Human immunodeficiency virus type 1 (HIV-1)-encoded Vif protein is important for viral replication and infectivity. Vif is a cytoplasmic protein that acts during virus assembly by an unknown mechanism, enhancing viral infectivity. The action of Vif in producer cells is essential for the completion of proviral DNA synthesis following virus entry. Therefore, Vif is considered to be an important alternative therapeutic target for inhibition of viral infectivity at the level of viral assembly and reverse transcription. To gain insight into this process, we developed a Vif-specific single-chain antibody and expressed it intracellularly in the cytoplasm. This intrabody efficiently bound Vif protein and neutralized its infectivity-enhancing function. Intrabody-expressing cells were shown to be highly refractory to challenge with different strains of HIV-1 and HIV-1-infected cells. Inhibition of Vif by intrabody expression in the donor cell produced viral particles that do not complete reverse transcription in the recipient cell. The anti-Vif scFv was shown to be specific for Vif protein because its function was observed only in nonpermissive cells (H9, CEM, and U38). Moreover, transduction of peripheral blood mononuclear cells with an HIV-derived retroviral vector expressing Vif intrabody was shown to confer resistance to laboratory-adapted and primary HIV strains. This study provides biochemical evidence for the role of Vif in the HIV-1 lifecycle and validates Vif as a target for the control of HIV-1 infection.

Functional Neutralization of HIV-1 Vif Protein by Intracellular Immunization Inhibits Reverse Transcription and Viral Replication

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Human immunodeficiency virus type 1 (HIV-1)1 is a complex
Here we have investigated the potential benefit of intracellular neutralization of Vif activity (36, 37, 40). We developed a Vif-specific single-chain antibody from immunized rabbits that was expressed intracellularly and confined to the cytoplasm. Cell lines and primary cells expressing the Vif intrabody were shown to be highly refractory to challenge with the HIV-1 virus or HIV-1-infected cells. Furthermore, replication of an HIV virus constructed to express anti-Vif scFv in cis was highly reduced after challenge with wild-type HIV-1. The formation of completed reverse transcripts was reduced when cells were infected with HIV-1 virus produced from intrabody-producing cells. These results suggest that gene therapy approaches, which deliver Vif intracellular antibodies, may represent a new therapeutic strategy for inhibiting HIV reverse transcription.

**EXPERIMENTAL PROCEDURES**

**Cells, Viruses, and Reagents**

Cells—H9, U38, Jurkat, SupT1, and CEM cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. All of the cell cultures were maintained at 37 °C in 5% CO2. Human PBMC from healthy donors were activated for 48 h with phytohemagglutinin (PHA) and then cultivated in RPMI 1640 medium supplemented with 20% fetal calf serum and 50 units of interleukin-2/ml (Roche Molecular Biochemicals). HeLa and 293T cells were grown in Dulbecco’s modified minimal essential medium supplemented with 10% fetal calf serum. Transfected cells were usually maintained in the presence of puromycin (0.5 μg/ml). The tissue culture media and reagents were from BioWhitaker.

Viruses—Plasmids coding for HIV-1NL4-3, pHIV-1NL4-3Δvif, and HIV-2ROD were obtained from the AIDS Research and Reference Reagent Program. HIV-1 primary strains Ac178 and strain J6524 isolated from HIV-1-infected children were obtained from Prof. M. Helena Lourengo (Faculdade de Farmácia de Lisboa, Lisboa, Portugal).

Plasmids—Plasmid pSVLif expresses the vif gene of the HXB2 clone of HIV-1 under the control of the SV40 promoter (15). Plasmids for the trans-complementation assay were described previously (15). Plasmid pComb3X is derived from pCombH3 (33). The coding sequence for anti-thyroglobulin scFv is cloned in plasmid pHEN obtained from Griffins et al. (41). The envelope plasmid pMD.G, the packaging plasmid pCMVR8.91, and the vector plasmid pRRL.SIN have been described previously (45, 46). HIV-1 protease was obtained from the AIDS Research and Reference Reagent Program.

Antibodies—Rabbit polyclonal Vif was described previously (15). For detection of Vif protein, rhodamine-conjugated sheep anti-rabbit immunoglobulin was used as a secondary antibody (Pierce). HRP-conjugated anti-mouse antibodies was obtained from Amersham Biosciences. FITC-conjugated, HRP-conjugated, and nonconjugated anti-HA high affinity antibody was purchased from Roche.

Proteins—Vif protein was derived from HIV-1NL4-3 strain and purified as described (45, 46). HIV-1 protease was obtained from the AIDS Research and Reference Reagent Program.

