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Inhibition of Human Immunodeficiency Virus Type 1 Replication *in Vitro* in Acutely and Persistently Infected Human CD4$^+$ Mononuclear Cells Expressing Murine and Humanized Anti-Human Immunodeficiency Virus Type 1 Tat Single-Chain Variable Fragment Intrabodies

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ABSTRACT

We have previously reported that a murine anti-Tat sFv intrabody, termed sFvtat1Ck, directed against the proline-rich N-terminal activation domain of HIV-1, is a potent inhibitor of HIV-1 replication [Mhashilkar, A. M., *et al.* (1995). EMBO J. 14, 1542–1551]. In this study, the protective effect of sFvtat1Ck expression on HIV-1 replication in both acutely infected and persistently infected CD4$^+$ cells was examined. Stably transfected CD4$^+$ SupT1 cells were resistant to HIV-1 infection at high MOI with both the laboratory isolate HxB2 and six syncytium-inducing (SI) primary isolates. Persistently infected U1 cells, which can be induced to increase HIV-1 mRNA synthesis on addition of PMA or TNF-α, showed decreased production of HIV-1 in the presence of sFvtat1Ck. In transduced CD4$^+$-selected, CD8$^+$-depleted, and total PMBCs, the sFvtat1Ck-expressing cells showed marked inhibition of HIV-1 replication. The anti-Tat sFv was subsequently humanized by substituting compatible human framework regions that were chosen from a large database of human V_H and V_L sequences on the basis of high overall framework matching, similar CDR length, and minimal mismatching of canonical and V_H/V_L contact residues. One humanized anti-Tat sFv intrabody, termed sFvhutat2, demonstrated a level of anti-HIV-1 activity that was comparable to the parental murine sFv when transduced PBMCs expressing the murine or humanized sFv intrabodies were challenged with HxB2 and two SI primary isolates. Because Tat is likely to have both direct and indirect effects in the pathogenesis of AIDS through its multiple roles in the HIV-1 life cycle and through its effects on the immune system, the strategy of genetically blocking Tat protein function with a humanized anti-Tat sFv intrabody may prove useful for the treatment of HIV-1 infection and AIDS, particularly when used as an adjuvant gene therapy together with highly active antiretroviral therapies that are currently available.

OVERVIEW SUMMARY

A murine anti-Tat sFv intrabody, termed sFvtat1Ck, is a known potent inhibitor of HIV-1 replication. In this study the breadth of this inhibition was demonstrated by challenging stably transfected CD4$^+$ SupT1 cells at different MOIs and with both the laboratory strain HxB2 and a panel of six syncytium-inducing (SI) primary isolates. A delay in HIV-1 replication was also seen in stably transfected, persistently infected U1 cells, which can be induced to upregulate HIV-1 mRNA synthesis in the presence of PMA and TNF-α. Transduced human CD4$^+$ mononuclear populations were also resistant to challenge with two SI primary isolates. A completely humanized version of sFvtat1Ck,

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termed sFvhutat2, was constructed and transduced into PBMCs. The transducted cells maintained resistance to infection, at a level comparable to that seen with transduced sFvtat1Ck-expressing cells, when challenged with HxB2 and two SI primary isolates. The humanized anti-Tat sFv intrabody can now be tested in a clinical gene therapy setting both to evaluate its antiviral activity in transduced cells and to determine whether induction of CTL activity against transduced $CD4^+$ mononuclear cells expressing the humanized anti-Tat sFv intrabody can be prevented.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS type 1 (HIV-1) encodes a 16-kDa regulatory protein, Tat, which is expressed early in the viral life cycle and is absolutely required for viral replication (Dayton, 1986; Fisher *et al.*, 1986). Tat acts as a potent transcriptional activator of viral gene expression through its binding to an RNA stem–loop structure called the *trans*-activation response element (TAR) that is located 40 bp downstream from the site of initiation of transcription in the 5′ long terminal repeat (LTR). Tat functions primarily to stimulate transcription initiation and increase transcriptional elongation (Kao *et al.*, 1987; Laspia *et al.*, 1989; Feinberg *et al.*, 1991; Yang *et al.*, 1997; Wei *et al.*, 1998). However, new evidence suggests that Tat may also be required for efficient HIV-1 reverse transcription (Huang *et al.*, 1994; Harrich *et al.*, 1997).

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 has been implicated in several immunosuppressive effects including increasing the expression of the potent immunosuppressive cytokine transforming growth factor β_1 (TGF-β_1) (Cupp *et al.*, 1993), suppressing antigen-induced proliferation of T cells (Viscidi *et al.*, 1989) and decreasing the activity of an MHC class I gene promoter, thereby providing a mechanism whereby HIV-1-infected cells may be able to avoid immune surveillance and recognition by specific cytotoxic T lymphocytes (Howcroft *et al.*, 1993). Other cellular genes such as those involved in G1 checkpoint control (p53) and in cellular defense against oxidative stress (Mn-superoxide dismutase) are also downregulated by Tat (Li *et al.*, 1995; Westendorp *et al.*, 1995).

Tat has additional functions in the pathogenesis of acquired immunodeficiency syndrome (AIDS), in part because of its ability to be released from HIV-1-infected cells through a nonclassic secretory pathway and to enter the nuclei of both infected and uninfected cells. Tat uptake not only enhances HIV-1 transcription in infected cells, it also affects a range of host cellular genes in both infected and uninfected cells. This includes activation of cellular genes such as those encoding tumor necrosis factor α and β (Sastry *et al.*, 1990; Buonaguro *et al.*, 1992, 1994) and interleukin 6 (IL-6) (Nakajima *et al.*, 1989; Scala *et al.*, 1994), which in turn may activate HIV-1 gene expression and replication leading to further spread of HIV-1 (Duh *et al.*, 1989; Poli *et al.*, 1990a,b; Popik and Pitha, 1993). Tat 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 response to costimulation with CD3 plus CD28 that is seen in HIV-1-infected primary T cells that are stimulated via CD3 and CD28 receptors (Ott *et al.*, 1997). Extracellular Tat has been shown to activate uninfected quiescent T cells *in vitro* and *in vivo*, thereby causing uninfected cells to become highly permissive to productive HIV-1-infection (Li *et al.*, 1997). Tat protein also differentially induces CXCR4, CCR5, and CCR3 expression in peripheral blood mononuclear cells and promotes infection with M-tropic (R5) and T-tropic (X4) strains of HIV-1 (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; Huang *et al.*, 1998).

The preceding examples provide strong evidence that Tat is likely to have both direct and indirect effects in the pathogenesis of AIDS through its multiple roles in the HIV-1 life cycle and on the immune system. 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, thus represents an important target for pharmacologically and genetically based therapeutic interventions to combat HIV-1 infection. While clinical results with the Tat antagonist Ro24-7429 showed no evidence of antiviral activity (Haubrich *et al.*, 1995) despite prolonged inhibition of HIV-1 replication *in vitro* (Hsu *et al.*, 1993), the results of a number of Tat-directed *in vitro* gene therapy studies have been encouraging (Sullenger *et al.*, 1990; Chang *et al.*, 1994; Zhou *et al.*, 1994; Aguilar-Cordova *et al.*, 1995; Vandendriessche *et al.*, 1995; Lisziewicz *et al.*, 1996; Rosenzweig *et al.*, 1997), particularly when combined with pharmacologic inhibitors of NF-κB (Biswas *et al.*, 1993; Mhashilkar *et al.*, 1997).

