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Targeted inhibition of immunodeficiency virus replication in lymphocytes through retroviral mediated gene transfer

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Summary. To test the feasibility of gene therapy for AIDS patients, an animal model is needed to evaluate the efficacy and safety of this approach. Antiviral genes (encoding antisense RNA or viral protein) derived from Simian immunodeficiency virus (SIV) were efficiently targeted into CD4+ lymphocytes through retroviral-mediated gene transfer. After challenging with infectious viruses, the transduced lymphocytes that received antiviral genes were not only protected from SIV infection, but also from infection with HIV, for at least 25 days. Furthermore, little or no cytolytic effect (syncytium formation) was observed in the protected cells. These data demonstrated that SIV or HIV replication could be effectively blocked by antisense sequence(s) or negative dominant factors which were introduced into targeted cells through retroviral-mediated gene transfer.

Introduction

Simian immunodeficiency virus (SIV) isolated from rhesus monkeys, has a lentivirus morphology, a CD4+ cell tropism, and serological crossreactivity with HIV-1 and HIV-2 [3, 8]. Furthermore, SIV causes an AIDS-like disease in experimentally infected rhesus monkeys [18]. SIV has, therefore, been employed as an animal model for studying potential treatments for human AIDS [9, 21].

Gene therapy has been proposed for treatment of AIDS [1]. The procedure would be initiated by taking bone marrow cells, including the hematopoietic stem cells, from an HIV-infected individual. The stem cells would be transduced with a recombinant virus that encoded an antiviral gene (an RNA or protein) which was capable of dominantly interfering with the intracellular growth of HIV. The modified stem cells would then be injected back into the patient

where they would return to the marrow. If only a small number of specific subsets of bone marrow cells are infected with HIV in the infected individual [16, 31], it is conceivable that, after targeting an antiviral gene, protected cells would be selected by a virus specific cytotoxic-effect to dominate the body's hematopoietic system. This approach would probably be suitable for already infected individuals where its purpose would be therapeutic. Inhibition of HIV replication in varying degrees by antisense RNA as well as sense RNA has been shown [15, 26, 29]. We now report that using a murine retroviral vector [10, 11, 13], an anti-SIV gene can be efficiently delivered into a cultured human CD4+ cell line in which it can effectively block SIV and HIV replication. This report represents the first step towards the application of antisense/sense gene therapy approach in an animal model.

Materials and methods

Cells and viruses

The SIV macaque isolate 239, HIV-2 and HIV-1 producing cell lines have been reported [22]. The porcine transmissible gastroenteritis coronavirus (TGEV) was grown on swine testicle cells (ST) as previously described [34]. The human CD4+ cell line HuT-78 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (penicillin and streptomycin, 100 units/ml each, Gibco, Bethesda, U.S.A.). NIH/3T3, amphotropic packaging cell line GP + envAM12 [19] and producer lines were grown in Dulbecco's Modified Eagle Medium supplemented with 10% donor calf serum (Gibco/BRL), 2 mM L-glutamine and antibiotics.

Construction of retroviral vectors and producer lines

The retroviral vectors were derived from the Moloney murine leukemia virus [2]. SIV molecular clone, SIVmac239 (GenBank database accession no. M33262), was used for constructing retroviral vectors. For vectors SIV gp+ and SIV gp−, a 5.6 kbp Nar I-Sph I viral DNA (1079 nt–6702 nt) was isolated (*gag-pol* region), blunt-ended and ligated to the Bam HI linearized and blunt-ended pZipNeoSV(×)1 vector. For vectors SIVenv+ and SIVenv−, a 3.5 kbp Sac I-Sac I DNA (6011 nt–9482 nt, *env* region) of SIVmac was isolated and added to a Bam HI linker, then ligated to the Bam HI linearized pZipNeoSV(×)1 vector. Producer lines were generated as described [19]. Briefly, packaging cell line, Gp + envAM12, was electroporated with 5 μg vector DNA, pSIVenv+, pSIVenv−, pSIVgp+, and pSIVgp−, respectively, and selected in the presence of 250 μg/ml active G418 (Gibco) for 10–14 days. The individual G418 resistant colonies were isolated with a cloning ring and expanded to a cell line. The virus titer of the producer line was determined on NIH/3T3 cells as described [20].