**DNA Transfections**

To produce large amounts of HIV-1 particles, 5 × 10^5 293T cells were transfected by FuGENE (Roche) with 2 μg of wild-type HIV-1NL4-3 or mutant pHIV-1NL4-3Δvif. After 24 h Jurkat and H9 cells were cocultured during 48 h in the presence of 50 μg/ml of dextran. The cells were maintained in RPMI medium plus 15% fetal calf serum. p24 antigen concentration was determined as recommended by the manufacturer (Innotest). For immunofluorescence and co-immunoprecipitation experiments, HeLa cells were co-transfected by the FuGENE reagent (Roche) according to the manufacturer’s protocol with 2 μg of pSVLif and 2-fold molar excess of plasmid encoding intrabodies or with control plasmid, pCDNA3.1Neo containing no insert. For co-immunoprecipitation, cell lysis was performed as described by Anderson (47). For immunoprecipitation, magnetic protein A beads were used as described by the manufacturer (Miltenyi Biotec).

**Replication Complementation Assay**

A transient complementation assay was used to provide a quantitative measure of the ability of wild-type or mutant Vif proteins to complement a single round of HIV-1 replication in trans. Briefly, H9 cells (10^5) were co-transfected by FuGENE (Roche), with 2 μg of either pHXB3ΔvifCAT or pHXB3ΔvifΔvifCAT, 2 μg of pSVLif, and plasmids encoding scFv as described (49). The ability of the intrabody to inhibit a single round of infection was measured by assaying for chloramphenicol acetyltransferase (CAT) activity in the transfected culture at 9 or 10 days after transfection. CAT assay was performed by ELISA (Roche).

**Immunofluorescence Staining**

HeLa cells transfected by FuGENE with 2 μg of plasmids were fixed in phosphate-buffered saline (PBS) with 4% paraformaldehyde for 10 min at room temperature, permeabilized with PBS plus 0.1% Triton X-100 for 5 min, and washed with PBS plus 2% fetal calf serum before fixation. The fixed cells were incubated with Vif antiserum (1:200) for 90 min at 37 °C, incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin antibody for 20 min at 37 °C, washed, and mounted on glass slides. For immunostaining of scFv, direct immunofluorescence was performed with FITC-conjugated anti-HA monoclonal antibody (1:40). For double immunofluorescence staining of intrabodies and Vif protein, FITC-conjugated anti-HA monoclonal antibody was used in combination with Vif antiserum at similar concentrations. The imaging setup consists of an Olympus IX-50 inverted microscope, Luddorf cooled filters wheels and dichroic mirror, and a CoolSnapCapture (Vysis) camera. Integrated control of filter wheel and image acquisition is achieved by Image-Pro Plus 4.0 and Scope-Pro 3.1 (Media Cybernetics). The settings for image acquisition (camera exposure time, filters, time interval, and storing modes) were determined by custom-made macros. The images were collected with Olympus 40× or 100× plan apo objectives (numerical apertures = 0.95 and 1.4, respectively).

**Rabbit Immunization**

A rabbit (New Zealand White) was treated with four subcutaneous injections containing 50 μg of purified Vif protein in a 1-ml emulsion of adjuvant (Ribi Immunocohm Research, Hamilton, MT). The injections were administered at 2–3-week intervals. Five days after the final boost, spleen and bone marrow were harvested and used for total RNA preparation (48, 50).

**RNA Isolation, cDNA Synthesis, Rabbit Antibody Library Construction, and Sfi Cloning**

Total RNA was prepared from rabbit bone marrow and spleen using TRI reagent from the Molecular Research Center (Cincinnati, OH) according to the manufacturer’s protocol and was further purified by lithium chloride precipitation. First strand cDNA was synthesized using the SUPERSCRIPT Preamplification System with oligo(dT) priming (Invitrogen). The protocol and the oligonucleotide primers for the construction of chimeric rabbit antibody libraries, where rabbit VL and VH sequences are combined with human Vx and CH1 sequences, have been described previously (32). Final PCR fragments encoding a library of antibody fragments were Sfi-cut, purified, and cloned into pComb3X vector. PComb3X contains a suppressor stop codon and sequences encoding peptide tags for purification (His6) and detection (HA) (50).

**Expression and Purification of Fab Fragments**

PCR fragments encoding Fab were generated by overlap extension PCR and cloned into pComb3X vector. To express Fab, phagemid DNA was transformed into the non suppressor Escherichia coli strain TOP 10F (50). Fab was purified from the concentrated supernatants of induced cultures by affinity chromatography. Purified Fab fragments were analyzed by SDS-PAGE followed by Coomassie Blue staining and Western blot with HRP-conjugated anti-HA monoclonal antibody. Protein concentration was determined by measuring the optical density at 280 nm by the classic Bradford method.