We have shown that a murine anti-Tat single-chain variable fragment (sFv) intrabody directed against the proline-rich N-terminal activation domain of HIV-1 Tat is a potent inhibitor of Tat-mediated LTR *trans*-activation and HIV-1 infection (Mhashilkar *et al.*, 1995, 1997; Poznansky *et al.*, 1998). In the presence study, we examine the protective effect of this intrabody at several HIV-1 challenge doses and with different syncytium-inducing primary isolates. Both acutely and persistently infected cell lines and transduced $CD4^+$ mononuclear cell populations were examined. To minimize evoking a cellular immune response against the murine anti-Tat sFv transgene in a clinical setting (Riddell *et al.*, 1996), CDR (complementarity-determining region) grafting experiments were performed to completely humanize the murine anti-Tat sFv. One humanized anti-Tat sFv intrabody demonstrated a level of anti-HIV-1 activity that was comparable to that of the parental murine sFv. These studies form the basis of a future clinical gene therapy trial in which the protective effect(s) of a humanized anti-Tat sFv intrabody can be examined.

MATERIALS AND METHODS

Stable cell lines and primary cell cultures

Stable clones of $CD4^+$ SupT1 cells expressing the anti-Tat sFv intrabody sFvtat1Ck (SupT-sFvtat1Ck cells) or pRc/CMV (InVitrogen, San Diego, CA) vector (SupT-vector cells) have been previously described (Mhashilkar *et al.*, 1995, 1997). Per-

sistently HIV-1-infected U1 promonocytic cells (Folks, 1987, 1988) (2×10^6 cells) were transfected with DEAE-dextran using 10–14 μg of pRc/CMV vector, pRc/CMV-sFvtat1, pRc/CMV-sFvtat1Ck, or pRc/CMV-sFvTac (leaderless sFv directed against the human interleukin 2 receptor) and were incubated at 37°C in RPMI medium. Forty-eight hours posttransfection, the cells were exposed to selection medium with G418 (500 μg/ml). Six to 8 days later, the bulk stable cells were thoroughly washed with phosphate-buffered saline (PBS) and exposed to different concentrations of phorbol myristate acetate (PMA, 0–10 ng/ml) or tumor necrosis factor α (TNF-α, 0–20 ng/ml) for 4 to 5 hr at 37°C. The cells were then washed and supernatants were collected on alternate days and analyzed for p24 antigen.

SupT1 cells, U1 cells, and peripheral blood mononuclear cells (PBMCs) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The PE501 ecotropic cell line and PG13 amphotropic retrovirus-producing packaging cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (GIBCO-BRL, Gaithersburg, MD). The same culture conditions were used for NIH 3T3 cells and COS-1 cells. All cells were grown at 37°C in a humidifed incubator with 5% CO_2.

Preparation of HIV-1 primary isolate stocks

For the preparation of virus stocks, $10–15 \times 10^6$ cells, either SupT1 or MT4 (for laboratory isolates) or phytohemagglutinin (PHA, 1 μg/ml)-activated PBMCs (for primary isolates) in 6 to 8 ml of PRMI medium, were challenged with 50–60 ng of p24 (or in some cases 100,000 to 120,000 cpm reverse transcriptase [RT]). Members of the panel of European syncytium-inducing (SI) primary isolates used in these studies were screened for their ability to infect SupT1 cells (a gift from J. Albert, Karolinska Institute, Stockholm, Sweden) but were passaged only in PBMCs. The cells were incubated at 37°C until postinfection days 7 to 9 and then centrifuged at 2000 rpm for 20 min at 4°C. The supernatants were removed and transferred to another tube and centrifuged again at 3000 rpm for 15 min at 4°C. The supernatants where then filtered through a 0.45–μm pore size filter and aliquoted. Virus titers (50% tissue culture infective dose [$TCID_{50}$]/ml) were determined with H9 cells by analysis of cytopathic effects as previously described (Johnson and Byington, 1990).

HIV-1 challenge experiments with stably transfected SupT-sFvtat1Ck cells

Stable clones of SupT-sFvtat1Ck and SupT-vector cells (2×10^6 cells/6 ml of RPMI medium) were challenged with six European SI primary isolates (20 ng of p24/ml) for 4 hr at 37°C and then washed once with RPMI medium and resuspended in 6 ml of medium. Every 2 to 3 days postchallenge, 5 ml of supernatant was removed and replenished with new medium of equal volume. The supernatants were analyzed for p24 levels using a radioimmunoassay kit for the HIV-1 p24 Gag protein (Du Pont, Wilmington, DE), according to manufacturer instructions. Briefly, the supernatants were inactivated with 5% Triton X-100 and then incubated with anti-p24 antibody (2 hr at room temperature), followed by an overnight incubation (room temperature) with ^{125}I tracer. The samples were then incubated with second antibody and centrifuged, and the resulting pellet was counted on a γ counter. The p24 levels were calculated using a standard curve generated with a kit viral lysate stock solution. For studies that analyzed the effect of HIV-1 challenge dose (Fig. 2), the same protocol was used except that the challenge dose was increasing amounts of p24.

Establishment of retrovirus-producing packaging cell lines

The different anti-Tat sFv intrabody genes were cloned into the LNCX moloney murine leukemia virus (Mo-MuLV) shuttle vector under the control of the cytomegalovirus immediate-early (CMVIE) promoter (Miller, 1994). The empty LNCX or LN-anti-Tat sFv intrabody vectors (10 μg) were transfected by calcium phosphate into the ecotropic cell line PE501 (10^6 cells/100-mm dish) (Miller, 1994). Twelve hours later, the cells were washed with PBS and 3 ml of fresh medium was added to the cells. After an additional 24 hr, the supernatants from the transfected cells were collected, cleared by low-speed centrifugation ($3000 \times g$; 1200 rpm), and filtered through a 0.45-μm pore size filter, and 3 ml was used to infect the amphotropic packaging cell line PG13 (ATCC CRL-10686) (Miller *et al.*, 1993) (10^6 cells/100-mm dish) in the presence of protamine sulfate (8 μg/ml). Forty-eight hours postinfection, the cells were washed and treated with selection medium containing G418 (800 μg/ml). Once producer cell lines were established, confluent monolayer cells were split and fresh medium was added. The cells were then incubated at 32°C, after which retrovirus-containing supernatants were harvested, filtered, and analyzed for viral titers by titration on COS-1 cells. Viral titers of 10^{-5} to 10^{-6} infectious particles/ml for individual subclones were routinely obtained. Anti-Tat sFv intrabody expression in the stable PG13 packaging cell line was confirmed by radioimmunoprecipitation (data not shown).

Transduction of PBMCs and $CD4^+$ mononuclear cells

PBMCs were isolated by Ficoll-Hypaque density centrifugation (Pharmacia, Uppsala, Sweden). Cells were stimulated for 60–70 hr at 1×10^6 cells/ml with PHA (1 μg/ml; Murex Diagnostics, Dartford, England) and IL-2 (20 U/ml; Collaborative Research, Bedford, MA) or in some experiments with anti-human CD3 (5 ng/ml), anti-human CD28 (5 ng/ml), and IL-2 (20 U/ml) (Fig. 7). In some studies, cells were either $CD4^+$ selected or $CD8^+$ depleted. $CD4^+$-selected cells were obtained by incubation of 20×10^6 PBMCs on CD4-coated Selector flasks (AIS, Santa Clara, CA) for 1 hr at room temperature. After incubation, flasks were washed six times with PBS (Ca^{2+} and Mg^{2+} free). After the washes, 4 ml of RPMI supplemented with 10% FCS, penicillin/streptomycin (P/S), PHA (1 μg/ml), and IL-2 (100 U/ml) was added to the flask and incubated at 37°C and 5% CO_2 for 60–70 hr. To obtain the $CD8^+$-depleted cells, 20×10^6 PBMCs were incubated on CD8-coated Selector flasks (AIS) for 1 hr at room temperature. After incubation, the supernatant was removed from the flask and centrifuged for 10 min at 1200 rpm. The cell pellet was resuspended in 4 ml of RPMI supplemented with 10% FCS, P/S, PHA (1 μg/ml), and IL-2 (100 U/ml) placed into a 25-cm^2 tissue culture flask, and incubated at 37°C and 5% CO_2 for 60–70 hr.

Transductions were performed on three consecutive days.