Transduction of HuT-78 cells and infection with SIV or HIV

The human CD4+ lymphoid cell line HuT 78 was co-cultured with producer lines in the presence of 8 μg/ml polybrene at a ratio of 1 : 1. The transduced HuT-78 cells were harvested and grown in the presence of G418 (500 μg/ml, active) and passaged 1 : 3 every 3 days for at least two weeks. Before challenging with uncloned SIVmac, HIV-1, and HIV-2 [4, 24], the HuT-78 cells were replenished with fresh medium in the absence or presence of G418 for at least 24 h. All transduced cells had similar growth rate prior to challenge with viruses. Approximately 10^6 viable cells in 5 ml medium were infected with 10 000 cpm reverse tran-

scriptase activity of uncloned SIVmac (approximately 400 $TCID_{50}$ titered on HuT-78 cells), HIV-1 or HIV-2. Untransduced and vector transduced HuT-78 were used as controls.

Polymerase chain reaction analysis of producer line and transduced HuT-78 cells

DNA was extracted from producer cell lines and transduced HuT-78 cells by proteinase K/phenol/chloroform method [14]. Standard PCR technique was used for amplification. In brief, 1 μg sample DNA was added to the 50 μl solution containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM each dNTPs, 2.5 Units Ampli Taq (Perkin Elmer) and 200 nM each primer. The following primers were used for PCR: For neomycin phosphotransferase gene, oligonucleotides
5′ ATGATTGAACAAGATGGATTGCAC and
5′ TCAGAAGAACTCGTCAAGAAGGC were used for 30 cycles. Each cycle consisted of 1 min of denaturation (94 °C), 1 min of annealing (60 °C) and 2 min of extention (72 °C). For β-globin gene, oligonucleotides 5′ TGAAGTTCTCAGGATCCACGTGC and
5′ TGAGTCCTTTGG GGATCTGTCCA were used with the condition of 1 min of denaturation (94 °C), 2 min of annealing (60 °C), and 1 min of extention (72 °C) for 30 cycles. For amplification of the "*env* region" of SIVmac, oligonucleotides
5′ AAGGCTTGGGGATATGTTATGAGC and
5′ GAAATAAGAGGGTGGGGAAGAGAACACTGG were used with the condition of 1 min of denaturation (94 °C), 1 min of annealing (60 °C), and 3 min of extention (72 °C) for 30 cycles. For detection of HIV-1 polymerase gene, the nested PCR was performed. The DNA of interest was subjected to an initial round of 15 cycles amplification using outer primer pair (5′ AAAATGATAGGGGGAATT and
5′ TACATCATTAGTGTGGGC), with each cycle consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 50 °C and 1 min of extention at 72 °C. Five microliters (1/10 volume) of the reaction mixture was then transferred to a second tube containing the inner primer pair (5′ GTAGGACCTACACCTGTCAACATAATTGGA and
5′ TATTTCTGCTATTTTAAGTCTTTTGATGGGTC) for another 30 cycles amplification, with the condition of 1 min of denaturation at 94 °C, 1 min of annealing at 65 °C and 2 min of extention at 72 °C. The PCR product (1/5 volume) was analyzed on a 1.2% agarose gel containing ethidium bromide.

Northern blot analysis of antisense and sense RNA expressed in transduced HuT-78 cells

Total RNA was extracted from transduced HuT-78 cells with a Stratagene RNA Extraction Kit. RNA was fractionated on a 1.2% formaldehyde gel and capillary blotted onto charged nylon paper (Micro Separation Inc.). The blot was probed with a [^{32}P]labeled DNA fragment prepared by PCR.

Reverse transcriptase (RT) activity assay

Viral replication was monitored by RT activity in the cell-free supernatant [5]. Two milliliter aliquots of cell-free supernatant were removed at different days postinfection for RT activity assay. Two milliliters of RPMI 1640 medium with 10% FBS were added into each cell culture after each removal.

Results

Construction of retroviral expression vectors

A 5.6 kbp DNA fragment of SIVmac 239 (Nar I-Sph 1) encompassing the primer binding site, *gag-pol, viv*, and 5′ *env* (*gag-pol* region) and a 3.5 kbp DNA frag-

ment of SIVmac 239 (Sac I-Sac I) encoding *vif, vpx, vpr, rev, tat,* and *env* (*env* region) were separately subcloned into the Bam HI site of the expression vector pZipNeoSV(×)1 in both sense (SIVgp+ and SIVenv+) and antisense (SIVgp− and SIVenv−) orientations (Fig. 1). The orientation of the inserts was determined by restriction mapping and DNA sequencing. These retroviral vectors were used to transfect the amphotropic packaging cell line, Gp + envAm12. The producer lines which produced a high titer of recombinant virus (approximately 10^6–10^7 cfu/ml) were isolated for transduction experiments. There was no evidence of replication competent virus in the transduced cells by checking the infectivity of culture medium on NIH/3T3 cells as described [20].

