initiated in either nonhuman primates or patients, since questions that relate to safety and disease progression can be best addressed in a relevant host/disease model. As noted previously, oncoretroviral and lentiviral vectors each have distinct advantages and disadvantages for delivery of inhibitory genes to hematopoietic cells. Although both classes of vectors can yield efficient transduction of hematopoietic cells *in vitro*, there may be important differences between these vectors with respect to their ability to efficiently transduce long-term multipotent repopulating hematopoietic stem cells. The limited *in vivo* studies that have been conducted to date do not allow a rigorous conclusion to be drawn as to whether lentiviral vectors will necessarily have a clear *in vivo* advantage over oncoretroviral vectors for their ability to transduce macaque hematopoietic stem cells (98-100). The most definitive way to address this question will be competitive repopulation studies in which CD34+ hematopoietic cells are transduced in equal proportions with either an oncoretroviral or a lentiviral vector. As noted above, due to the relative endogenous block to transduction of macaque cells with HIV-1 vectors (58), SIV-based vectors are likely to prove a better choice for these studies than HIV-1-based vectors. The relative percentage of cells marked with each vector will then be compared long-term, a strategy that has been proved quite effective in optimizing results from previous stem cell gene therapy trials in nonhuman primates (76, 101). Another key question relating to vector design is whether lentiviral vectors should employ a SIN design or maintain an intact LTR in the integrated provirus. While Tat-induced upregulation of inhibitory gene expression may offer some advantages in minimizing expression of potentially toxic transgenes in uninfected cells, increasing concerns about limiting the number of integration events and avoiding potential insertional activation of host genes may dictate greater use of SIN lentiviral vectors. Again, these questions can be addressed via competitive repopulation experiments in the same animal.

Given the diversity of RNA-based inhibitors, addressing the choice of the specific inhibitor used for *in vivo* gene therapy experiments probably represents one of the most important questions to try to address in nonhuman primates. Initial nonhuman primate studies should focus on use of the most promising RNA inhibitors based on the criteria detailed above to determine whether candidate inhibitory genes are able to inhibit viral replication *in vivo* and increase the survival of genetically-modified cells. This issue is best addressed by experiments using autologous CD4+ T lymphocytes that have been transduced with either a vector containing the inhibitory RNA or a carefully matched control vector and examining whether cells containing the inhibitory vector have a relative survival advantage *in vivo*. Since many HIV-infected patients enrolled in clinical gene therapy trials are receiving some antiretroviral therapy, the ability to observe a clear survival benefit of cells containing an inhibitor gene may be limited. In contrast, macaque studies can be carried out in animals with relatively high levels of viremia, a setting that should optimize the ability to observe expansion of genetically-modified CD4+ T cells. If such an effect is demonstrated, subsequent experiments should examine whether a similar effect can be obtained using transduced hematopoietic stem cells.

The level of *in vivo* gene marking necessary to provide a therapeutic benefit represents one of the key unanswered questions in AIDS gene therapy that can be addressed in nonhuman primates. As noted above, the highest level of *in vivo* gene marking that have been obtained in nonhuman primates or humans with either adoptive transfer of T cells or stem cells are generally in the range of 1% to 5% (63, 76, 78, 95). In contrast, most *in vitro* studies have evaluated the ability of genetic inhibitors to inhibit viral replication in the setting where essentially 100% of the cells are transduced. Based on the site in the viral life cycle at which inhibition occurs and other effects such as activation of the type I interferon pathway, different RNA inhibitors are likely to vary in their ability to confer a survival advantage on genetically-modified cells at the low levels of gene marking that are currently obtainable *in vivo*. Another factor to bear in mind is that adoptive T cell therapy and stem cell gene therapy will lead to introduction of genetically-modified cells into different subsets of T lymphocytes. *Ex vivo* expansion and transduction of T lymphocytes results in relative depletion of naive T cells. Thus, adoptive T cell therapy protocols are only able to introduce a candidate inhibitor gene into a small fraction of the memory CD4+ T cell pool. Many of these T cells are relatively short-lived after reinfusion and only a small subset of these *ex vivo* expanded and transduced cells are able to persist long-term *in vivo* (102). In contrast, stem cell gene therapy will initially lead to genetic, modification of naive recent thymic emigrants, which will only slowly convert to memory T cells. In light of the differential tropism of CCR5-tropic and CXCR4-tropic HIV (and SIV

strains) for memory and naive CD4+ T cells respectively (103, 104), the tropism of clinical HIV strains may affect whether the CD4+ T cell subset targeted by a specific lentivirus carries an inhibitory gene. This feature also has implications for the design of nonhuman primate studies. Studies examining the efficacy of genetically-modified T cells may best be tested using CCR5-tropic strains such as SIVmac or SHIV162p3, whereas approaches examining the potential efficacy of inhibitors introduced into stem cells may best be tested using CXCR4-tropic viruses such as SHIV89.6p or DH12.