The cells were washed and placed in an RPMI phosphate-free medium supplemented with 10% dialyzed FCS (GIBCO, Grand Island, NY) for 6 hr at 37°C. Cells were washed, resuspended in complete RPMI medium, and mixed with retroviral supernatant to obtain a final multiplicity of infection (MOI) of 1.0 in the presence of protamine sulfate (8 μg/ml; Sigma, St. Louis, MO). The cells were centrifuged for 1 hr at (1000 $\times$ *g*; 2600 rpm) at 32°C. The cells were then incubated overnight at 32°C (Bunnell *et al*., 1995). This procedure was repeated for two more consecutive days. After this incubation, the cells were washed with fresh medium and resuspended in medium supplemented with IL-2 (20 U/ml) and G418 (800 μg/ml) and incubated at 37°C. The medium was changed on day 5 with continued G418 selection. After 10 days in culture, the cells were washed and stimulated with irradiated human PBMCs (5000 rads) at a ratio of 1:10 (transduced cells:feeders) and PHA (1 μg/ml). Four days later the cells were fed with medium supplemented with IL-2 (20 U/ml) and G418 (800 μg/ml). When cells started to expand (on about days 10–14) they were washed and prepared for HIV-1 challenge.

Transduction efficiency of the PBMCs was determined by polymerase chain reaction (PCR) amplification with appropriate primers of the neomycin gene (Morgan *et al*., 1990). Twenty-four hours after the last transduction, 20,000 cells were collected and PCR band intensity was compared with the intensity of a quantitation curve. The transduction efficiency was estimated as about 10–15%.

HIV-1 challenge of transduced PBMCs and CD4$^+$ mononuclear cells

For the PBMC and CD4$^+$ mononuclear cell challenge experiments, cells were incubated in six-well plates containing 1 $\times$ 10^6 cells/3 ml of medium. Cells were incubated for 4 hr or in some cases overnight at 37°C with HIV-1 challenge doses (MOIs of 0.1–0.5). The cells were then washed and resuspended in 3 ml of fresh medium. Every 2 to 3 days, 2 ml of supernatant was collected for p24 assay and replaced with the same volume of fresh medium.

FACS analysis of PBMCs

Cells (transduced PBMCs and nontransduced PBMCs) were washed twice with PBS supplemented with 2% fetal calf serum (PBS–2% FCS) and resuspended at 4 $\times$ 10^6 cells/ml. 50 microliters of cell suspension (2 $\times$ 10^5 cells) was used for each antibody staining. Cells were pipetted into 12 $\times$ 75 polystyrene round-bottom tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). One microgram of antibody in 50 μl of PBS–2% FCS was added to the appropriate tube and incubated for 1 hr at 4°C. After incubation, the cells were washed three times with 1 ml of PBS–FCS per tube (1200 rpm for 3 min). After the washes were completed, 50 μl of a 1:50 dilution of the secondary antibody (fluorescein isothiocyanate [FITC]-labeled goat anti-mouse IgG) was added to each of the appropriate tubes and incubated for 30 min at 4°C. After incubation, the cells were washed three times with 1 ml of PBS–FCS per tube (1200 rpm for 3 min). Cell pellets were resuspended in 500 μl of PBS–FCS and analyzed by FACScan flow cytometer (Becton Dickinson, San Jose, CA). The monoclonal antibodies (MAbs) directly labeled with FITC and obtained from Becton Dickinson were mouse anti-human CD4, mouse anti-human CD8, mouse anti-human CD15, and mouse anti-human CD19. Other MAbs were obtained from the following sources: Endogen (Cambridge, MA)—mouse anti-human CD2, mouse anti-human CD31, and mouse anti-human ICAM; Immunotech (Westbrook, ME)—mouse anti-human CD3, mouse anti-human CD28, mouse anti-human MHC-I, and mouse anti-human β_2-microglobulin: Biosource (Camarillo, CA)—mouse anti-human CD48 and mouse anti-human MHC-II; Ancell (Bayport, MN)—mouse anti-human CD74, mouse anti-human CD80, and mouse anti-human CD86; Sigma—goat anti-mouse conjugated with FITC. FITC-labeled goat anti-mouse IgG alone served as a nonspecific control since in some cases the isotype of the commercial primary antibody was not known.

Construction of humanized anti-Tat sFv intrabodies

The anti-Tat sFv was humanized by substituting compatible human framework regions chosen from a database constructed in Microsoft (Redmond, WA) Excell 5. The database contained 1287 human V_H sequences and 1041 human V_L sequences downloaded from the Andrew C.R. Martin KabatMan web page (http://www.biochem.ucl.ac.uk/~martin/abs/simkab.html) or obtained by sequencing of human antibody V region clones at Chiron (Emeryville, CA). The anti-Tat V_H and V_L amino acid sequences were aligned and mismatched residues were highlighted and scored using Excel macros. "Best matched" human V_H and V_L sequences for anti-Tat were chosen on the basis of high overall framework matching, similar CDR length, and minimal mismatching of canonical and V_H/V_L contact residues. Framework sequences from these matches were then concatenated with CDR sequences from murine anti-Tat to design humanized anti-Tat heavy and light chain sequences. Complete DNA sequence analysis of each sFv was performed in both directions.

DNA fragments encoding desired humanized sequences were constructed by the PCR approach of Jayaraman *et al*. (1991). Sets of complementary single-stranded oligonucleotides of 39 to 69 bases were synthesized with staggered overlaps that could be annealed to create the desired fragments. Sixteen oligonucleotides (8 pairs) were used to generate each heavy chain sequence followed by a $(Gly_4Ser)_3$ linker, and 12 oligonucleotides (6 pairs) were used to generate each light chain sequence. The oligonucleotides for each fragment were mixed and annealed, and then amplified by PCR using a terminal oligonucleotide from each fragment as primer. The heavy chain/linker fragment was spliced to the light chain fragment via a *Sac*I site incorporated into the first two codons of the light chain, and the completed humanized constructs were transferred into expression vectors using flanking restriction sites.

RESULTS

Dose-dependent inhibition of HIV-1 replication in stably transfected CD4$^+$ SupT cells expressing the sFvtat1Ck intrabody

The anti-Tat sFv intrabody genes used in this study are depicted in Fig. 1. The parent genes V_H and V_L are derived from the hybridoma cell line 1D9D5 that produces a murine MAb