PCR analysis of producer cell line and transduced HuT-78 cells

After G 418 selection (more than two weeks), total cellular DNA was extracted from the producer lines and pooled transduced cells for PCR analysis. The results showed positive for "*env* region" for DNA extracted from producer cells, SIVenv− and SIVenv+ (Fig. 2 A), but only positive for DNA extracted

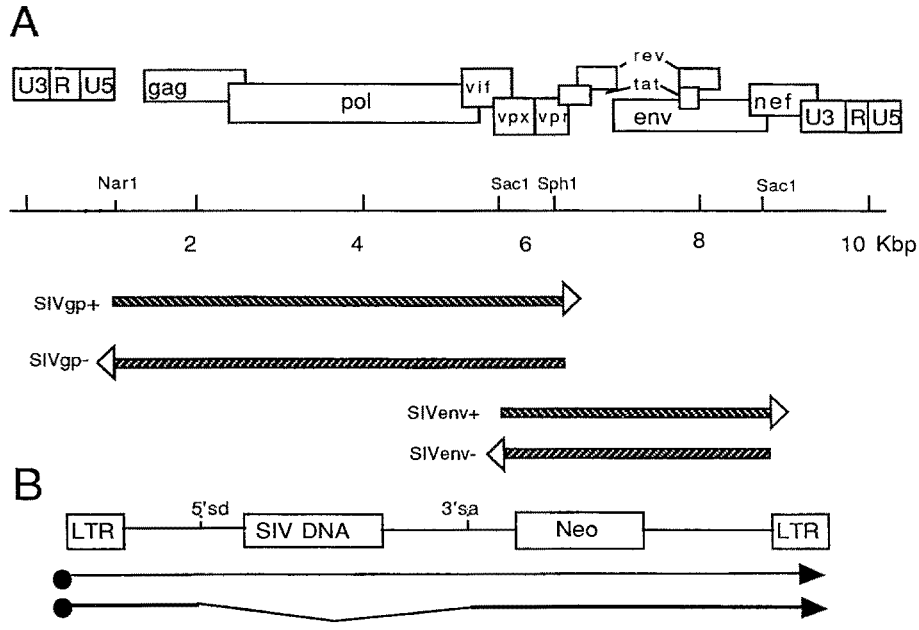

Fig. 1. Construction of retroviral expression vectors. **A** Genomic organization of SIVmac and restriction endonuclease sites that were involved in construction of the expression vectors. For SIVgp+ and SIVenv+, the viral DNA was inserted in the sense orientation. For the SIVgp− and SIVenv−, the viral DNA was inserted in the antisense orientation. **B** Structure and RNA transcripts of expression vectors. The inserted genes are under the control of Moloney murine leukemia virus *LTR*. The marker gene (*Neo*) is expressed by splicing and is under the control of the viral promoter in the Moloney LTR. *5'sd* Splicing donor site; *3'sa* 3' splicing acceptor site

