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Human anti-HIV-1 tat sFv intrabodies for gene therapy of advanced HIV-1-infection and AIDS

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Abstract

The early successes of highly active anti-retroviral therapies (HAART) for the treatment of HIV-1-infection and AIDS have raised the question as to whether there is a legitimate role for gene therapy in the treatment of this chronic infectious disease. However, in many patients the profound suppression of viral replication is short lived, particularly if patients have been treated with sequential monotherapies in the past, have been infected with a highly drug resistant isolate of HIV-1, or have temporarily discontinued therapy as a ''holiday'' or because of drug intolerance. In addition, life-long adherence to maintenance HAART will probably be required even in responding patients with undetectable viremia because of the reservoirs of latently infected cells that can persist for years. Gene therapy through the introduction of anti-retroviral ''resistance'' genes into $CD4^+$ T cells is one approach that could give long term protection to these HIV-1 susceptible cells in vivo. We have explored this approach by developing intrabodies to the critical HIV-1 transactivator protein, Tat that is absolutely required for HIV-1 replication. This provocative treatment approach, that will be tested in a clinical gene therapy trial, sets the groundwork for determining if anti-Tat intrabody gene therapy together with HAART can provide a treatment strategy for the immune reconstitution of HIV-1-infected patients with advanced disease.

Keywords: AIDS; Gene therapy; Intrabodies; Intracellular antibodies; HIV infection; AD, activation domain of Tat; ARM, arginine-rich motif of Tat

Abbreviations: HAART, Highly active anti-retroviral therapy; sFv, Single-chain variable region antibody fragment (single-chain antibody); sFv intrabody, Intracellular sFv antibody; sFvtat1Ck, Murine anti-tat intrabody with an additional carboxy-terminal human C_{kappa} domain; sFvhutat2, Humanized version of sFvtat1Ck without the additional carboxy-terminal human C_{kappa} domain; TAR, Transactivation response element; P-TEFb, Positive transcription elongation factors; CTD, Carboxy-terminal domain of RNA polymerase II; CDR, Complementarity determining region; NGFR, Human nerve growth factor receptor; ΔNGFR, Truncated human nerve growth factor receptor; LNCΔNGFR, LNCX vector modified to express ΔNGFR in place of neomycin; LNCΔNGFRsFvhutat2, Same modified LNCX vector with sFvhutat2 expression off an internal CMVIE promoter; ADA, Adenosine deaminase deficiency; MAb, Monoclonal antibody; TCR, T-cell receptor

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1. Introduction

Early in the viral life cycle, human immunodeficiency virus type 1 (HIV-1) encodes a 16-kDa regulatory protein Tat, that is absolutely required for viral replication (Dayton et al., 1986; Fisher et al., 1986). Efficient transcription of human immunodeficiency virus (HIV) genes is known to be dependent on the viral transactivator protein Tat (Kao et al., 1987; Laspia et al., 1989; Feinberg et al., 1991). Tat acts to enhance the processivity of RNA polymerase II complexes that would otherwise terminate transcription prematurely at random locations downstream of the viral RNA start site. The mechanism of Tat transactivation is unique in that the *cis*-acting transactivation response element (TAR) is a stable RNA stem-loop structure that forms at the 5′ end of nascent viral transcripts (Jones and Peterlin, 1994).

The Tat protein contains two regions that are important for its function — an arginine-rich motif (ARM) that mediates the binding to TAR RNA and an activation domain (AD) that mediates the interaction with the cellular transcription machinery. Tat recruits the human transcription elongation factor P-TEFb complex, consisting in part of Cdk9 and cyclin T1, to the HIV-1 promoter via cooperative binding to the nascent HIV-1 TAR element (Fig. 1). The binding of Tat protein to the regulatory subunit cyclin T1, which is mediated by residues in the core and cysteine-rich motifs of the Tat activation domain, is the first critical step in TAR RNA recognition and Tat-mediated transactivation. The interaction of Tat protein with cyclin T1 strongly enhances the affinity and specificity of the Tat:TAR RNA interaction (Garber et al., 1998; Wei et al., 1998). The catalytic subunit Cdk9, which binds to an independent site within the cyclin domain of cyclin T1, has been shown to be essential for P-TEFb to hyperphosphorylate the carboxy-terminal domain (CTD) of RNA polymerase and as a consequence increases its ability to make full-length viral RNA transcripts (Herrmann and Rice, 1995).

Apart from its role in viral replication, Tat protein also has an effect on cellular genes that may aid in the dissemination of virus infection. For example, Tat protein is released extracellularly by infected cells and is taken up by neighboring cells causing them to be activated and highly permissive for productive HIV-1-infection (Frankel and Pabo, 1988; Helland et al., 1991; Westendorp et al., 1995; Chang et al., 1997; Li et al., 1997). Tat protein has also been shown to upregulate IL-2 secretion in activated T cells (Westendorp et al., 1994) and to recapitulate the phenotype of increased IL-2 secretion in re-

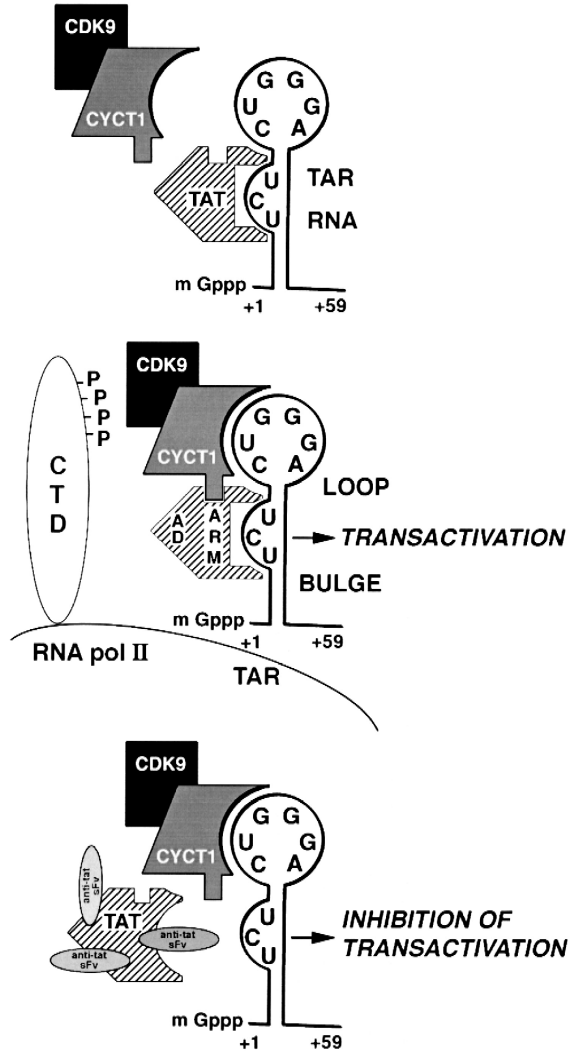

Fig. 1. Schematic view of Tat transactivation. In the absence of cellular factors, Tat binds poorly to TAR RNA (Top panel). Binding of CyclinT1 to Tat induces a conformational change in Tat such that high affinity binding to the bulge in TAR occurs. In concert with cdk9, transactivation of TAR containing nascent viral transcripts occurs by cdk9 mediated phosphorylation of the carboxy-terminal domain of RNA polymerase II (middle panel). In the presence of the anti-tat sFv intrabodies, inhibition of Tat-mediated transactivation occurs (lower panel).