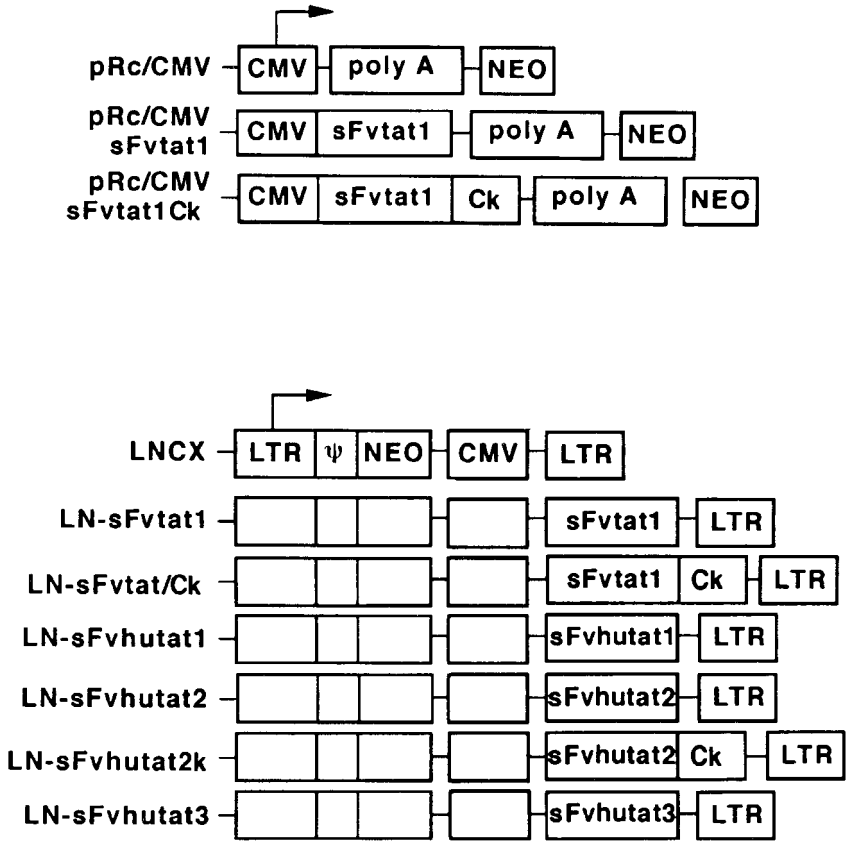

FIG. 1. Schematic representation of anti-Tat sFv intrabody expression vectors. Murine sFvtat1 and sFvtat1Ck were cloned into pRc/CMV (InVitrogen). Murine sFvtat1, sFvtat1Ck, and the humanized (hu) versions sFvhutat1, sFvhutat2, sFvhutat2Ck, and sFvhutat3 were cloned into the retroviral vector pLNCX. All of the constructs were confirmed by DNA sequencing. Empty vectors pRc/CMV and LNCX were used as negative controls throughout the studies. (See Fig. 6 for structural details on the humanized anti-Tat sFv genes.)

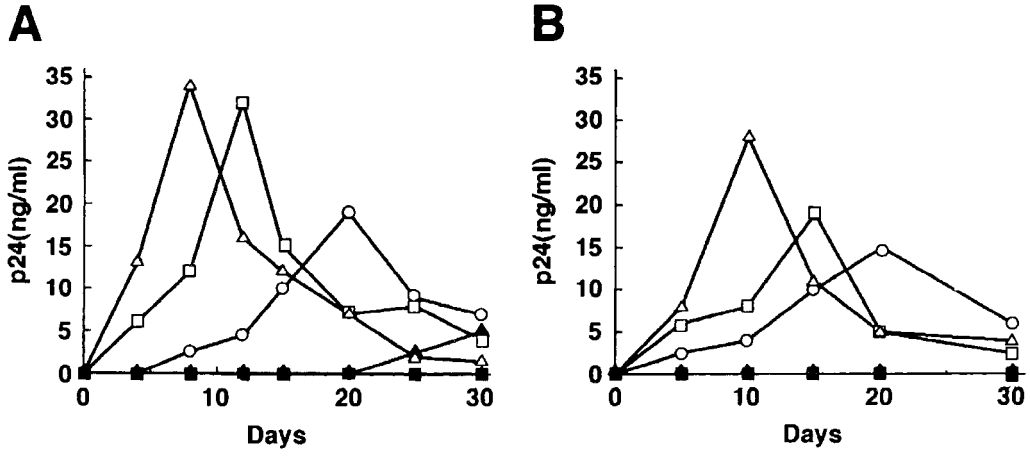

FIG. 2. Effects of HIV-1 challenge dose on resistance of sFvtat1Ck-expressing cells to HIV-1 infection. Stably transfected SupT1-sFvtat1Ck and SupT-vector cell clones were challenged with different amounts of either HIV-1_{HxB2} (**A**) or European SI primary isolate 1 (**B**). SupT-vector cells (open symbols) and SupT-sFvtat1Ck cells (closed symbols) were challenged with various MOIs of HIV-1. Circles, MOIs of 0.075 and 0.0062; squares, MOIs of 0.75 and 0.62; triangles, MOIs of 7.5 and 6.2 for HxB2 and SI primary isolate 1, respectively. The cell-free supernatant samples obtained on different days postinfection were analyzed for p24 using a commercial RIA kit (Du Pont).

directed against the proline-rich N-terminal activation domain of HIV-1 Tat to produce the anti-Tat sFv intrabody, termed sFvtat1 (Mhashilkar *et al.*, 1995).

We have previously reported that the addition of the human C_{kappa} domain to produce the anti-Tat sFv-human C_{kappa} intrabody, termed sFvtat1Ck, results in a more potent inhibitor of HIV-1 replication than sFvtat1, presumably owing to C_{kappa}-induced intrabody dimerization (Mhashilkar *et al.*, 1995, 1997; Poznansky *et al.*, 1998). However, the effect of HIV-1 challenge dose on this sFvtat1Ck-mediated protection was not reported. In the present study, stably transfected SupT-sFvtat1Ck cells were challenged with different amounts of HIV-1 to determine the effect of HIV-1 challenge dose on the degree of inhibition of HIV-1 replication. As shown in Fig. 2A, SupT-vector cells challenged with the laboratory strain HIV-1_{HxB2} showed a progressively earlier and higher level of p24 production as the challenge dose was increased over a 100-fold range. In contrast, with SupT-sFvtat1Ck, only the highest challenge dose of HIV-1_{HxB2} resulted in detectable p24 production and this was delayed until day 25.

Inhibition of replication of syncytium-inducing primary isolates in SupT-sFvtat1Ck cells

Members of a panel of syncytium-inducing primary isolates were screened for their ability to infect SupT1 cells and were subsequently used in HIV-1 challenge experiments. Similar to the results described above and as shown in Fig. 2B, when SupT-sFvtat1Ck or control SupT-vector cells were challenged with primary isolate 1, the SupT-vector cells again demonstrate a progressively earlier and higher level of p24 production as the challenge dose was increased over a 100-fold range. Over the time course of this experiment, p24 production remained undetectable in the SupT-sFvtat1Ck cell cultures.

The SupT-sFvtat1Ck and SupT-vector cells were next challenged with six different SI primary isolates at a fixed challenge dose of HIV-1 (20 ng of p24/ml). As shown in Fig. 3, infections of SupT-vector cells with all six SI-primary isolates resulted in p24 detection by day 5 and reached a peak by day 15–20. In contrast, the SupT-sFvtat1Ck cells were protected to varying degrees but in all cases, a marked delay in p24 production was noted. With primary isolates 4 and 5, there was a delay of about 40–45 days before p24 production was observed.

Resistance of persistently infected and stably transfected U1 promonocytic cells to production of HIV-1

The persistently infected U1 promonocytic cell line contains two copies of HIV-1 proviral DNA and can be induced by PMA and TNF-α to upregulate HIV-1 mRNA synthesis (Folks *et al.*, 1987, 1988, Poli *et al.*, 1990b). This cell line serves as a model for latent infection and cytokine-inducible HIV-1-replication. Sequence analysis of *tat* cDNAs from the U1 cell line identified two distinct forms of *tat*: one *tat* cDNA lacks an ATG ini-