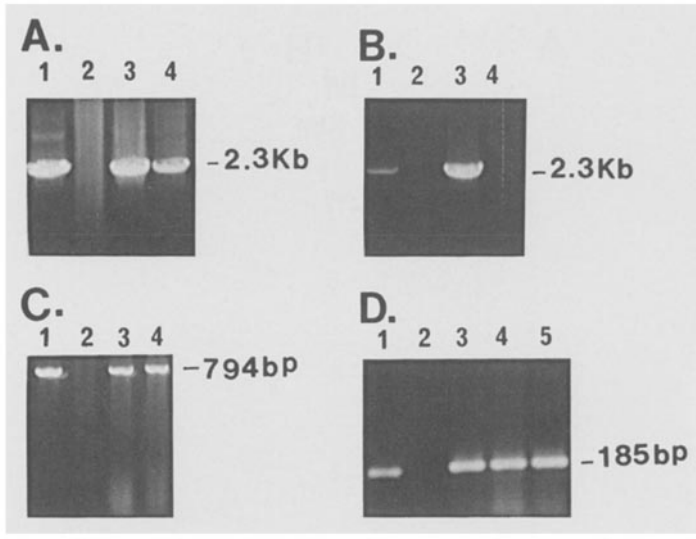

Fig. 2. PCR analysis of producer lines and transduced HuT-78 cells. **A, B** DNA were analyzed for *env* gene (2.3 kbp). One ng plasmid DNA (pSIVenv−) was used as positive control (*1*). The packaging cell line (**A,** *2*) or untransduced HuT-78 (**B,** *2*) was used as negative control. DNA isolated from producer cell lines SIVenv− and SIVenv+ (**A,** *3* and *4*) and from SIVenv− transduced HuT-78 cell were positive for *env* gene (**B,** *3*), but DNA isolated from SIVenv+ transduced HuT-78 cell was negative (**B,** *4*). **C** DNA were analyzed for *neo* gene (794 bp). One ng plasmid DNA, pZipneoSV(×) was used as a positive control (*1*). The results were positive for SIVenv− (*3*) and SIVenv+ (*4*) transduced HuT-78 cells, but negative for untransduced cells (*2*). **D** DNA was analyzed for human β-globin gene (185 bp). The human cellular DNA (1 μg) was used as a positive control (*1*). DNA isolated from NIH/3T3 cells was used as a negative control (*2*). The results were positive for untransduced (*3*), SIVenv− transduced (*3*), and SIVenv+ (*4*) transduced HuT-78 cells. One kbp ladder (BRL) was used as a molecular marker for gel electrophoresis

from SIVenv− transduced HuT-78 cells (Fig. 2 B). When DNA extracted from SIVenv+ transduced HuT-78 before G 418 selection was used for PCR, weak positive band for *env* region was also observed (data not shown). However, the PCR analysis of *neo* gene showed positive for both SIVenv− and SIVenv+ transduced HuT-78 cells (Fig. 2 C). This data suggested that both the full-length and spliced RNA transcripts (Fig. 1) were transferred into the SIVenv+ transduced HuT-78 cells. A possible explanation is that splicing donor (nt 6853) and acceptor (nt 9062) sites in the sense RNA of *env* region resulted in the spliced RNA being more favourably packaged into viral particles. The Northern RNA analysis also supports this hypothesis (Fig. 3).

Northern analysis of transduced HuT-78 cells

Total cellular RNA was extracted from transduced HuT-78 cells which were selected with G 418 and maintained in the presence or absence of G 418 (500 μg/ml, active). The RNA blots were probed with [^{32}P]labeled *env, pol,* and *neo*

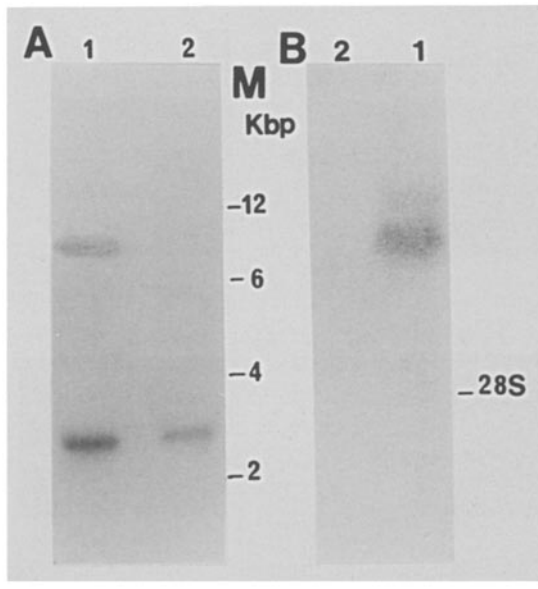

Fig. 3. Northern blot analysis of transduced HuT-78 cells. Total RNA was extracted from SIVenv− (*1*) and SIVenv+ (*2*) transduced HuT-78 cells (grown in the absence of G418), and probed with [^{32}P]labeled *neo* specific probe (**A**) or *env* probe (**B**) as described in Materials and methods. The full-length (6.5 kb, upper band) and spliced (2.5 kb, lower band) RNA transcripts were shown Undenatured one kbp ladder (BRL) was used as migration indicator. *28S* Migration position of 28S ribosomal RNA