sponse to costimulation of HIV-1-infected primary T-cells via CD3 and CD28 receptors (Ott et al., 1997). Extracellular Tat also favors transmission of both macrophage-tropic and T cell-tropic HIV-1 strains by inducing CCR5 and CXCR4 co-receptors (Huang et al., 1998). In this way, Tat protein is unique among the HIV-1 proteins in not only being critical for viral transcriptional activation but also for its role in evolving a self-perpetuating mechanism to actively generate cells permissive to productive and cytopathic infection (Goldstein, 1996; Li et al., 1997). Tat may also have profound effects in AIDS pathogenesis through its various immunosuppressive effects (Viscidi et al., 1989; Cupp et al., 1993; Howcroft et al., 1993) and through its role in promoting the growth of Kaposi sarcoma cells (Ensoli et al., 1990).

Thus, Tat protein through both its direct and indirect effects on the host's immune system is an attractive target for anti-retroviral therapies. Disruption of Tat protein interaction with TAR RNA or the cellular factors that bind Tat protein, and of Tat protein release from HIV-1-infected cells could potentially have a significant therapeutic benefit. By inhibiting HIV-1-infected T cells from upregulating viral transcription, the cells would remain latently infected and unable to support HIV-1 replication. The goal of our studies has been to develop intrabody gene therapy for the treatment of HIV-1-infection and AIDS using the Tat protein as our target (Mhashilkar et al., 1995, 1997, 1999; Poznansky et al., 1998, 1999). In this manuscript, we will review our studies to date, present some of our recent pre-clinical data with a humanized intrabody sFvhutat2 and the design of our clinical gene therapy trial.

2. Construction and characterization of two anti-Tat single chain (sFv) antibodies

Two murine anti-Tat hybridoma cell lines, termed 1D9D5 and 2D9D5 that were derived from a mouse immunized with recombinant Tat protein, were initially chosen for these studies. The 1D9D5 MAb has been epitope-mapped and is known to recognize amino acids 1–20 of Tat protein (Valvatne et al., 1996). This proline-rich region containing three acidic residues has been shown to be critical in Tat-mediated transactivation (Kuppuswamy et al., 1989; Ruben et al., 1989; Rice and Carlotti, 1990; Carroll et al., 1991). This region of Tat protein is also highly conserved among different HIV-1 subtypes (Korber et al., 1995; Goldstein, 1996). MAb 1D9D5 can detect Tat protein by Western blot in $pExtat_{III}$ transfected COS-1 cells (Valvatne et al., 1996). 1D2C9 Mab (formally called 2D9D5 in Mhashilkar et al., 1995) has been epitope mapped to exon 2 of Tat (D.G. Helland, personal communication).

Two single-chain antibodies termed sFvtat1 (derived from 1D9D5) and sFvtat3 (derived from 1D2C9) were constructed. The cDNA nucleotide and deduced amino acid sequence of sFvtat1 and sFvtat3 are shown in Tables 1 and 2. The sFvtat1 and sFvtat3 genes were cloned into pHEN1 for bacterial expression (Mhashilkar et al., 1995). Periplasm preparations were used to demonstrate binding of sFvtat1 and sFvtat3 to Tat protein by ELISA (Mhashilkar et al., 1995). In these studies, we observed that the binding activity of sFvtat1 was approximately twice as strong as sFvtat3.

3. Design and biological activity of anti-Tat sFv intrabodies

For expression in mammalian cells, an initiation methionine and strong Kozak sequence were introduced before the first amino acid of VH framework 1 (FR1) of sFvtat1 and sFvtat3. Three additional sFvtat1 and sFvtat3 fusion proteins were constructed that contained a carboxyl-terminal C_{kappa} domain (Ck) to promote dimerization (McGregor et al., 1994), an SV40 nuclear localization signal or a combination in a C_{kappa}-SV40 format. These studies demonstrated that: (1) nuclear targeting of sFvtat1 and sFvtat3 was not required to inhibit Tat-mediated HIV-1 LTR transactivation; (2) sFvtat1 had greater inhibitory effect than sFvtat3 on both Tat-mediated HIV-1 LTR transactivation and blockade of Tat protein entry into the nucleus; and 3) in some cell lines, the addition of the C_{kappa} domain increased the