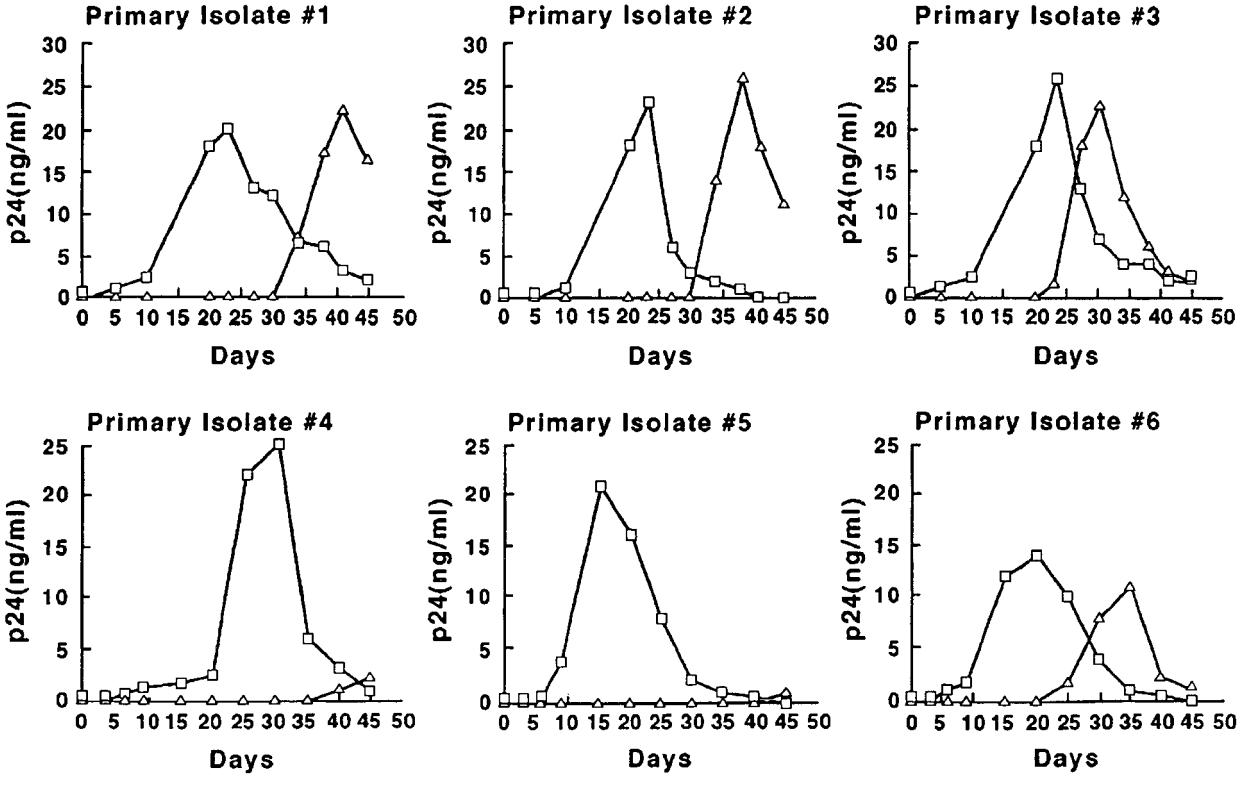

FIG. 3. Resistance of stably transfected SupT-sFvtat1Ck cells to infection with SI primary isolates of HIV-1. Stably transfected SupT1-sFvtat1Ck and SupT-vector cells were challenged, in parallel, with six different European SI primary isolates (20 ng of p24 per milliliter). (□) SupT-vector cells; (△) SupT-sFvtat1Ck cells. The cell-free supernatant samples obtained on different days postinfection were processed as described in Fig. 2.

tiation codon, while the other contains an H-to-L mutation at amino acid 13, which causes a severe reduction in transcriptional activation and defines *tat* mutations as a mechanism for HIV postintegration latency (Emiliani *et al*., 1998). Importantly, other reports have established that a reservoir of latently infected cells persists in HIV-1-infected patients that have clinically responded to highly active antiretroviral therapy (HAART) (Finzi *et al*., 1997; Wong *et al*., 1997).

Bulk populations of stably transfected U1-sFvtat1, U1-sFvtat1Ck, U1-sFvTac, and U1-vector cells were treated with different concentrations of PMA and TNF-α and cell-free supernatants were harvested on alternate days and analyzed for p24 levels. As shown in Fig. 4A the PMA-stimulated sFvtat1- and sFvtat1Ck-expressing U1 cells showed a reduction and delay in p24 production at 8 days poststimulation, with PMA (5 ng/ml) compared with irrelevant sFvTac-expressing cells or vector cells. However, by 12 days poststimulation this reduction in p24 production was no longer observed. When cells were stimulated with TNF-α (1 ng/ml), a marked reduction in p24 production was seen over the 8-day experiment (Fig. 4B). At higher concentrations of PMA (10 ng/ml) and TNF-α (10 ng/ml), neither a delay nor a reduction in p24 production was seen (data not shown). Thus under some conditions of stimulation, latently infected U1 cells expressing anti-Tat sFv intrabodies can be protected against activation of latent HIV-1 even when a critical H-to-L mutation exists within the Tat protein epitope to which sFvtat1 and sFvtat1Ck are directed.

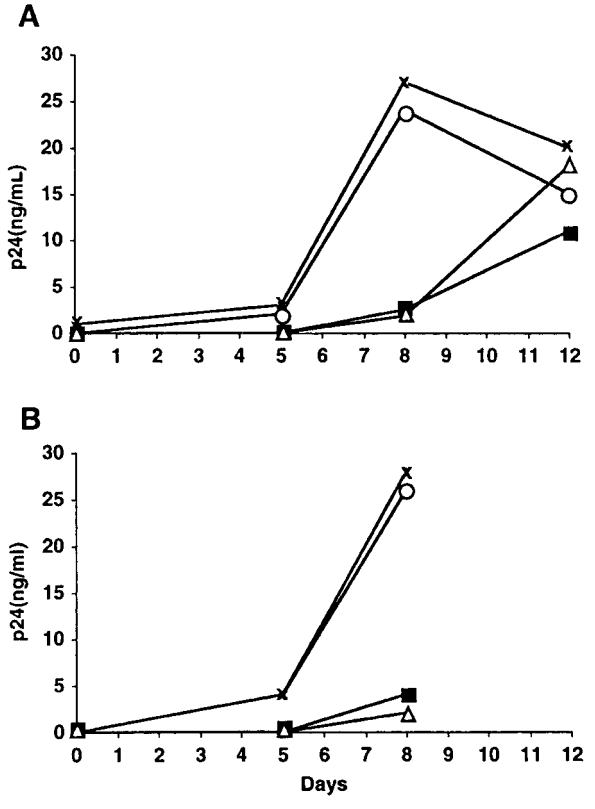

FIG. 4. Resistance of stably transfected U1 promonocytic cells to production of HIV-1. Bulk stably transfected or parental cells were treated with different concentrations of PMA (**A**) or TNF-α (**B**) and cell-free supernatants were harvested for analysis of p24 levels. (○) U1-vector cells; (×) U1-sFvTac cells; (■) U1-sFvtat1 cells; (△) U-sFvtat1Ck cells.

HIV-1 challenge of retrovirally transduced human PBMCs expressing sFvtat1Ck

HIV-1 challenge experiments with two SI primary isolates were carried out on G418-resistant bulk populations of $CD4^+$-selected and $CD8^+$-depleted peripheral blood mononuclear cells as well as on total PBMCs. In these studies, cells were transduced with retroviral supernatants from one or two different PG13 packaging cell lines producing either empty vector or vectors expressing the sFvtat1 or sFvtat1Ck intrabodies. As shown in Fig. 5, the transduced $CD4^+$-selected (Fig. 5A) and $CD8^+$-depleted (Fig. 5B) peripheral blood mononuclear cells expressing the sFvtat1Ck intrabody showed marked resistance to infection with both SI primary isolates when challenged at an MOI of 0.1. In contrast, the sFvtat1 intrabody-expressing cells have only a small inhibitory effect on HIV-1 production in agreement with our previous reports (Fig. 5A and B). HIV-1 challenge of transduced PBMCs (Fig. 5C) showed greater protection against primary isolate 2 than against primary isolate 1.

Transduced PBMCs expressing sFvtat1Ck were also tested for surface phenotype by fluorescence-activated cell sorting (FACS) analysis. As shown in Table 1, the sFvtat1Ck-expressing PBMCs were similar in their expression of a large number of cell surface molecules except for CD4 and CD86, which were expressed at 2- and 3.6-fold higher levels in transduced PBMCs compared with nontransduced PBMCs, and CD31, which was expressed at about 3-fold lower levels in transduced PBMCs.

Inhibition of HIV-1 replication in transduced PBMCs expressing humanized anti-Tat sFv intrabodies

The primary amino acid sequences encoded by the murine anti-Tat heavy and light variable region genes were compared with 1238 heavy and 1041 light human variable immunoglobulin genes, respectively. "Best matched" human V_H and V_L sequences for anti-Tat were chosen on the basis of high overall framework matching, similar CDR length, and minimal mismatching of canonical and V_H/V_L contact residues. On the basis of these criteria, human immunoglobulin genes K5B8 and TR1.6 were chosen for framework humanization (Figs. 1 and 6). Three different versions of humanized anti-Tat were constructed. sFvhutat1 retained five murine amino acids at framework positions and sFvhutat3 retained one; sFvhutat2 contained strictly human framework residues.