**DNA Constructs**

For the conversion of a Vif-specific Fab into a scFv, specific oligonucleotides primers were used to amplify Vh and VL gene segments from purified phagemid DNA isolated from J4, a Fab fragment specific for the Vif protein. This Fab protein was isolated from an immunized rabbit by using the phage display approach (50). The following primers were used: Vh, RSCVX1, 5′-GAGCGCCAGCGGCCAGCTGCTGATGGCAGCTACCTCA-3′; and RKB608-B, 5′-GGAAGATCTAGAGGAACCACCC- TAGACATCATCGCTGGGAGCATGAGAGGAGGT-3′; and VL, RSCVH3, 5′-GGCTGGTT- CCTTAGATCTCCATGCTGTTGAGAGGATCAGG-3′; and RSCVH4, 5′-TCCTGGGCGCTGCCCAGCTAATGACCAGTGCCCCTTTTGGARGC-3′. The purified PCR products were assembled by another PCR using the following primers: RSC-F, 5′-GAGGAGGAGGAGGAGAGGGGCGGGCGCCAGCTAGGAGTAGG-3′; and RSC-B, 5′-GAGGAAGGAGGAGGCGCCAGCAGCTAGGAGTAGG-3′. The resulting PCR product encodes an scFv in which the N-terminal
$V_r$ region is linked with the $V_h$ region through a $7$-amino acid peptide linker (GGGSGRRSS) and a $18$-amino acid peptide linker (SSGGSSGGG-GGGSSSRSS), named scFv $4B$ and $4BL$, respectively. The DNA fragment was gel-purified, digested with the restriction endonuclease SfiI, and cloned into the appropriately cut plasmid vector pComb3X, a variant of pComb3H. The binding activity of the expressed scFv was confirmed, and the genes encoding the scFv were transferred to pCDNA3.1/Neo (Invitrogen), pBabePuro vectors, and pRRL.SIN. A methionine initiation codon sequence was added into the $4BL$ and $4B$ scFv variant of pComb3H. The binding activity of the expressed scFv was and cloned into the appropriately cut phagemid vector pComb3X, a cloning anti-Vif scFv in pBabePuro and pRRL.SIN: Vector/scFv5, 5′-GGCATCGGGGCCCAGGAGCTC-3′/H11032 3′-GCCACCACCCTCCTAAGAAGC-5′. Similar primers were used for cloning anti-Vif scFv in pBabePuro and pRRL.SIN. Vector/scFv5, 5′-ACCGCTGACGATCGTATCGCCGCCGGCAGAAGC-CACCACCCTCCTAAGAAGC-3′. Downstream of the sequence, we maintained a sequence encoding the HA tag sequence (YPYDVPDYA) followed by a stop codon. Control scFv anti-thyroglobulin was amplified from pHEN2 and Sn-cloned in pComb3X. A methionine was introduced at the $N$ terminus, and the DNA fragment with a HA-tag at the $C$ terminus was cloned into the appropriately digested vector DNAs. The modified pHEN3.1/Neo plasmid encoding 4BL, 4B, and anti-thyroglobulin was designated p4BL, p4B, and pThr. Anti-Vif scFv 14BL and anti-thyroglobulin was cloned in pHxNcFLAP-IREs-N2+ by inserting the fragment in place of FLAP-IREs-S-n. The primers used for cloning anti-thyroglobulin were scFv5/HIV, 5′-ATAAGATGCCTGGCCGCAGTAATATTGGGGGCGCCAGGCGGCCGACGTC-3′, and scFv3/HIV, 5′-CGGCTCAGGCCGGCCCGAGCTC-3′. The gene encoding the antibody in place of PLAP-IRES-nef. The primers used for cloning anti-Vif scFv were used to challenge 1 × 10^6 H9 cells and PBMC in a volume of 1 ml. For infection, a stock of 2 ng of soluble p24 antigen was used. The PCR procedure to analyze reverse transcription was described previously (23, 52). The primers used in this assay (M667/M661-LTR/gag, nucleotides 496–695) amplify the late DNA products in reverse transcription. Similar reaction conditions were used, except only the 24 h time point was analyzed. The products of all PCR reactions were electrophoresed through 1.5% agarose gels, transduced to Hybond N membranes (Amersham Biosciences), subjected to Southern hybridization by using random-primed radiolabeled DNA probes (Amersham Biosciences), and visualized by autoradiography. The DNA used for radiolabeling were obtained following PCR amplification of segments of pHV-1NL4-3 or a human β-globin expression vector with the relevant primer pairs.

**In Vitro Studies of Anti-Vif Antibody Fragments**

The methods for bacterial expression of anti-Vif scFv were described before (50). Briefly, scFv were cloned in pComb3X that allows the expression of the supernatant and purification by the Nt-NTA column, causing by the presence of a His-tag tag at the C-terminal end. After induction of scFv expression by growth in 1 ml isopropyl-β-D-thiogalactopyranoside for 20 h at 30 °C, analysis of supernatants indicated that both anti-Vif scFv were expressed. For assays of expression of antibody fragments, 1 ml of culture supernatant was concentrated (Centricon 30), and Nt- and C-terminal HA-tag were used for detection (CLONTECH). Relative binding affinities of anti-Vif ScFv were determined via ELISA after coating of the wells with 200 ng of purified recombinant HIV-1 Vif protein. Purified anti-Vif scFv and Fab were diluted at various concentrations starting at 5 µg/ml and added to the wells for further incubation. After washing the wells with PBS, horseradish-conjugated anti-HA monoclonal antibody was applied for detection. Recognition of Vif by anti-Vif scFv and Fab was next demonstrated by Western blot analysis. In vitro studies, virus and HIV-1 protease (200 ng) were separated by PAGE and transferred to nitrocellulose membranes. After blocking, the proteins were probed with anti-Vif Fab and scFv antibody fragments and then with horseradish-conjugated anti-HA monoclonal antibody as a secondary antibody. The proteins were visualized using the ECL system (Amersham Biosciences).