Table 1
Nucleotide and deduced amino acid sequences of murine sFvtat1

	V_H																
ATG	CCG	GTG	AAA	CTG	CAG	GAG	TCT	GGA	CCT	GGC	CTG	GTG	GCG	CCC	TCA	CAG	AGG
Met	Pro	Val	Lys	Leu	Gln	Glu	Ser	Gly	Pro	Gly	Leu	Val	Ala	Pro	Ser	Gln	Arg
													←–––––––––**CDR1**––––→				
CTG	TCC	ATC	ACA	TGC	ACC	GTC	TCA	GGG	TTC	TCA	TTA	ACT	**AGC**	**TAT**	**GGT**	**GTA**	**CAC**
Leu	Ser	Ile	Thr	Cys	Thr	Val	Ser	Gly	Phe	Ser	Leu	Thr	**Ser**	**Tyr**	**Gly**	**Val**	**His**
														←–––––**CDR2**–––––––			
TGG	GTT	CGC	CAG	CCT	CCA	GGA	AAG	GGT	CTG	GAG	TGG	CTG	GTA	**GTG**	**ATA**	**TGG**	**AGT**
Trp	Val	Arg	Gln	Pro	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Leu	Val	**Val**	**Ile**	**Trp**	**Ser**
––→																	
GAT	**GGA**	**AGC**	**ACA**	**ACC**	**TAT**	**AAT**	**TCA**	**GCT**	**CTC**	**AAA**	**TCC**	AGA	CTG	AAC	ATC	AGC	AAG
Asp	**Gly**	**Ser**	**Thr**	**Thr**	**Tyr**	**Asn**	**Ser**	**Ala**	**Leu**	**Lys**	**Ser**	Arg	Leu	Asn	Ile	Ser	Lys
GAC	AAC	TCC	AAG	AGC	CAA	GTT	TTC	TTA	AAA	ATG	AAC	AGT	CTC	CAA	ACT	GAT	GAC
Asp	Asn	Ser	Lys	Ser	Gln	Val	Phe	Leu	Lys	Met	Asn	Ser	Leu	Gln	Thr	Asp	Asp
								←––––––––––––––**CDR3**–––––––––––––→									
ACA	GCC	ATG	TAC	TAC	TGT	GCC	AGA	**GAG**	**CCT**	**CCC**	**ACG**	**ACG**	**TAC**	**GTT**	**TGC**	**TTA**	CTG
Thr	Ala	Met	Tyr	Tyr	Cys	Ala	Arg	**Glu**	**Pro**	**Pro**	**Thr**	**Thr**	**Tyr**	**Val**	**Cys**	**Leu**	Leu
GGC	CAA	GGG	ACC	TCG	GTC	ACC	GTC	TCC	TCA	GGT	GGC	GGT	GGC	TCG	GGC	GGT	GGT
Gly	Gln	Gly	Thr	Ser	Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly
							V_K										
GGG	TCG	GGT	GGC	GGC	GGA	TCT	GAG	CTC	GTG	CTC	ACC	CAG	TCT	CCA	CTC	TCC	CTG
Gly	Ser	Gly	Gly	Gly	Gly	Ser	Glu	Leu	Val	Leu	Thr	Gln	Ser	Pro	Leu	Ser	Leu
												←––––––––**CDR1**–––––––––					
CCT	GTC	AGT	CTT	GGA	GAT	CAT	GCC	TCC	ATC	TCT	TGC	**AGA**	**TCT**	**AGT**	**CAG**	**AGC**	**CTT**
Pro	Val	Ser	Leu	Gly	Asp	His	Ala	Ser	Ile	Ser	Cys	**Arg**	**Ser**	**Ser**	**Gln**	**Ser**	**Leu**
––––––––––––––––––––––––––––––––––––→																	
GTA	**CAC**	**AGT**	**AAT**	**GGA**	**ATC**	**ACC**	**TAT**	**TTA**	**CAT**	TGG	TAC	CTG	CAG	AAG	CCA	GGC	CAG
Val	**His**	**Ser**	**Asn**	**Gly**	**Ile**	**Thr**	**Tyr**	**Leu**	**His**	Trp	Tyr	Leu	Gln	Lys	Pro	Gly	Gln
							←––––––––––––**CDR2**––––––––→										
TCT	CCA	AAG	CTC	CTG	ATC	TAC	**AAA**	**GTT**	**TCC**	**AAC**	**CGA**	**TTT**	**TCT**	GGG	TTC	CCA	GAC
Ser	Pro	Lys	Leu	Leu	Ile	Tyr	**Lys**	**Val**	**Ser**	**Asn**	**Arg**	**Phe**	**Ser**	Gly	Phe	Pro	Asp
AGG	TTC	AGT	GGC	AGT	GGA	TCA	GGG	ACA	GAT	TTC	ACA	CTC	AAG	ATC	GGC	AGA	GTG
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Lys	Ile	Gly	Arg	Val
										←––––––––––––**CDR3**–––––––––––––							
GAG	GCT	GAG	GAT	CTG	GGA	GTT	TAT	TTC	TGC	**TCT**	**CAA**	**AGT**	**ACA**	**CAT**	**ATT**	**CCG**	**TGG**
Vlu	Ala	Glu	Asp	Leu	Gly	Val	Tyr	Phe	Cys	**Ser**	**Gln**	**Ser**	**Thr**	**His**	**Ile**	**Pro**	**Trp**
––– →																	
ACG	TTC	GGT	GGA	GGC	ACC	AAG	CTG	GAA	ATA	AAA	CGG	GCT	TAG				
Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	Ala	STOP				

Underlined area indicates interchain linker.

inhibitory activity of the sFvtat1 and sFvtat3 intrabodies (Mhashilkar et al., 1995).

4. Some anti-Tat sFv intrabodies can block a spreading HIV-1 infection

Based on these results, HIV-1 challenge experiments were performed on transfected $CD4^+$ SupT cells that stably expressed the different anti-Tat sFv intrabodies. Both syncytia formation and cell-free p24 levels were determined throughout the month long experiment. These results demonstrated that neither sFvtat3 nor sFvtat3Ck expressing cells were protected from HIV-1 infection compared to cells expressing an irrelevant intrabody. The surprising findings that exon 2 specific sFvtat3 intrabodies had partial inhibitory activity for Tat-mediated transacti-

Table 2
Nucleotide and deduced amino acid sequences of sFvtat3

V_H

ATG GAC GTG AAG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG TCC
Met Asp Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Gly Ser

←--------**CDR1**------→

CTG AAA CTC TCC TGT GCA GCC ACT GGA TTC ACT TTC AGT **GAC CAT TAC ATG TAT**
Leu Lys Leu Ser Cys Ala Ala Thr Gly Phe Thr Phe Ser **Asp His Tyr Met Tyr**

←------**CDR2**----

TGG GTT CGC CAG ACT CCG GAA AAG AGG CTG GAG TGG GTC GCA **ACC ATT AAT GAA**
Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu Trp Val Ala **Thr Ile Asn Glu**

----------→

GGT GGA AGT TAC ACC TAC TAT CCA GAC AAT TTG AAG GGG CGA TTC ACC ATC TCC
Gly Gly Ser Tyr Thr Tyr Tyr Pro Asp Asn Leu Lys Gly Arg Phe Thr Ile Ser

AGA GAC AAT GCC AAG AAC AAC CTG TAC CTG CAA ATG AGC AGT CTG AAG TCT GAG
Arg Asp Asn Ala Lys Asn Asn Leu Tyr Leu Gln Met Ser Ser Leu Lys Ser Glu

←----------**CDR3**----------

GAC ACA GCC ATG TAT TAC TGT GCA AGA **GAT GGC TAC TAT GAT TAC GAC ACG GAT**
Asp Thr Ala Met Tyr Tyr Cys Ala Arg **Asp Gly Tyr Tyr Asp Tyr Asp Thr Asp**

----------→

TAC TAT GCT ATG GAC TAC TGG GGT CAA GGG ACC ACG GTC ACC GTC TCC TCA GGT
Tyr Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly

V_K

GGC GGT GGC TCG GGA GGC GGC GGT GGC TCG GGC GGT GGT GGG TCG GAG CTC GTG
Gly Gly Gly Ser Gly Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Glu Leu Val

CTC ACC CAG TCT CCA GCA CTC ATG GCT GCA TCT CCA GGG GAG AAG GTC ACC ATC
Leu Thr Gln Ser Pro Ala Leu Met Ala Ala Ser Pro Gly Glu Lys Val Thr Ile