Identification of such a survival benefit would also likely be dependent on the assumption that direct virus-mediated cytopathicity represents a dominant mechanism of CD4+ T cell death *in vivo*. If however, indirect mechanisms of cell death play a dominant role in depletion of CD4+ T lymphocytes, then presence of an inhibitory gene may not confer a survival benefit on that cell.

If the relatively low levels of genetically-modified cells that can be currently obtained *in vivo* via adoptive T cell transfer, or via the transduction of hematopoietic stem cells, are not sufficient to mediate a protective effect against HIV or SIV infection, then alternative strategies to increase the level of genetically-modified cells will have to be pursued. For adoptive T cell therapy, depletion of the host T cell reservoir with agents such as cyclophophamide and fludarabine, coupled with systemic administration of IL-2, has proved effective in increasing the *in vivo* expansion and frequency of adoptively transferred cells (105). However, given the toxicity of such regimens, proof-of-principle demonstrations of their benefit for adoptive T cell transfer for AIDS in primates would be helpful in evaluating whether the additional benefit would outweigh the risk for human clinical trials. An alternative strategy to increase the frequency of genetically-modified cells is the use of *in vivo* selection, a strategy in which the retroviral vector encodes either a gene conferring resistance against the cytotoxic drug or a gene that induces proliferation, generally in a regulated fashion. Recent studies have demonstrated the feasibility of these approaches to yield transient levels of gene-modified repopulating cells of 80% to 90% in large animal models that received infusions of transduced HSC (106, 107). Again, in light of the potential toxicity and potential disadvantages for vector delivery of these *in vivo* selection approaches, proof-of-principle demonstrations in nonhuman primates should prove very helpful in evaluating their potential efficacy for AIDS gene therapy.

Given the well-documented ability of HIV and SIV to mutate to escape pharmacologic, immunologic and genetic inhibition, the question is not whether resistance to RNA-based inhibitors will occur, but rather how frequently it will occur and how it can be minimized. Several factors are likely to affect the ability of the virus to develop resistance, including the efficacy of the inhibitor, the viral target (or host target) of inhibition and the mechanism of inhibition. RNA inhibitors such as aptamers that bind to viral proteins with multiple sites of interaction and/or target key conserved sites are less likely to develop resistant viruses (108). In contrast, inhibitors that have relatively short points of contact such as ribozymes and RNAi are more likely to be affected by single base mutations. Because of inherent limitations in the number of replication cycles and the size of the virus population studied, which differ on several orders of magnitude from those observed in *in vivo* settings, *in vitro* studies are likely to be a relatively insensitive means to rigorously assess the potential development of resistance. If sufficient levels of genetically-modified cells can be obtained *in vivo* in order to observe an antiviral effect of a given inhibitor, studies in nonhuman primates should prove quite helpful to assess the potential evolution of resistance for a given RNA inhibitor.

Nonhuman primate studies should also prove useful in addressing some of the important safety questions related to gene therapy for AIDS. Some safety or potential toxicity issues have already been addressed in nonhuman primate models. Immunogenicity has been demonstrated for the marker gene GFP (21) and for fetal bovine serum (109). The reports of leukemia in patients who have undergone autologous stem cell transplants for SCID-X1 (80) should prompt additional studies to help determine the root cause of this tumorgenicity (e.g. insertional mutagenesis or over-expression of the common gamma chain) in nonhuman primates. Although induction of malignancy attributable to retroviral vector insertion has only rarely been observed in monkeys undergoing stem cell gene therapy protocols (110), none of the vectors used in these studies to date conferred an *in vivo* survival benefit on the genetically-modified hematopoietic progeny comparable to that observed in the SCID-X1 trial. If such dramatic *in vivo* selection is observed in nonhuman primate trials for stem cell gene therapy for AIDS, this model may offer the opportunity to more rigorously examine whether retroviral insertional mutagenesis significantly increases the risk of malignancy in the setting of extensive proliferation of genetically-modified cells.

All of these questions lead to the most important question: will a gene therapy strategy for AIDS provide therapeutic benefits? This question can only be answered in an *in vivo* system either as a proof-of-principle experiment in the macaque model or ultimately in human clinical trials. Given the advances over the past decade in gene transfer technology and in the design and delivery of genes able to inhibit HIV and SIV replication, experiments in nonhuman primates are well-poised to help address some of these basic questions.

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