**Retroviral Gene Transfer:**

Generation of Transduced H9 and Jurkat Cells

The amphotropic packaging cell line PhoenixAmpho was transfected with pBabe Puro plasmids encoding the anti-Vif scFv inserts (51). For control purposes pBabe Puro plasmids encoding scFv specific to thyroglobulin were also used (41). These packaging cell lines were used to generate a luciferase medium for two rounds of infection of H9 and Jurkat cells in the presence of 10 µg/ml Polybrene (Calbiochem). Two days after the last infection, transduced H9 and Jurkat cells were selected in puromycin (0.5 µg/ml) by limiting dilution cloning. After 15 days of selection, analysis of cells for anti-Vif expression and infectability was started. The untransduced parental H9 and Jurkat cell lines were named H9-P and Jurkat-P, respectively, and the H9 and Jurkat cells transduced to express anti-Vif intrabodies were named H9-14BL, H9-14B, Jurkat-14BL, and Jurkat-14B. The cell lines transduced to express the intrabody anti-thyroglobulin were named H9-thyr and Jurkat-thyro.

**HIV-1 Infection of H9 and Jurkat Cells**

Transduced and untransduced H9 and Jurkat cells were infected at a multiplicity of infection of 0.01–0.05 for 6 h at 37 °C, washed, and then cultured for up to 30 days. To monitor infection, aliquots were taken from the cultures at the indicated time points, and the p24 levels were determined in an HIV-1 ELISA (Innotest). The amphotropic packaging cell line PhoenixAmpho was transfected and Jurkat cells were infected at a multiplicity of infection of 0.01–0.05 for 6 h at 37 °C, washed, and then cultured for up to 30 days. To monitor infection, aliquots were taken from the cultures at the indicated time points, and the p24 levels were determined in an HIV-1 ELISA (Innotest). The relative binding affinities of anti-Vif Fab antibody fragments were determined via ELISA after coating of the wells with 200 ng of purified recombinant HIV-1 Vif protein. Purified anti-Vif scFv and Fab were diluted at various concentrations starting at 5 µg/ml and added to the wells for further incubation. After washing the wells with PBS, horseradish-conjugated anti-HA monoclonal antibody was applied for detection. Recognition of Vif by anti-Vif scFv and Fab was next demonstrated by Western blot analysis. In vitro studies, virus and HIV-1 protease (200 ng) were separated by PAGE and transferred to nitrocellulose membranes. After blocking, the proteins were probed with anti-Vif Fab and scFv antibody fragments and then with horseradish-conjugated anti-HA monoclonal antibody as a secondary antibody. The proteins were visualized using the ECL system (Amersham Biosciences).
Anti-Vif scFv Gene Expression in E. coli and in Vitro Interaction with HIV-1 Vif—The antibody fragment 4J4, that binds purified Vif protein was derived from a Fab phage display library. To express it in the cytoplasm in a lower molecular weight form, 4J4 Fab was converted to a single-chain fragment. Anti-Vif scFv was constructed by PCR amplification of VL and VH fragments and covalently linked with a short peptide linker of 7 amino acids (4B, GGSSRSS) and a long peptide linker of 18 amino acids (4BL, SSGGGSSGGGGGGRSSS). It was expected that the short linker peptide would result in a dimeric scFv protein (33). To determine whether the binding efficiency of recombinant scFv to the HIV-1 Vif would correlate with those of the parental Fab clone, both scFv 4BL and 4B were expressed in E. coli, and binding to HIV-1 Vif was assessed by ELISA and Western blotting. Both 4B and 4BL anti-Vif scFv, together with J4 Fab, were expressed in bacterial cell culture supernatant and purified by Ni²⁺ affinity columns. As the data in Fig. 2 demonstrate, 4B and 4BL scFv have different binding patterns. Binding of anti-Vif scFv prepared with a long linker (4BL) binds Vif protein in ELISA at similar levels to Fab J4. In contrast, anti-Vif scFv prepared with a short linker (4B) demonstrates comparatively a very low affinity. In contrast, nearly background signals indicate very low nonspecific binding of scFv 4BL to control proteins HIV-1 protease and bovine serum albumin, a profile similar to that of Fab J4.