←----------**CDR1**----------→

ACC TGC **AGT GTC AGC TCA AGT ATA AGT TCC AGC TAC TTG CAC** TGG TAC CAG CAG
Thr Cys **Ser Val Ser Ser Ser Ile Ser Ser Ser Tyr Leu His** Trp Tyr Gln Gln

←----------**CDR2**----------→

AAG TCA GGA ATC TCC CCC AAA CCC TGG ATT TAT **GGC ACA TCC AAC CTG GCT TCT**
Lys Ser Gly Ile Ser Pro Lys Pro Trp Ile Tyr **Gly Thr Ser Asn Leu Ala Ser**

GGA GTC CCT ACT CGC TTC AGT GGC AGT GGA TCT GGG ACC TCT TAC TCT CTC ACA
Gly Val Pro Thr Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr

←--------**CDR3**----

ATC AGC AGC ATG GAG GCT GAA GAT GCT GCC ACT TAT TAC TGT **CAA CAG TGG AGT**
Ile Ser Ser Met Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys **Gln Gln Trp Ser**

----------→

AGT TCC CCA TAC ACG TTC GGA GGG GGG ACC AAG CTG GAA ATA AAA CGG GCT TAG
Ser Ser Pro Tyr Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala STOP

Underlined area indicates interchain linker.

vation and blockade of Tat nuclear import and essentially no inhibitory activity on HIV-1 infection may be explained as being secondary to Rev-dependent single exon coded Tat being produced during the late phage of HIV-1 infection, while Rev-independent two exon coded Tat is produced in both early and late phases of infection (Malim et al., 1988). However, we did not conduct studies to rule out the contribution of differences in sFvtat affinity to explain these opposing results.

In striking contrast, sFvtat1 expressing cells were significantly resistant to infection until circa two weeks after challenge, when syncytia and cell-free p24 were detected. However, in the sFvtat1Ck expressing cells, viral replication could not be detected over the month long experiment. To determine

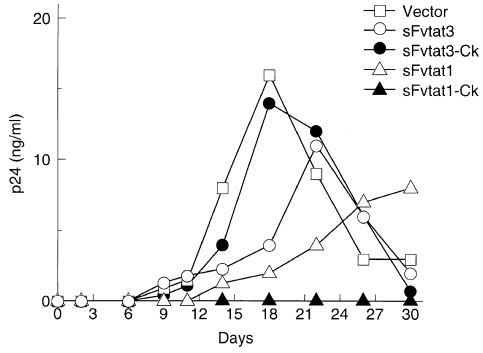

Fig. 2. HIV-1 infection of stably transfected $CD4^+$ SupT1 cells expressing different anti-Tat sFv intrabodies using virus recovered from infected sFvtat1 cells. For these studies, stably transfected SupT cells (1×10^6) were infected with $HIV\text{-}1_{IIIB}$ (25,000 c.p.m./RT activity) that was recovered from previously infected SupT-sFvtat1 cells (Mhashilkar et al., 1995). The kinetics of infection was similar to the original infection with SupT-sFvtat1 cells showing a delay in infection and SupT-sFvtat1Ck cells showing complete resistance to infection.

whether the transient inhibition of HIV-1 production in the sFvtat1 expressing cells was due to the development of HIV-1 resistance to the sFvtat1 intrabody, virus recovered from infected SupT-sFvtat1 expressing cells was used to infect fresh stocks of the different anti-tat intrabody expressing SupT cells. The results of these studies are shown in Fig. 2. The kinetics of infection were similar compared to the original infection (data not shown) thereby providing evidence that resistance to the sFvtat1 intrabody did not occur. Because of the profound inhibition of HIV-1 replication that was seen in sFvtat1Ck expressing cells, we decided to investigate this intrabody further.

5. Enhanced inhibition of HIV-1 replication by a combination of sFvtat1Ck gene therapy and NF-κB antagonists

Expression of Tat in human cells in culture leads to transactivation and overexpression of cellular genes encoding cytokines such as tumor necrosis factor alpha (TNF-α), TNF-β, and interleukin-2 (IL-2) (Sastry et al., 1990; Buonaguro et al., 1992, 1994; Westendorp et al., 1994; Ott et al., 1997), some of which in turn can activate viral transcription through their activation of NF-κB (Buonaguro et al., 1992, 1994; Westendorp et al., 1994). NF-κB binding to enhancer elements in the HIV-1 LTR can lead to TAR-independent (NF-κB dependent) activation of viral transcription by Tat (Liu et al., 1992; Biswas et al., 1995). This superactivation of HIV-1 LTR-driven gene expression is thus induced by the concerted interaction of the cellular transactivator NF-κB and Tat (Kamine and Chinnadurai, 1992; Liu et al., 1992; Biswas et al., 1993, 1995; Alcami et al., 1995).

We next investigated whether combining sFvtat1Ck gene therapy with pharmacologic inhibitors of NF-κB could increase the inhibition of HIV-1 replication that was seen after treatment of cells with either of these strategies alone. Retroviral mediated gene transfer with the MuLV shuttle vector LNCX was used to introduce the sFvtat1Ck gene into human PBMCs and $CD4^+$ mononuclear cells. The results of HIV-1 challenge experiments with transduced human PBMCs and $CD4^+$ mononuclear cells demonstrated that the combined treatment resulted in more durable inhibition of HIV-1 replication than was seen with the NF-κB inhibitors alone or the anti-Tat sFv intrabodies alone (Mhashilkar et al., 1997). These studies provide evidence that a combined pharmacologic and genetic strategy may improve the survival of transduced cells and prolong clinical benefit.

6. Inhibition of HIV-1 replication and growth advantage of transduced $CD4^+$ T cells from HIV-1-infected individuals that express sFvtat1Ck

We examined the efficiency and stability of transduction of $CD4^+$ T cells derived from 48 HIV-1-infected patients at different stages in the progression of their disease, from seroconversion to AIDS. Efficiency and stability of retroviral transduction varied during the course of HIV-1 infection; $CD4^+$ T cells derived from asymptomatic patients were transducible at higher efficiencies and stabilities than $CD4^+$ T cells from patients with AIDS. Remarkably, in experiments with transduced $CD4^+$ T cells from patients with asymptomatic HIV-1 infection that were either challenged with $HIV\text{-}1_{MN}$ strain or co-cultured

with activated PBMCs from uninfected donors, HIV-1 replication was significantly inhibited in transduced cells expressing sFvtat1Ck compared to mock transduced or empty vector transduced cells. Interestingly, these studies also demonstrated that although only circa 20% of the population of cells treated with the MuLV vector encoding the sFvtat1Ck gene were transduced, this minority population of cells was able to inhibit HIV-1 replication in the mixed cultures. These surprising results suggested that either the $CD4^+$ T cells that are permissive to productive HIV-1 infection are preferentially transduced by the vector encoding the sFvtat1Ck gene and/or that the sFvtat1Ck intrabody blocks Tat release from HIV-1-infected cells and as a result, blocks the neighboring uninfected or quiescently infected cells from becoming activated and permissive to HIV-1 infection (Poznansky et al., 1998).