Transduced and G418-selected bulk populations of PBMCs expressing empty vector, murine sFvtat1Ck, or the humanized sFvhutat1, sFvhutat2, sFvhutat2Ck, or sFvhutat3 intrabodies were challenged with HxB2 and SI primary isolates 1 and 2. As shown in Fig. 7, cells transduced with empty vector, sFvhutat1, or sFvhutat3 were similar in susceptibility to HIV-1 infection, with high levels of cell-free p24 being observed as early as 3–5 days postchallenge with the laboratory strain and two SI primary isolates. In contrast, cells transduced with murine sFvtat1Ck and the humanized sFvhutat2 and

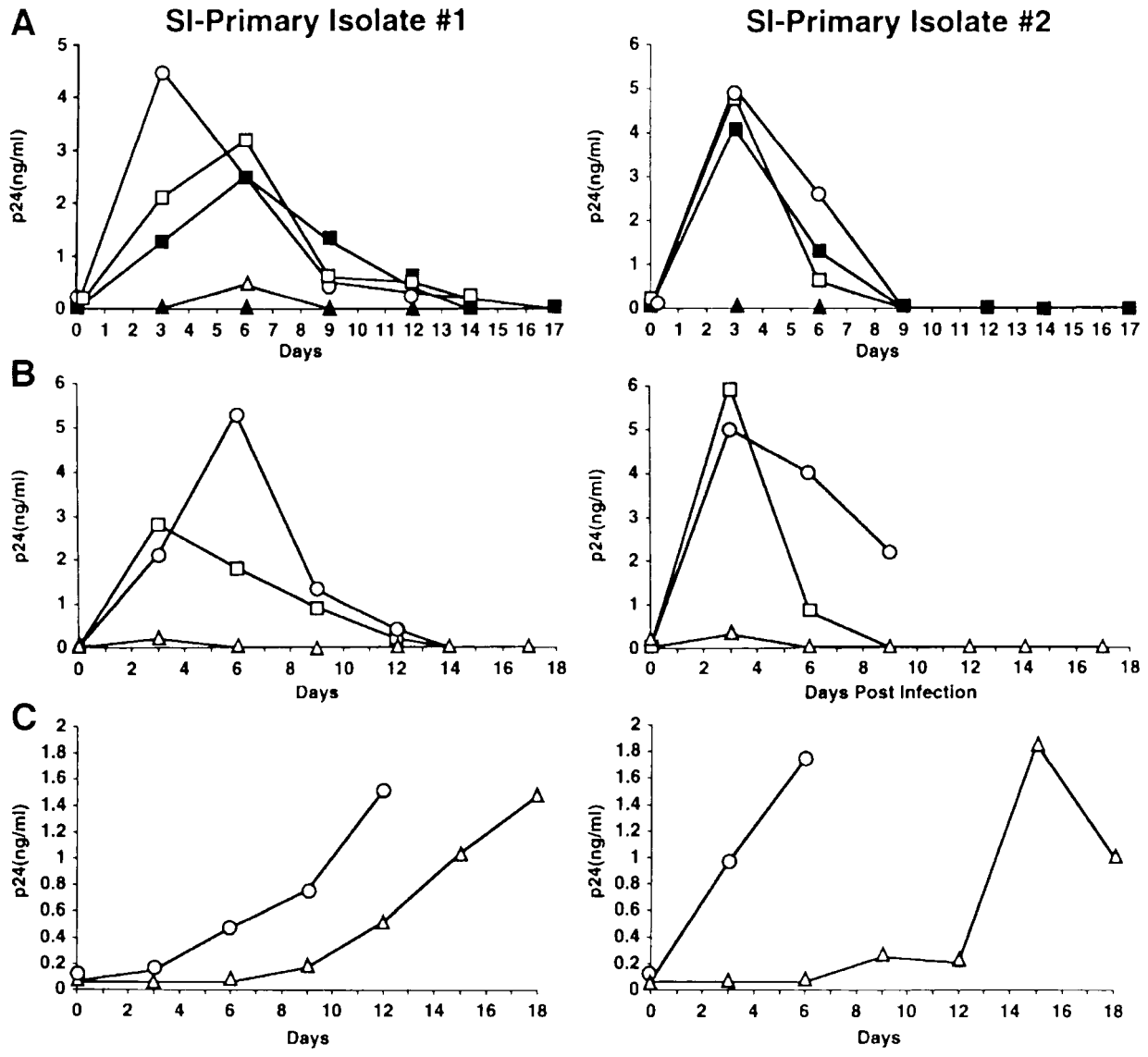

FIG. 5. Resistance of selected and total bulk populations of transduced $CD4^+$ PBMCs to HIV-1 infection. $CD4^+$-selected cells (**A**), $CD8^+$-depleted cells (**B**), and total PBMCs (**C**) were transduced with retrovirus-contain ing supernatants from one or more PG13 packaging cell lines expressing empty vector (○), or vectors expressing sFvtat1 (□, ■) or sFvtat1Ck (△, ▲). Transduced cells were selected for G418 resistance and bulk populations of cells were challenged with either SI primary isolate 1 (left panels) of SI primary isolate 2 (right panels). Cell-free supernatants were harvested for analysis of p24 levels.

sFvhutat2Ck were resistant to HIV-1 infection and showed similar efficacy at protecting the cells as long as 17–21 days after viral challenge. Of interest and in contrast to our results with murine sFvtat1 (Mhashilkar, 1995; and Fig. 5A and B), sFvhutat2 appears to be equal to or slightly better than sFvhutat2Ck in inhibiting HIV-1 replication.

DISCUSSION

In this study, the protective effect of stable expression of the murine sFvtat1Ck intrabody on HIV-1 replication in both acutely infected and persistently infected $CD4^+$ cells was examined. Stably transfected $CD4^+$ SupT1 cells were resistant to HIV-1 infection at high MOI with both the laboratory isolate HxB2 and six SI primary isolates. Persistently infected U1 cells, which can be induced by PMA and TNF-α to significantly increased HIV-1 mRNA synthesis, showed a delay in production of HIV-1 in the presence of the anti-Tat sFv intrabody. In transduced $CD4^+$-selected, $CD8^+$-depleted, and total PMBCs, the murine sFvtat1Ck-expressing cells showed marked inhibition of HIV-1 replication. Moreover, when transduced PBMCs expressing the murine sFvtat1Ck or the humanized sFvhutat2 and sFvhutat2Ck intrabodies were challenged with HxB2 and two SI primary isolates, comparable levels of inhibition of HIV-1 inhibition were observed. However, in contrast to our observations with murine sFvtat1, the addition of a C_{kappa} domain to sFvhutat2 did not consistently improve its antiviral activity, thus raising the possibility that framework residues on murine sFvtat1 may be responsible for mediating this C_{kappa} effect.

TABLE 1. FACS ANALYSIS OF NONTRANSDUCED PBMCs AND TRANSDUCED PBMCs EXPRESSING sFvtat1Ck INTRABODY: MEAN FLUORESCENCE INTENSITY MEASUREMENTS[a]

Antibody	*Nontransduced PBMCs*	*Transduced PBMC sFvtat1Ck (murine)*
Minus 1° and 2° antibody	5	9
Minus 1°, plus 2° antibody	3	10
CD2	854	839
CD3	193	190
CD4	24	48
CD8	199	137
CD15	16	28
CD19	4	7
CD28	45	55
CD31	230	78
CD58	143	97
CD74	10	31
CD80	39	31
CD86	57	205
ICAM	194	304
MHC-I	783	552
MHC-II	1185	671
β_2-Microglobulin	1854	1232

[a]Flow cytometry analysis of nontransduced PBMCs and transduced PBMCs (sFvtat1Ck) using a panel of cell surface phenotype markers. The transduced PBMCs have been under G418 selection whereas the nontransduced PBMCs were processed identically except for treatment with G418.