The specificity of binding of anti-Vif scFv 4BL was next investigated by immunoblotting and immunofluorescence (Fig. 3). Vif protein and HIV-1 protease were separated by SDS-PAGE and probed with anti-Vif Fab J4 and recombinant anti-Vif scFv 4BL. In the Western blot the major band of 23 kDa corresponding to HIV-1 Vif was recognized strongly by the scFv 4BL and Fab J4. The recombinant antibody fragment, as expected from the ELISA results, did not recognize HIV-1 protease. Immunofluorescence studies also show a specific recognition of Vif protein by anti-Vif Fab and scFv 4BL (Fig. 3). Thus, in opposition to scFv 4B, Vif protein is specifically recognized in vitro by scFv with a long linker.

Cellular Expression of Anti-Vif scFv Co-immunoprecipitates Vif Protein and Co-localizes with HIV-1 Vif in the Cytoplasm—To express the antibody fragments 4BL and 4B in the cytoplasm of human T lymphoid cells, an initiation codon was appended to the N terminus of the proteins, and an HA epitope was maintained at the C terminus. Expression plasmids were constructed in pCDNA3.1Neo (p14BL and p14B). Because 4B scFv was unable to bind Vif protein in ELISA, we anticipated that its expression in the cell would not effectively trap Vif in the cytoplasm. As a control for the activity of scFv, the antibody fragment anti-thyroglobulin (41) was cloned in the same eukaryotic vector and expressed in the human T lymphoid cells.

The efficacy of intrabody expression for binding HIV-1 Vif protein in the cell was examined by co-immunoprecipitation and fluorescence microscopy studies (Fig. 4). HeLa cells were co-transfected with intrabodies and pSVLvif, and then lysed 48 h after transfections. The intrabody fragment I4BL was immunoprecipitated with an anti-HA high affinity monoclonal antibody and protein A magnetic beads. Cell lysis and immunoprecipitation were carried out using mild detergent and salt conditions to allow co-immunoprecipitation of binding proteins. Upon immunoprecipitation of cells transfected with pSVLvif and pI4B, no Vif protein was detected by Western blot, whereas co-transfection of pSVLvif with pI4BL resulted in co-immunoprecipitation of Vif protein (Fig. 4). This result is consistent with previous data in ELISA showing Vif binding. As a control, anti-thyroglobulin scFv expressed in HeLa cells did not bind Vif because this protein did not co-immunoprecipitate with the antibody fragments (data not shown).

To evaluate the cellular localization of the antibody fragment I4BL expressed together with the antigen Vif, intracellular staining of co-transfected HeLa cells using an antibody specific to the HA tag and a polyclonal serum against Vif was performed in HeLa cells (Fig. 4). 48 h after co-transfection with pI4BL and pSVLvif, HeLa cells were fixed in paraformaldehyde
and observed by fluorescence microscopy. Microscopy showed a predominant punctate pattern of co-localization of these two proteins in the cytoplasm, with nucleoplasm exclusion (Fig. 4). In addition, Vif was consistently detected in the nucleus, and scFv was only observed in the cytoplasm in the majority of cells evaluated. When Vif and scFv were expressed alone, its pattern of localization is different from the co-localization staining. In contrast, when anti-thyroglobulin scFv was expressed together with anti-Vif scFv, no obvious co-localization was detected (data not shown). These results demonstrate that Vif protein is specifically recognized in the cytoplasm by the intracellular expression of anti-Vif scFv and that its interaction co-localizes Vif and scFv 4BL throughout the cytoplasm.

I4BL Inhibits Vif Function in a trans-complementation Assay—To provide a qualitative and quantitative measure of the biological activity of the anti-Vif scFv, a transient complementation assay in nonpermissive cells was used to examine the abilities of the anti-Vif scFv and control scFv to inhibit a single round of HIV-1 replication in trans (27). In a previous study, we showed that the expression of Vif in trans in this assay restores the efficiency of a single round of replication of a vif-defective virus to the wild-type level (15). The Vif expresser plasmid is co-transfected with a vif-positive (pHXBΔenvCAT) or vif-negative env-defective (pHXBΔAvrΔenvCAT) HXB2 provirus plasmid that expresses CAT and an HIV-1 envelope expresser plasmid. The HIV-1 virus particles produced in this assay result in only a single round of infection, because the packaged viral genome is defective for Env production. The efficiency of a single round of virus replication is quantitated by measuring the level of CAT enzyme activity in the infected cultures after 9–10 days, the minimum time for a detectable signal above background. The ability of the anti-Vif scFv to inhibit a single round of HIV-1 replication in trans was examined. In the absence of Vif, replication of the vif-negative virus was 10-fold lower than that of the vif-positive virus (Fig. 5). Coexpression of I4BL scFv in H9 cells reduced trans-complementation to 15% of the wild-type level, as shown in Fig. 5. In contrast, the expression of I4B scFv, which does not bind Vif, caused a much less significant reduction in trans-complementation to 85% of the wild-type level. Expression of anti-thyroglobulin scFv caused no significant reduction in trans-complementation. Thus, the biological activities of the anti-Vif scFv correlated directly with their Vif binding activities in ELISA and co-immunoprecipitation assays, indicating that I4BL scFv is likely to inhibit HIV-1.