7. Expression of sFvtat1Ck can protect cells against acute infection with syncytium-inducing (SI) primary isolates of HIV-1 and can decrease production of HIV-1 in persistently infected cells

We established that expression of sFvtat1Ck could protect stably transfected $CD4^+$ T cell lines and transduced primary human $CD4^+$ T cells from acute infection with a number of primary isolates of HIV-1 (Mhashilkar et al., 1999). Moreover, persistently infected U1 cells, which can be induced to increase HIV-1 mRNA synthesis on addition of PMA or TNF-α, also showed decreased production of HIV-1 in the presence of sFvtat1Ck. These latter results support the idea that sFvtat1Ck could protect latently infected $CD4^+$ T cells that act as a reservoir for the continued spread of HIV-1 even in HIV-1-infected patients that are being treated and responding to HAART (Chun et al., 1997; Finzi et al., 1997, 1999; Wong et al., 1997; Markowitz et al., 1999).

8. Humanization of the murine sFvtat1 intrabody

Like its normal cellular protein counterparts, the sFvtat1Ck protein will be degraded by the 20S and 26S proteasome and presented by MHC-I to antigen-presenting cells (Goldberg, 1995; Rock, 1996). When the MHC-I-presented peptides are recognized as foreign, a subsequent cellular immune response can be elicited against the transduced cells. Indeed, there is growing evidence that a cytotoxic T lymphocyte (CTL) response can limit long-term protein transgene expression (Riddell et al., 1996). Because of these considerations, humanization of murine sFvtat1Ck was considered a prerequisite before the protective effects of the anti-Tat sFv intrabody gene therapy approach could be examined in a clinical trial.

The sFvtat1 gene was humanized by substituting compatible human framework regions that were chosen from a database of more than 1200 human V_H sequences and more than 1000 V_L sequences. Best-matched V_H and V_L sequences were chosen on the basis of high overall framework matching, similar CDR (complementarity-determining region) length, and minimal mismatching of canonical and V_H/V_L contact residues. Through HIV-1 challenge experiments of transduced primary $CD4^+$ T cells, only the completely humanized anti-Tat sFv intrabody demonstrated a level of anti-HIV-1 activity that was comparable to murine sFvtat1Ck. This intrabody termed sFvhutat2 was chosen for clinical studies (Table 3). Interestingly and in contrast to our results with murine sFvtat1Ck, the addition of a human C_{kappa} domain to sFvhutat2 did not consistently improve its antiviral activity, thus raising the possibility that framework residues on murine sFvtat1 may have contributed to this C_{kappa} effect (Mhashilkar et al., 1999).

9. Proceeding to a human clinical gene therapy trial

Highly active anti-retroviral therapies (HAART) have lead to encouraging results in clinical trials of HIV-1-infected individuals, however, these trials have also provided a snapshot into the limitations of HAART. For example, an increasing number of patients who initially had profound anti-viral responses to HAART are now experiencing recurrence of plasma viremia (Collier et al., 1996; Hammer et al., 1997). Rapid rebound of plasma HIV-1 RNA

Table 3
Nucleotide and deduced amino acid sequences of sFvhutat2

V_H

ATG	CAG	GTG	CAA	CTG	AAG	CAG	TCT	GGA	CCT	GGC	CTG	GTG	CAT	CCC	TCA	CAG	AGC
Met	***Gln***	Val	***Gln***	Leu	***Lys***	***Gln***	Ser	Gly	Pro	Gly	Leu	Val	***His***	Pro	Ser	Gln	***Ser***
													←------CDR1-------→				
CTG	TCC	ATC	ACA	TGC	ACC	GTC	TCA	GGG	TTC	TCA	TTA	ACT	**AGC**	**TAT**	**GGT**	**GTA**	**CAC**
Leu	Ser	Ile	Thr	Cys	Thr	Val	Ser	Gly	Phe	Ser	Leu	Thr	**Ser**	**Tyr**	**Gly**	**Val**	**His**
														←--------CDR2---→			
TGG	GTT	CGC	CAG	TCT	CCA	GGA	AAG	GGT	CTG	GAG	TGG	CTG	GGA	**GTG**	**ATA**	**TGG**	**AGT**
Trp	Val	Arg	Gln	***Ser***	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Leu	***Gly***	**Val**	**Ile**	**Trp**	**Ser**
----------→																	
GAT	**GGA**	**AGC**	**ACA**	**ACC**	**TAT**	**AAT**	**TCA**	**GCT**	**CTC**	**AAA**	**TCC**	AGA	CTG	AAC	ATC	ACC	AAG
Asp	**Gly**	**Ser**	**Thr**	**Thr**	**Tyr**	**Asn**	**Ser**	**Ala**	**Leu**	**Lys**	**Ser**	Arg	Leu	Asn	Ile	***Thr***	Lys
GAC	AAC	TCC	AAG	CGC	CAA	GTT	TTC	TTC	AAA	ATG	AAC	TCC	CTC	CAA	GCT	GAT	GAC
Asp	Asn	Ser	Lys	***Arg***	Gln	Val	Phe	***Phe***	Lys	***Met***	Asn	Ser	Leu	Gln	***Ala***	Asp	Asp
								←----------CDR3----------→									
ACA	GCC	ATC	TAC	TAC	TGT	GCC	AAA	**GAG**	**CCT**	**CCC**	**ACG**	**ACG**	**TAC**	**GTT**	**TGC**	**TTA**	TGG
Thr	Ala	***Ile***	Tyr	Tyr	Cys	Ala	***Lys***	**Glu**	**Pro**	**Pro**	**Thr**	**Thr**	**Tyr**	**Val**	**Cys**	**Leu**	Trp
GGC	CAA	GGG	ACC	TCG	GTC	ACC	GTC	TCC	TCA	GGT	GGC	GGT	GGC	TCG	GGC	GGT	GGT
Gly	Gln	Gly	Thr	Ser	Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly	Gly	Gly
												V_L					
GGG	TCG	GGT	GGC	GGC	GGA	TCT	GAG	CTC	GTG	ATG	ACC	CAG	TCT	CCA	CTC	TCC	CTG
Gly	Ser	Gly	Gly	Gly	Gly	Ser	Glu	Leu	Val	***Met***	Thr	Gln	Ser	Pro	Leu	Ser	Leu
												←----------CDR1--------					
CCT	GTC	ACT	CCT	GGA	GAG	CCT	GCC	TCC	ATC	TCT	TGC	**AGA**	**TCC**	**AGT**	**CAG**	**AGC**	**CTT**
Pro	Val	***Thr***	***Pro***	Gly	***Glu***	***Pro***	Ala	Ser	Ile	Ser	Cys	**Arg**	**Ser**	**Ser**	**Gln**	**Ser**	**Leu**
----------→																	
GTA	**CAC**	**AGT**	**AAT**	**GGA**	**ATC**	**ACC**	**TAT**	**TTA**	**CAT**	TGG	TAC	TTG	CAG	AAG	CCA	GGC	CAG
Val	**His**	**Ser**	**Asn**	**Gly**	**Ile**	**Thr**	**Tyr**	**Leu**	**His**	Trp	Tyr	Leu	Gln	Lys	Pro	Gly	Gln
							←--------CDR2---------→										
TCT	CCA	CAG	CTC	CTG	ATC	TAC	**AAA**	**GTT**	**TCC**	**AAC**	**CGA**	**TTT**	**TCT**	GGG	GTC	CCA	GAC
Ser	Pro	***Gln***	Leu	Leu	Ile	Tyr	**Lys**	**Val**	**Ser**	**Asn**	**Arg**	**Phe**	**Ser**	Gly	***Val***	Pro	Asp
AGG	TTC	AGT	GGC	AGT	GGA	TCA	GGG	ACA	GAT	TTC	ACA	CTC	AAG	ATC	AGC	AGA	GTG
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Lys	Ile	***Ser***	Arg	Val
										←----------CDR3----------							
GAG	GCT	GAG	GAT	GTG	GGA	GTT	TAT	TAC	TGC	**TCT**	**CAA**	**AGT**	**ACA**	**CAT**	**ATT**	**CCG**	**TGG**
Glu	Ala	Glu	Asp	***Val***	Gly	Val	Tyr	***Tyr***	Cys	**Ser**	**Gln**	**Ser**	**Thr**	**His**	**Ile**	**Pro**	**Trp**
---→																	
ACG	TTC	GGT	CAA	GGC	ACC	AAG	CTG	GAA	ATC	AAA	CGG	GCT	TAG				
Thr	Phe	Gly	***Gln***	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	Ala	STOP				