Intracellular immunization strategies that are aimed at inhibiting target gene expression can be RNA (antisense, ribozymes, RNA decoys) or protein (intrabodies, dominant-negative mutants) based and each group of inhibitors has distinct advantages and limitations. While RNA-based strategies are often limited by the inability to achieve high levels of inhibitor expression or to allow accurate subcellular localization, protein-based strategies may be limited by their potential immunogenicity, particularly when the genetic strategy is to treat a chronic infection such as AIDS. Like its normal cellular protein counterparts, the intracellularly expressed protein transgene 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, while results of several cancer gene therapy marking studies (Brenner *et al*., 1993a,b) and gene replacement studies (Blaese *et al*., 1995; Bordignon *et al*., 1995) that have used the neomycin selection marker have shown persistence of the marker gene and on HIV-1 revM10-based intracellular immunization study has shown preferential survival of the revM10-expressing cells compared with the non-expressing frameshift revM10-transduced cells (Woffendin *et al*., 1996), 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 the murine anti-Tat sFv was considered a prerequisite before the protective effects of the anti-Tat sFv intrabody could be examined in a clinical trial.

The anti-Tat sFv 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 human V_H and V_L sequences were chosen on the basis of high overall framework matching, similar CDR length, and minimal mismatching of canonical and V_H/V_L contact residues. In the first humanized construct, sFvhutat1, murine amino acids were retained at three heavy chain and two light chain framework positions. The second humanized construct, sFvhutat2, contained completely human frameworks. In sFvhutat3, only a single murine amino acid was retained at the heavy chain CDR4/FR4 boundary. Our results show that only sFvhutat2 was as active as the original murine sFvtat1Ck in several assays, while the less humanized versions sFvhutat1 and sFvhutat3 were considerably less protective. The unusual W → L substitution at the first heavy chain framework 4 (frm4) residue of murine sFvtat1 seems most likely (based on DNA sequence not shown) to have resulted from paired single-base deletion/insertion events such that the leucine in question is actually encoded by the last base of the last CDR codon and the first two bases of the first framework 4 codon. Maintaining this unusual change (in sFvhutat3) was not sufficient to maintain activity, emphasizing the difficulties that are often encountered in humanization procedures, particularly since modeling at the CDR3/FR4 boundary is imprecise (Chothia *et al*., 1989), and the need for some degree of trial and error to obtain optimal humanization of the sFv is often required (Queen *et al*., 1989; Carter *et al*., 1992).

Advances in antiretroviral therapies coupled with the encouraging results of clinical studies of HIV-1-infected individuals who are being treated with HAART raise the question of whether gene therapy by intracellular immunization has a legitimate role in the treatment of this chronic disease (Carpenter *et al*., 1997). However, an increasing number of patients who initially had profound antiviral responses to HAART are

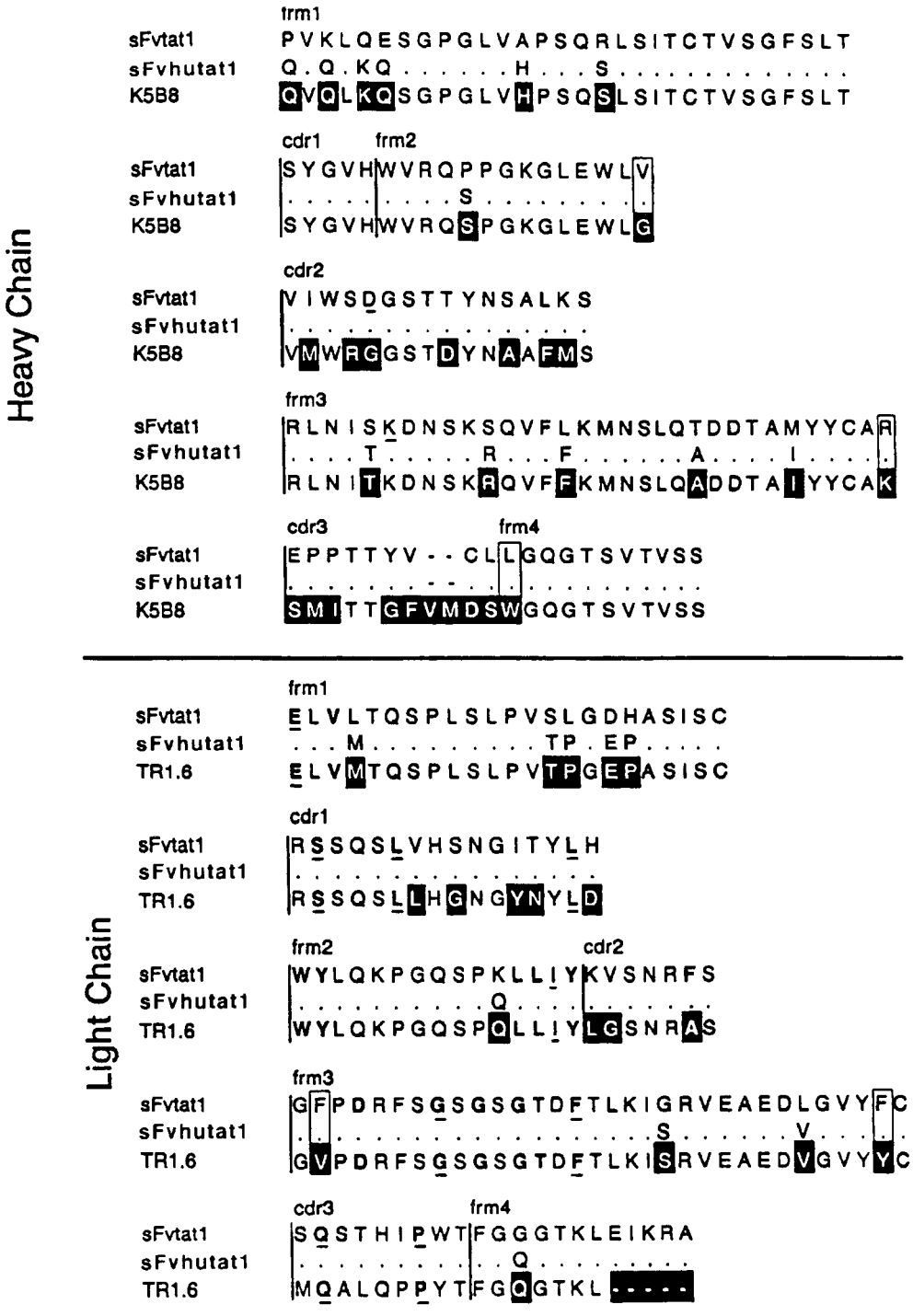

FIG. 6. Construction of humanized anti-Tat sFv intrabodies: Sequences used for generating sFvhutat intrabodies. Human V_H gene K5B8 and V_L gene TR1.6 were used for humanization procedures. *Top*: Heavy chain sequence comparisons and changes. *Bottom*: Light chain sequence comparisons and changes. Shaded boxes contain framework amino acids that are different between the murine and human genes. In the first humanized version, sFvhutat1, murine amino acids were retained at three heavy chain and two light chain framework positions (open boxes). The second humanized version sFvhutat2 was made by changing all murine framework amino acids in both heavy and light chains to the corresponding human sequences. In the third humanized version, sFvhutat3, only a single murine amino acid (L) was retained at the heavy chain CDR3/FR4 boundary. The sFvhutat2 version was further modified by adding a complete human C_{kappa} sequence at its C terminal.