H9 Cells Expressing Vif-specific Intrabody I4BL Are Protected from HIV-1 Infection—The experiments described above examined the biological activity of anti-Vif scFv in a transient assay under conditions in which most virus transmission occurs by cell-to-cell spread (15, 49). To investigate whether anti-Vif scFv can inhibit Vif function during several rounds of
HIV-1 replication, permissive (Jurkat) and nonpermissive (H9) cell lines expressing intrabodies were generated. For these experiments recombinant murine leukemia virus (MLV)-derived retroviral vectors encoding anti-Vif intrabody I4BL and control intrabody anti-thyroglobulin were used to transduce the human T cell lines H9 and Jurkat. Transduced cells were established through puromycin selection and limiting dilution cloning. Retroviral transduced cell lines were analyzed for intrabody and anti-thyroglobulin expression by Western blot (Fig. 6). Intrabodies were also detected by staining permeabilized transduced H9 and Jurkat cells with an anti-HA antibody (data not shown). The resultant H9-I4BL and Jurkat-I4BL cell lines showed homogeneous and stable expression of intrabody after puromycin selection during at least 3 months in cell culture. Subsequent studies on virus replication were performed in these intrabody-expressing cell lines.

Parental and transduced permissive and nonpermissive cell lines were challenged with HIV-1 utilizing the highly cytopathic viral strain HIV-1NL-3. Infectivity assays were performed with an MOI of 0.05 or 0.1 to mimic natural infections (35). Parental nontransduced H9 and Jurkat cell lines supported vigorous replication of HIV-1, as shown by the initial increases in HIV-1 p24 antigen, which peaked at −14−20 days. In contrast, low levels of HIV-1 p24 antigen were observed in the supernatants of H9-I4BL and Jurkat-I4BL as well as parental cell lines infected with HIV-1NL-3 at early time points after infection. On days 14−20 post-infection, H9-I4BL cells showed −94−98% inhibition of HIV-1 p24 antigen production compared with the parental H9 cells. Similar results were obtained in H9 cells infected with HIV-1NL-3ΔVif. In contrast, at the same time points of viral replication, Jurkat-I4BL cells showed similar levels of HIV-1 p24 antigen production compared with parental Jurkat cells. These results indicate that HIV-1 replication and infectivity was dramatically reduced in nonpermissive H9 cells by expression of a Vif-specific intrabody and were consistent in multiple independent experiments. In addition, expression of control anti-thyroglobulin scFv in the same cells had no significant inhibitory effect on HIV-1 replication, indicating that anti-Vif scFv is specific for Vif inhibition in other nonpermissive cell lines (Fig. 6).

Previous studies have shown that intracellular antibody expression has no obvious negative effects on cell viability or proliferation. Nevertheless, we quantified cell proliferation and cell viability of transduced H9-I4BL and Jurkat-I4BL cells compared with parental cells. The assay consists of a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche). The kinetics of the metabolism of WST-1 reagent showed that H9-I4BL and Jurkat-I4BL cells have similar proliferation curves compared with parental cell lines (Fig. 6). The evaluated time points were the same as those used for p24 antigen detection. Therefore, expression of anti-Vif single-chain antibody stably expressed in the cell inhibits HIV-1 replication in multiple rounds of infection.

**Proviral DNA Synthesis Is Impaired for Virus Produced from H9 Cells Expressing I4BL Intrabody**—The synthesis of viral DNA in H9 cells exposed to HIV-1NL-3 produced from H9-I4BL and Jurkat-I4BL cells was analyzed by PCR, with prim-
Inhibition of HIV-1 Viral Infectivity by Anti-Vif I4BL Is Specific for Nonpermissive Cells—Vif modulates HIV-1 infection in cultured T-cell lines in a cell-dependent manner (2, 4, 6, 8, 9). To evaluate the specificity of viral inhibition in different cell lines, a recombinant HIV-1 expressing I4BL or anti-thyroglobulin scFv, generating pHXB-I4BL and pHXB-thyro. In this experiment, 293T cells were transfected, and a high titer supernatant of HXB-I4BL, HXB-thyro and HXB2avif was obtained. HIV-1 supernatants normalized for the same TCID50 were used to infect permissive (Jurkat and SupT1), semi-permissive (CEM), and nonpermissive cells (H9, U38, and PBMC), and their ability to replicate in these cells was assessed by performing standard virus growth curves. Three patterns of results were obtained (Fig. 8). All of the nonpermissive cells tested in this experiment did not support the spread of HXB-I4BL. Similar results were obtained with HXB2avif. In contrast, permissive cell lines supported replication of all viruses used in this experiment. In CEM cells, replication of HXB-I4BL exhibited a intermediate behavior, although in some experiments its replication was higher than expected. As a control, HXB-thyro was able to replicate at wild-type levels in all cells tested (Fig. 8). HXB-I4BL was also co-cultivated with HIV-1NL4-3 in nonpermissive cells, and infection was monitored by p24 antigen production. In this coculture experiment MOI of HIV-1NL4-3 were similar and five times higher than HXB-I4BL. Compared with infection of HIV-1NL4-3 alone in nonpermissive cells, p24 antigen production was dramatically reduced from 9 to 97% at similar MOI and from 87 to 95% with higher MOI (data not shown). Thus, the HXB-I4BL can control replication of wild-type virus. These results show that the specificity of Vif inhibition by the intrabody correlates with the cellular requirements for Vif function, confirming the specificity of anti-Vif scFv.