Underlined area indicates interchain linker. Italic and bold amino acids in framework regions were changed in the humanization procedures (Mhashilkar et al., 1999).

levels after the temporary discontinuation of antiviral therapy has been reported (Daar et al., 1998; Markowitz et al., 1999). These observations underscore the importance of the persistence of proviral DNA despite the successful suppression of viral replication in peripheral blood and GALT (Chun et al., 1997; Finzi et al., 1997, 1999; Wong et al., 1997; Markowitz et al., 1999). Indeed, maintenance regimens with HAART will likely need to be life long (Havlir et al., 1998; Pialoux et al., 1998). These studies have clearly provided evidence that new and improved treatments for this chronic infection disease are required.

We are initiating a clinical gene therapy trial to examine the ability of sFvhutat2 to protect $CD4^+$ T cells from HIV-1-infected patients in vivo. New

antiretroviral pharmacologic therapies directed against Tat protein are not currently available and therefore the potential clinical benefits of the addition of anti-Tat therapy to HAART are unknown. In this regard, this trial will supply vitally important information and at the same time will add a new dimension to the therapies that are being tested in the group of patients that are in need of additional therapeutic options. In this study, we will evaluate in 10 HIV-1 infected individuals with advanced disease (plasma viral RNA levels >1000 copies/ml and $CD4^+$ counts $>100/mm^3$ while on maximal antiretroviral therapy) whether sFvhutat2 intrabody gene therapy together with HAART can prolong the survival of the genetically treated cells after they are returned to the patients. The principal objectives of gene therapy trial are to: (1) evaluate the safety of infusing autologous lymphocytes which have been transduced ex vivo with a retroviral vector encoding sFvhutat2; (2) assess the in vivo kinetics and survival of sFvhutat2-transduced cells in each patient in comparison with cells transduced with the vector alone as a control (identical except for the sFvtat cassette); (3) evaluate the in vivo expression of sFvhutat2 in human transduced lymphocytes; and (4) investigate whether immune responses directed against the sFvhutat2-transduced cells are generated by the host in vivo. A secondary objective of this trial is to make preliminary observations on the effects of gene therapy using the sFvhutat2 intrabody on in vivo plasma viral RNA and $CD4^+$ lymphocyte levels. Several other secondary objectives of the trial are related to the vector design used in this study and are discussed below.

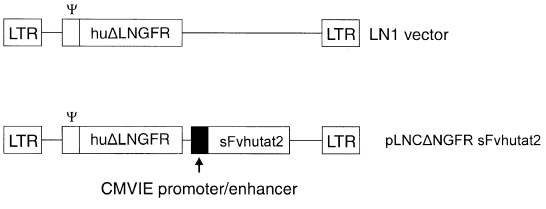

Fig. 3. Retroviral vector design. The vectors are derived from LNCX (Miller et al., 1993). The neomycin selection marker was replaced with truncated human nerve growth factor receptor (ΔNGFR) to produce LNCΔNGFR and sFvhutat2 expression is driven from an internal CMV promoter in LNCΔNGFRsFvhutat2.

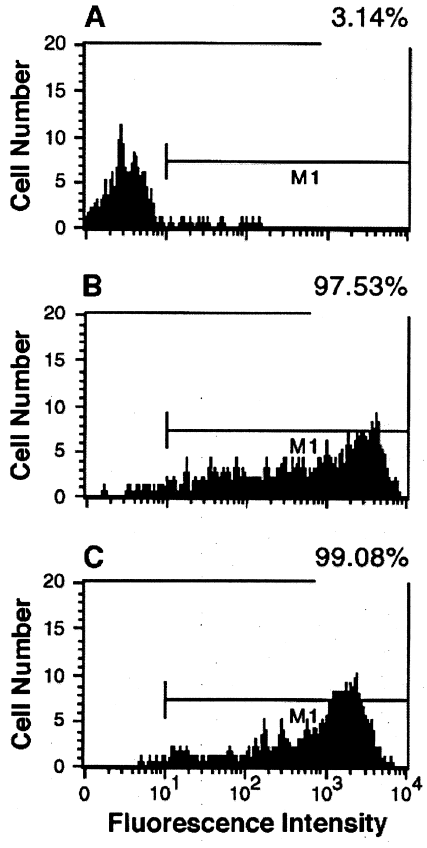

Fig. 4. FACS analysis of transduced cells. Human PBMCs depleted of $CD8^+$ cells were transduced for two consecutive days. Three days following transduction, the cells were selected for nerve growth factor receptor (NGFR) expression using anti-NGFR MAb and goat anti-mouse IgG coated high-density nickel particles. This enrichment procedure was performed two times. After the enrichment procedure, the cells were analyzed by flow cytometry. Panel A, mock transduced cells; panel B, cells transduced with LNCΔNGFR vector supernatants; panel C, cells transduced with LNCΔNGFRsFvhutat2 vector supernatants. The percentage of positive cells for each cell population is shown. *X* axis, fluorescence intensity; *Y* axis, cell number.