now experiencing recurrence of plasma viremia (Deeks *et al.*, 1997; Gulick *et al.*, 1997). It has also been suggested that lifelong maintenance HAART will probably be required following successful induction of HAART to suppress HIV-1 replication (Havlir *et al.*, 1998; Pialous *et al.*, 1998). Furthermore, a reservoir of latently infected cells persists in these HIV-1 infected individuals (Finzi *et al.*, 1997; Wong *et al.*, 1997). As shown in Fig. 4, anti-Tat sFv intrabody gene therapy can inhibit HIV-1 replication in a transduced population of latently infected cells. Indeed, in this patient population, the genetic inhibition of Tat protein function combined with pharmacologic inhibitors of HIV-1 reverse transcriptase and protease may represent a powerful and important adjuvant therapy to inhibit the development of multiple drug-resistant viruses in these patients. Our

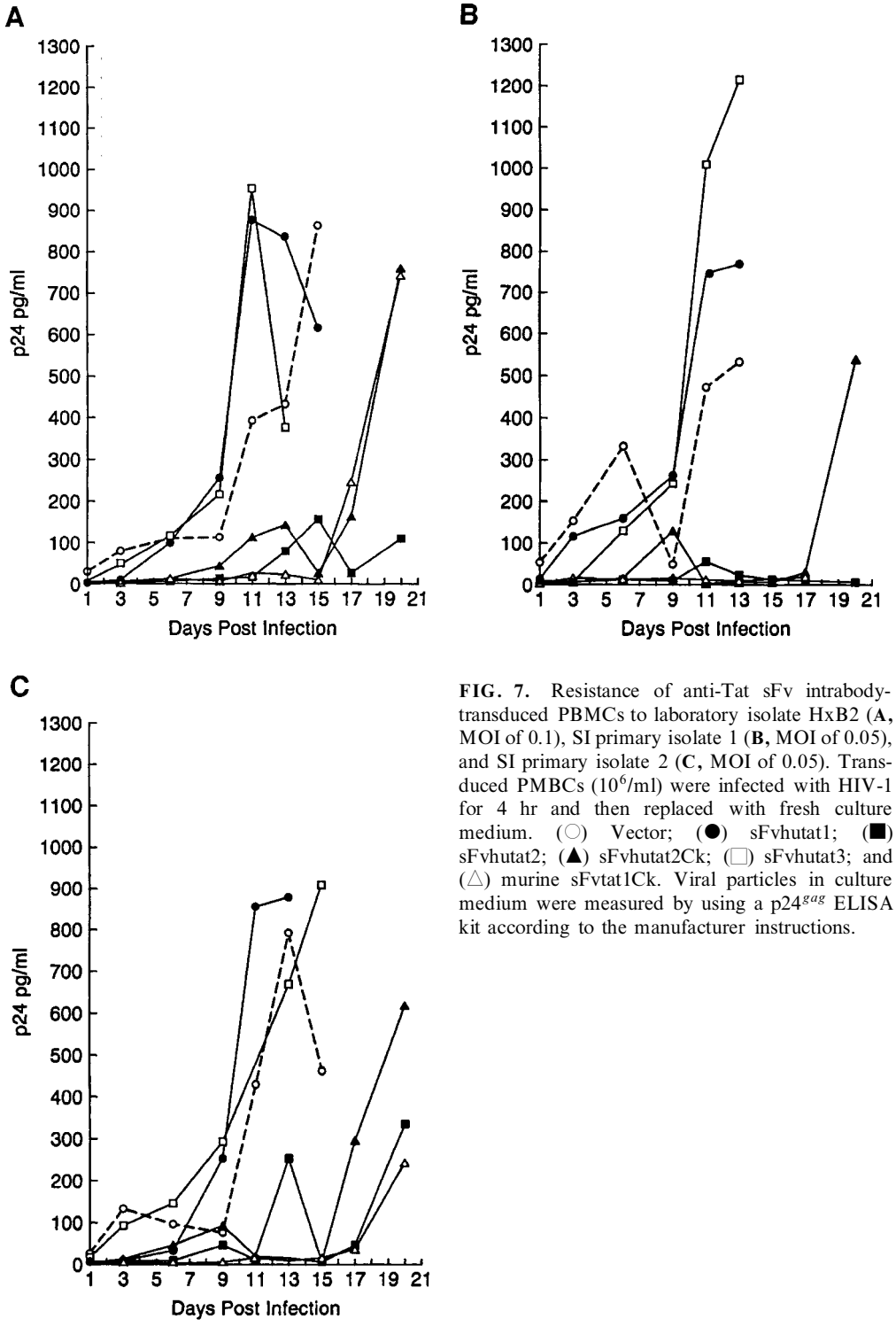

FIG. 7. Resistance of anti-Tat sFv intrabody-transduced PBMCs to laboratory isolate HxB2 (**A,** MOI of 0.1), SI primary isolate 1 (**B,** MOI of 0.05), and SI primary isolate 2 (**C,** MOI of 0.05). Transduced PMBCs (10^6/ml) were infected with HIV-1 for 4 hr and then replaced with fresh culture medium. (○) Vector; (●) sFvhutat1; (■) sFvhutat2; (▲) sFvhutat2Ck; (□) sFvhutat3; and (△) murine sFvtat1Ck. Viral particles in culture medium were measured by using a $p24^{gag}$ ELISA kit according to the manufacturer instructions.

report that pharmacologic inhibitors of NF-κB combined with anti-Tat sFv intrabody gene therapy resulted in more durable inhibition of HIV-1 replication than did treatment with either inhibitor alone supports the notion that a combined pharmacologic and genetic strategy may improve the survival of transduced cells and prolong clinical benefit in a gene therapy setting (Mhashilkar *et al*., 1997).

Finally, the introduction into susceptible cells of a humanized anti-Tat sFv intrabody that interferes with viral replication would represent an attractive approach to the treatment of HIV-1 infection if $CD4^+$ mononuclear cells from HIV-1-infected patients could be successfully transduced with retroviral vectors to express the anti-Tat sFv intrabody and the intrabody were active against a wide range of primary isolates. The data presented in Fig. 3, combined with our studies, which demonstrate that transduced and sFvtat1Ck-expressing $CD4^+$-selected mononuclear cells from HIV-1-infected patients at different stages of disease can be protected and expanded in culture (Poznansky *et al*., 1998), suggest that the humanized sFvhutat2 will be active against a wide range of primary isolates and against the quasi-species of HIV-1 that are commonly found within HIV-1-infected individuals with advanced disease. Furthermore, the N-terminal epitope to which the anti-Tat sFv intrabody is directed is highly conserved (Goldstein, 1996). Last, in several experiments in which we have examined the viruses that have escaped sFvtat1Ck intrabody suppression and have used these viruses to challenge freshly transduced cells, the viruses have remained sensitive to sFvtat1Ck suppression, suggesting that the limitations of this therapy may be more a matter of the long-term level of transgene expression *in vivo* rather than due to the development of anti-Tat sFv intrabody escape mutants (Mhashilkar *et al*., 1995; and data not shown).

In summary, the critical role that the Tat protein is likely to play both directly and indirectly in the pathogenesis of AIDS through its multiple roles in the HIV-1 life cycle and on the immune system suggests that it is an excellent target for the gene therapy of HIV-1-infection and AIDS. A role for Tat protein in HIV-1-related malignancies (Vogel *et al*., 1988; Ensoli *et al*., 1994; Albini *et al*., 1996; Dhawan *et al*., 1997; Mitola *et al*., 1997) and in upregulating other viral promoters (Ho *et al*., 1990; Tada *et al*., 1990; Sieczkowski *et al*., 1995) has also been proposed. Humanization through CDR grafting has resulted in an anti-Tat sFv intrabody that is directed against the same critical epitope of Tat protein and maintains potent antiviral activity. These studies form the basis of a future clinical gene therapy trial in which both the protective effect(s) of the sFvhutat2 intrabody on $CD8^+$-depleted, transduced PBMCs and the development of CTL activity against the intrabody-expressing cells will be examined in HIV-1-infected individuals with advanced disease.

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