Inhibition of HIV Replication by Anti-Vif scFv in Transduced Human PBMC—To evaluate the potential of anti-Vif scFv for clinical application, PHA-stimulated human PBMC were transduced with a retroviral vector that expresses anti-Vif scFv I4BL. Lentiviruses are attractive vectors for gene therapy because of their ability to integrate into nondividing cells (42, 43, 44). The retroviral vectors used in this study are derived from the HIV SIN system described previously by Naldini et al. (42). This delivery system has been shown to be highly effective in transduction of primary cells and offers significant advantages for its predicted biosafety (42). Intrabodies were cloned in the self-inactivating vector pRRLSIN and expressed under the cytomegalovirus promoter. Replication-defective retroviral particles were generated by transient co-transfection of 293T human kidney cells with the combination of three plasmids as described under “Experimental Procedures.” The p24 antigen concentration was determined, and the amount corresponding to 100 ng was used to transduce PBMC. The transduction protocol consisted of the daily infection of stimulated human PBMC with fresh 100 ng of viral p24 supernatants for 3 days, leading to transduction efficiencies, as measured by anti-HA immunofluorescence, ranging from 40 to 48% for three different experiments. The efficiency of transduction was significantly higher than previous experiments with MLV vectors, which may reflect the higher capability of HIV-based vectors for infecting human cells.

PBMC were challenged with HIV-1NL4-3 and two clinical isolates of HIV-12 in the range of MOI 0.05–0.1. Fig. 9 shows a dramatic inhibition of HIV-1NL4-3 production by anti-Vif I4BL transduction. Similar values of inhibition are obtained for the HIV-1 clinical isolates. In contrast, HIV-1 replication in PBMC transduced with anti-Vif I4B and anti-thyroglobulin showed less inhibition or no inhibition, respectively. These results show the potential for anti-Vif scFv for inhibiting HIV-1 replication in primary cells by ex vivo transduction.

\[\text{Inhibition of HIV Replication by Anti-Vif scFv in Transduced Human PBMC—}
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\[\text{To evaluate the potential of anti-Vif scFv for clinical application, PHA-stimulated human PBMC were transduced with a retroviral vector that expresses anti-Vif scFv I4BL.}
\]

\[\text{Lentiviruses are attractive vectors for gene therapy because of their ability to integrate into nondividing cells (42, 43, 44).}
\]

\[\text{The retroviral vectors used in this study are derived from the HIV SIN system described previously by Naldini et al. (42).}
\]

\[\text{This delivery system has been shown to be highly effective in transduction of primary cells and offers significant advantages for its predicted biosafety (42). Intrabodies were cloned in the self-inactivating vector pRRLSIN and expressed under the cytomegalovirus promoter.}
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\[\text{Replication-defective retroviral particles were generated by transient co-transfection of 293T human kidney cells with the combination of three plasmids as described under “Experimental Procedures.”}
\]

\[\text{The p24 antigen concentration was determined, and the amount corresponding to 100 ng was used to transduce PBMC.}
\]

\[\text{The transduction protocol consisted of the daily infection of stimulated human PBMC with fresh 100 ng of viral p24 supernatants for 3 days, leading to transduction efficiencies, as measured by anti-HA immunofluorescence, ranging from 40 to 48% for three different experiments. The efficiency of transduction was significantly higher than previous experiments with MLV vectors, which may reflect the higher capability of HIV-based vectors for infecting human cells.}
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\[\text{PBMC were challenged with HIV-1NL4-3 and two clinical isolates of HIV-12 in the range of MOI 0.05–0.1. Fig. 9 shows a dramatic inhibition of HIV-1NL4-3 production by anti-Vif I4BL transduction. Similar values of inhibition are obtained for the HIV-1 clinical isolates. In contrast, HIV-1 replication in PBMC transduced with anti-Vif I4B and anti-thyroglobulin showed less inhibition or no inhibition, respectively. These results show the potential for anti-Vif scFv for inhibiting HIV-1 replication in primary cells by ex vivo transduction.}
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DISCUSSION

The action of Vif is essential for the completion of proviral DNA synthesis after virus entry, most likely as a result of its effect during virus production (6, 25, 28). ScFv-based strategies directed against Vif may be an effective approach to inhibit these two crucial steps of the viral replication cycle (36–38). The genetic approach described here abrogates viral infectivity and reverse transcription by functional deletion of Vif, thereby inhibiting HIV-1 replication prior to integration of viral DNA in the host genome. We showed that anti-Vif scFv binds to Vif in

FIG. 8. Cell-specific inhibition of HIV-1 replication in presence of anti-Vif scFv. Replication of HIV-encoding anti-Vif scFv I4BL and anti-thyroglobulin was assessed in permissive and nonpermissive cells. In the left panels, permissive and semi-permissive cells Jurkat, SupT1, and CEM cells, were infected with HIV-14BL (A) and HIV-thyro (B). In the right panels, nonpermissive cells H9, U38, and PHA-stimulated PBMC were infected with HIV-I4BL (C) and HIV-thyro (D). Notice in the upper right panel that the lower values of HIV-1 p24 concentration were measured. The PBMC data were obtained with cells from three different HIV-1 seronegative donors. The data are representative of two independent experiments.