The design of the retroviral vectors that will be used in this study are shown in Fig. 3. The truncated human nerve growth factor receptor (ΔNGFR) is used as a phenotypic selection marker (Rudoll et al., 1996; Bonini et al., 1997; Fehse et al., 1997; Verzeletti et al., 1998). Standard separation techniques using MAb coated high-density nickel particles (Kenyon et al., 1998) will be used to first deplete $CD8^+$ T cells from the lymphopheresis prod-

uct by negative selection and then to enrich for transduced cells by positive selection using high-density nickel particles coated with MAb against NGFR. Using these techniques, we have achieved

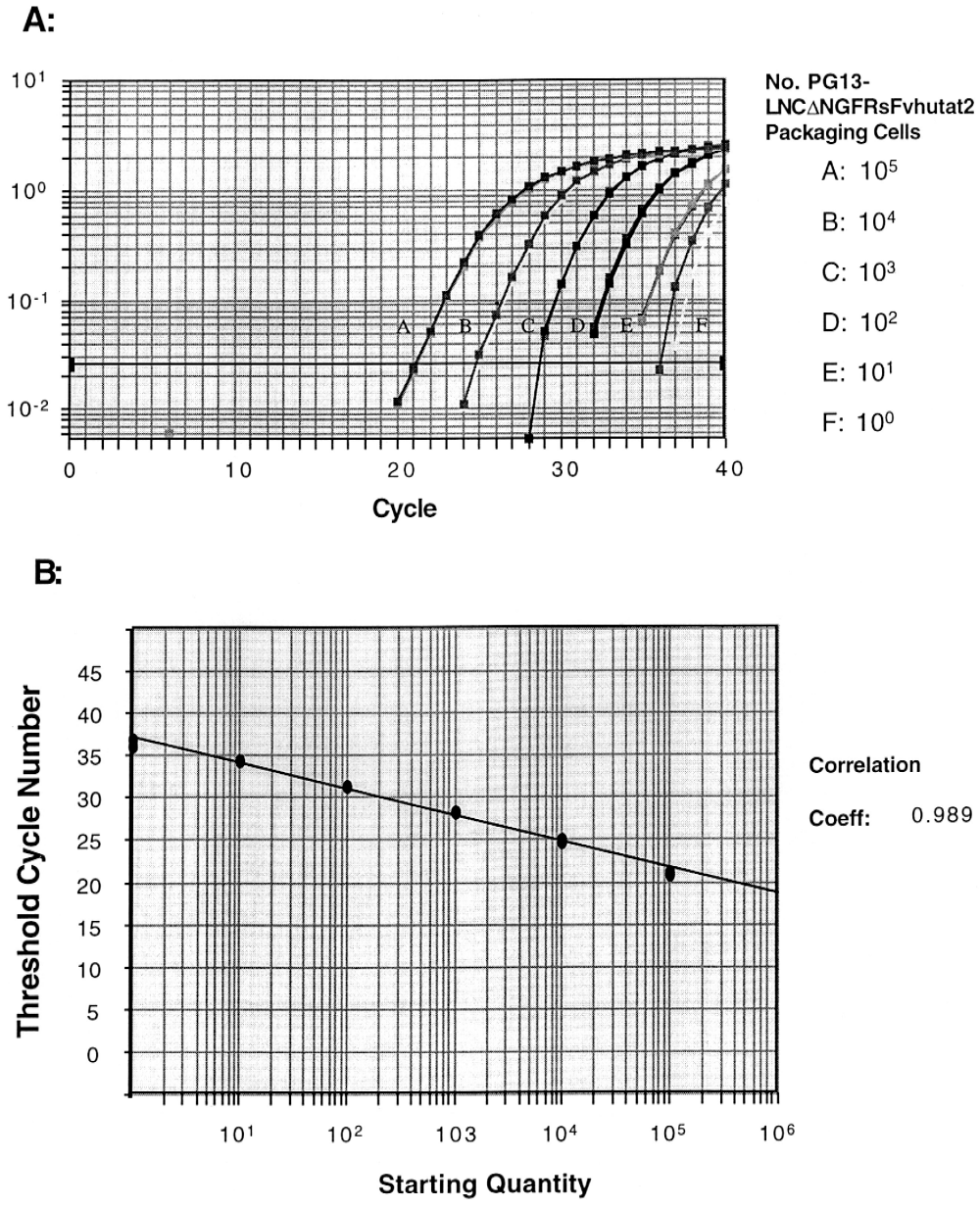

Fig. 5. Real-time Quantitative PCR. Panel A, fluorescence data generated with Sequence Detector (Perkin Elmer Biosystems) software showing release of reporter dye from the sFvhutat2-specific probe during each cycle of PCR amplification of a region of the intrabody gene. Overlapping lines contain data for duplicate wells of the 1:10 dilution curve of PG13-LNCΔNGFRsFvhutat2 packaging cell DNA from 10^5 to 10^0 cells extracted. The dark horizontal line indicates the fluorescence threshold for generation of a standard curve; Panel B, standard curve of starting copy number vs. cycle number at which fluorescence threshold is exceeded for amplification plot in panel A. This type of plot will be used to quantitate sFvhutat2 copy number from a known quantity of post-gene therapy patient PBMCs.

highly purified populations of $CD8^+$ T cell depleted transduced cells (Fig. 4). We are using anti-CD3/anti-CD28 coated beads to maximize ex vivo expansion of the transduced cells. This approach has the advantage of inducing a HIV-1-resistant phenotype for macrophage-tropic isolates of HIV-1 (Levine et al., 1996; Carroll et al., 1997; Riley et al., 1997). Our goal is to reinfuse 5×10^8–1×10^9 transduced cells/kg, a target number that has been used in gene therapy trials for the treatment of ADA deficiency (Blaese et al., 1995).