FIG. 9. Inhibition of HIV infection by transduction with an HIV-derived retrovector encoding anti-Vif scFv. Purified PBMC from three different HIV-1/HIV-2 seronegative donors were PHA-stimulated and transduced with SIN18-I4BL using the protocol described under “Experimental Procedures.” Left panel, nontransduced PHA-stimulated PBMC were infected with HIV-1NL4–3 (MOI = 0.05–0.1); two HIV-1 clinical isolates (strain Ac178 and strain Je524, Prof. H. L., Faculdade de Farmácia de Lisboa), 1 ng of HIV-1 p24 antigen/10⁶ PBMC. Right panel, transduced PHA-stimulated PBMC was challenged with similar HIV strains as in the left panel. The data are representative of at least two sets of independent experiments.
the cytoplasm. We also showed that scFv co-immunoprecipitates Vif, probably because of its high affinity. Anti-Vif scFv expressed transiently, constitutively, or by retroviral transduction abrogated viral replication in primary human T-lymphocytes. Wild-type viral particles produced from nonpermissive cells expressing anti-Vif scFv have a defect in infectivity at the level of reverse transcription because of neutralization of Vif in trans. These results confirm previous studies demonstrating that Vif acts in the producer cell and affects virus assembly. Consistent with this model, the expression of anti-Vif scFv in target cells had no effect on the infectivity of wild-type HIV-1.

The transducing vector used in this study was derived from HIV-1 (42, 43, 44), which can transduce dividing and nondividing human T-lymphocytes. Comparative studies using a vector derived from MLV demonstrated that the HIV-derived vector is more efficient at protecting human T-lymphocytes from HIV infection (data not shown). The effectiveness of neutralization was assessed by challenging intrabody-transduced PBMC with an HIV-1 laboratory-adapted strain and HIV-1 primary isolates. The Vif epitope recognized by the 4BL intrabody may be dominant, because it neutralizes both laboratory adapted and primary HIV-1 strains. Effective intracellular immunization must be designed for highly divergent proteins that share only a few peptide motifs because HIV-1 rapidly evolves compensatory mutations in response to selective pressures. Extended in vitro challenge with HIV-1 is necessary to determine whether Vif will acquire escape mutations associated with resistance.

Vif is predominantly localized in the cytoplasm of infected cells. A small fraction is localized in the nucleus, reflecting a possible role of Vif in this compartment. Our results show that scFv expressed in the cytoplasm co-localizes with Vif. Thus, the result of our study predicts that the cytoplasm is where Vif functions during HIV-1 replication.

The requirement for Vif differs among cell lines (2, 4, 6, 8, 9). Recent studies suggest that Vif enhances infectivity by overcoming an inhibitory factor present in nonpermissive cells (53, 54). When anti-Vif scFv was cloned in HIV-1 in place of the nef gene, viral replication occurred only in permissive cells, whereas in nonpermissive cells replication was similar to wild-type HIV-1. The activity of the anti-Vif intrabody was shown to be cell-specific, because in cis and in trans it had inhibitory effects only in nonpermissive cells. Anti-Vif scFv will help to validate candidates for HIV-1 cell-specific inhibitors that can be neutralized by Vif (20, 54).

The results presented here extend previous studies demonstrating that high affinity single-chain antibodies can be stably expressed in the cytoplasm (31, 34, 36, 35, 37). Folding of the scFv in the reducing environment of the cytoplasm can form functional binding sites as demonstrated by co-immunoprecipitation. Furthermore, the toxicity of the scFv was low as assessed by cell viability in intrabody-expressing human T-lymphocytes. We constructed a single-chain antibody by ligating VL with VH using a short and long linker. It was anticipated that a short linker peptide would result in a dimer scFv with two sites for antigen binding (33). Nevertheless, we observed a lack of binding of the 4B antibody. The reason for this result might be reduced stability or folding yield at the growth temperature. The short linker peptide may inhibit the coherent structure of the framework regions, thereby altering the binding affinity.

A large panel of scFvs against Vif is being constructed to map epitope-binding regions and putative functional sites. Using a panel of anti-Vif scFvs directed to specific intracellular sites, it may be possible to gain novel insights into Vif function. In addition, this strategy will facilitate the development of rational combinatorial gene therapies that simultaneously target different functional motifs.

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