Detection of transduced cells in vivo will be achieved using real-time quantitative PCR (Lie and Petropoulos, 1998; Orlando et al., 1998). With this technique we will measure the percentage of vector and sFvhutat2 transduced PBMCs in patient's blood samples from different time points during the year long clinical trial. In this assay, the 5′ nuclease activity of Taq Polymerase cleaves a fluorochrome-labeled gene-specific nucleotide probe to release the fluorochrome from quenching. Starting copy number is thus estimated by the kinetics of fluorochrome accumulation during subsequent PCR cycles rather than end-point DNA quantitation. These data are then normalized by starting cell number using a commercially available β-actin-specific quantitative PCR and another standard curve. An example of this assay as applied to one of our retroviral packaging cell lines is demonstrated in Fig. 5. DNA is extracted from of 10^5 PG13 packaging cell lines expressing the LNCΔNGFRsFvhutat2 vector. As can be seen, this assay has a sensitivity that is capable detecting transduced cells at the level of a single cell.

10. Can immune reconstitution be achieved using sFvhutat2 intrabody gene therapy?

The incorporation of a phenotypic selection marker into the vectors should also allow us to perform some important immunologic studies to understand whether immune reconstitution can be documented using this gene therapy approach. For example, we should be theoretically able to recover transduced $CD4^+$ T cells from the patient's peripheral blood by selection for NGFR expression. Thus, additional secondary goals of our study are to examine longitudinal changes in $CD4^+$ T cell surface marker phenotype in terms of naïve and memory status (Connors et al., 1997). Likewise, changes in antigen receptor (TCR) repertoire can be examined both by analyzing TCR Vβ gene usage and by analyzing the varying lengths of the β-chain CDR3 hypervariable region following sFvhutat2 gene therapy (Connors et al., 1997; Gorochov et al., 1998). Finally, HIV-1-specific (Pitcher et al., 1999) and CMV-specific (Komanduri et al., 1998) $CD4^+$ T cell responses can be examined using a multiparameter flow cytometric assay of antigen-specific $CD4^+$ memory T cells based on the concominant induction of intracellular cytokine(s) and intracellular CD69 (a rapidly induced activation marker) after very short-term (6 h) stimulation of golgi transport-inhibited lymphocytes with antigen.

11. Future directions

The future directions of this gene therapy approach will obviously depend on the outcome of our clinical trial. A successful trial would ideally demonstrate that sFvhutat2 transduced $CD4^+$ T cells show a reduced ability to support HIV-1 replication as evidenced by their extended survival in vivo. For this to occur, the sFvhutat2 intrabody would have to be active against a suspected wide range of quasispecies of HIV-1 with differing Tat genes that are likely to exist in these patients with advanced HIV-1-infection (Meyerhans et al., 1989; LeGuern et al., 1993; Neuveut and Jeang, 1996). In addition, despite our inability to demonstrate the development of resistance to sFvhutat2 in vitro (Fig. 2), resistance to the sFvhutat2 intrabody may still occur in vivo. Indeed, one characteristic of primate lentivirus infections is the propensity for the virus to rapidly undergo genomic variations. Consequently, viruses isolated from the same patient with AIDS, at different stages of disease, often show sequence changes that phenotypically reflect differences in replication and cytopathicity (Connor et al., 1997; Lu et al., 1997; Glushakova et al., 1998; Kimata et al., 1999). This may occur by adaptive evolution in vivo, a natural selection process that results in selection of progeny

viruses that have escaped immunologic recognition through mutations (Koenig et al., 1995; McKnight et al., 1995; Nowak et al., 1995; Wolinsky et al., 1996; Goulder et al., 1997). Similarly, drug resistance (to protease inhibitors, nucleoside inhibitors and non-nucleoside reverse transcriptase inhibitors are good examples) evolves through the accumulation of replication errors (mutations) in the viral population and the preferential survival of the mutants best able to replicate in the presence of drug. This replication sets the stage for new rounds of mutation and selection, increasing the level of drug resistance over time (Coffin, 1995; Havlir and Richman, 1996; Condra, 1998; Shafer et al., 1998). However, the addition of anti-Tat intrabody gene therapy could add an important new component to divergent combination therapy; the addition of another distinct therapeutic class of drugs with nonoverlapping sets of resistance determinants should raise the genetic barrier to resistance for the viral population (Condra, 1998).

Equally important to the reduced ability of sFvhutat2 transduced $CD4^+$ T cells to support HIV-1 replication in vivo is whether the truncated human NGFR and sFvhutat2 proteins are recognized as host or as foreign proteins. Our ability to avoid eliciting an immune response to the protein transgenes is critical if we are to circumvent the otherwise inevitable consequence that the transduced cells will be killed prematurely by CTLs (Riddell et al., 1996).

As with all new therapies, we are likely to experience successes and failures; the latter will be a learning tool for refinement of future gene therapy trials. However, the successful passage over these clinical hurdles would open up the opportunity to extend this type of therapy significantly. For example, HIV-1-infected patients could be retreated with additional infusions of sFvhutat2 transduced cells in an effort to ''boost'' the numbers of $CD4^+$ T cells expressing sFvhutat2. Another scenario could involve the transduction, ex vivo expansion and reinfusion of $CD34^+$ hematopoietic stem cells that retain pluripotent and self-renewing capabilities. Indeed, we have recently demonstrated that adult bone marrow and umbilical cord blood derived $CD34^+$ stem cells can be transduced with a retroviral vector encoding sFvtat1Ck with transduction efficiencies of circa 25% (Poznansky et al., 1999). Furthermore, when the transduced stem cells are induced to differentiate into monocytes or T-cells in vitro and then subsequently challenged with monocytotropic or T-cell tropic HIV-1 isolates respectively, marked inhibition of HIV-1 replication was observed. In addition, the sFvtat1Ck expressing cells have a selective growth advantage in culture. We conclude from these studies that stem cell gene therapy using sFvhutat2 to protect progeny $CD4^+$ monocytes and T-cells may hold promise in future clinical trials.

Other intrabody approaches that are under development involve targeting the cellular proteins that interact with Tat. Indeed, Herrmann et al. (1998) reported that induction of Cdk9 and cyclin T1 protein expression occurs in purified primary $CD4^+$ T cells that are activated by a variety of stimuli that promote HIV-1 replication. These results suggest that under conditions where P-TEFb is inactive, Tat may fail to stimulate viral gene expression, causing the virus to enter into a transcriptionally latent state and escape from immune surveillance (Emerman and Malim, 1998). Interestingly, alanine-scanning mutagenesis of human cyclin T1 resulted in the identification of a 12 amino acid region at the carboxy-terminal edge of the cyclin domain that is critical for Tat:TAR recognition (Garber et al., 1998). Intrabodies directed to this region of cyclin T1 in particular may be expected to block Tat-mediated transactivation. Targeting intrabodies to these cellular cofactors may have an advantage in that resistance is not likely to occur through genetic changes in the cellular gene(s). This is in striking contrast to the genetic changes that occur in the viral genome. These combined efforts should provide key insights into the pathogenesis of HIV-1 disease and allow us to determine if intrabody gene therapy will have a significant clinical role in the treatment of HIV-1-infection and AIDS in the next millennium.

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