specific probes, respectively. The full-length RNA encoding antisense of *env* region, as well as spliced *neo* RNA, was seen in the SIVenv− transduced HuT-78 cells, but only spliced RNA encoding *neo* gene was seen in the SIVenv+ transduced HuT-78 cells (Fig. 3). For SIVgp+ and SIVgp− transduced cells, both full-length (8 kb) and spliced RNA (2.5 kb) were seen (Fig. 4). The ratio of full-length and spliced RNA was different in the SIVgp− transduced HuT-78 in the presence or absence of G418. In the presence of G418, the spliced RNA encoding the *neo* gene became dominant (Fig. 4, lane 1). A 2.7 kb RNA, which comigrated with *neo* spliced RNA, also hybridized to the *pol* probe in the SIVgp+ transduced HuT-78 cells (Fig. 4 B, lane 3). This 2.7 kb RNA could be the splicing intermediate of the full-length transcript of the SIVgp+. The slightly different size of spliced RNA transcripts could be due to the distortion by 28S ribosomal RNA during blotting, or different lengths of polyadenylation. Repeated experiments with prolonged electrophoresis showed that the size difference was minimal. This data indicated that the presence of G418 in the growth medium influenced the steady state level of primary RNA transcripts. In the presence of G418, the cells maintained a high steady state level of spliced transcripts.

Inhibition of SIV and HIV replication in the transduced HuT-78 cells

After infecting fractions of transduced cells with uncloned SIVmac or HIV-1, RT activities (Fig. 5) and syncytium formation (Fig. 6) were monitored. The

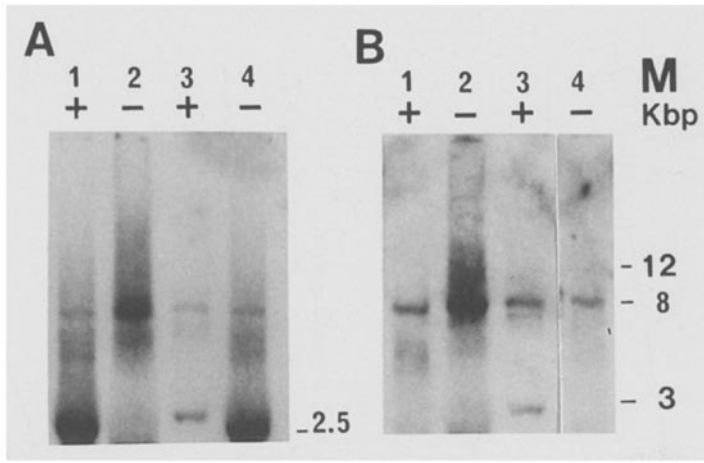

Fig. 4. The effect of G 418 in the steady state of RNA expression. Total RNA was extracted from SIVgp− (*1* and *2*) and SIVgp+ (*3* and *4*) grown in the presence (+) or absence (−) of G 418 (1 mg/ml, 50% active). The same quantity of each RNA sample (5 µg) was loaded in **A** and **B.** **A** was probed with [^{32}P]labeled *neo* probe. **B** was probed with [^{32}P]labeled SIVmac *pol* specific probe. *M* Undenatured 1 kbp ladder (BRL) was used as migration indicator

HuT-78 cells transduced with antisense constructs of *gag-pol* and *env* regions were effectively protected (no RT activity) from SIVmac infection for more than 20 days when compared to cells transduced with the corresponding sense constructs. The viral activity (RT) from cells transduced with sense construct of *gag-pol* region was lower than that from cells transduced with sense construct of *env* region (Fig. 5 A). The partial interference could be due to the *gag* and polymerase proteins expressed in SIVgp+ transduced cells (data not shown). The mechanism of partial interference is unclear. The higher RT in the control, untransduced HuT-78 cells, could be due to slightly higher cell number and/or G 418 selection in transduced cells. There was little or no cytopathic effect (syncytium formation) in protected cells (cells transduced with SIVenv− and SIVgp−) compared to that in unprotected cells (high degree of syncytium formation in cells transduced with SIVenv+), at 20 days postinfection. Interestingly, there was little or no inhibition of SIVmac replication and strong cytopathic effect in SIVgp− transduced HuT-78 cells when culture was maintained in G 418 selection (Figs. 5 B and 6).

When the fraction of pooled transduced cells was infected with HIV-1, cells transduced with sense and antisense constructs of *gag-pol* region were effectively protected from viral infection. Almost no viral replication was observed in SIVgp+ transduced cells and minimal viral replication was observed in SIVgp− transduced cells (Fig. 5 C). The slightly higher background RT activity (approximately 3 000 cpm/ml) in SIVgp+ transduced cells was derived from the empty SIVmac viral particles (data not shown). Cells transduced with antisense and sense constructs of the *env* region were not effectively protected. It should

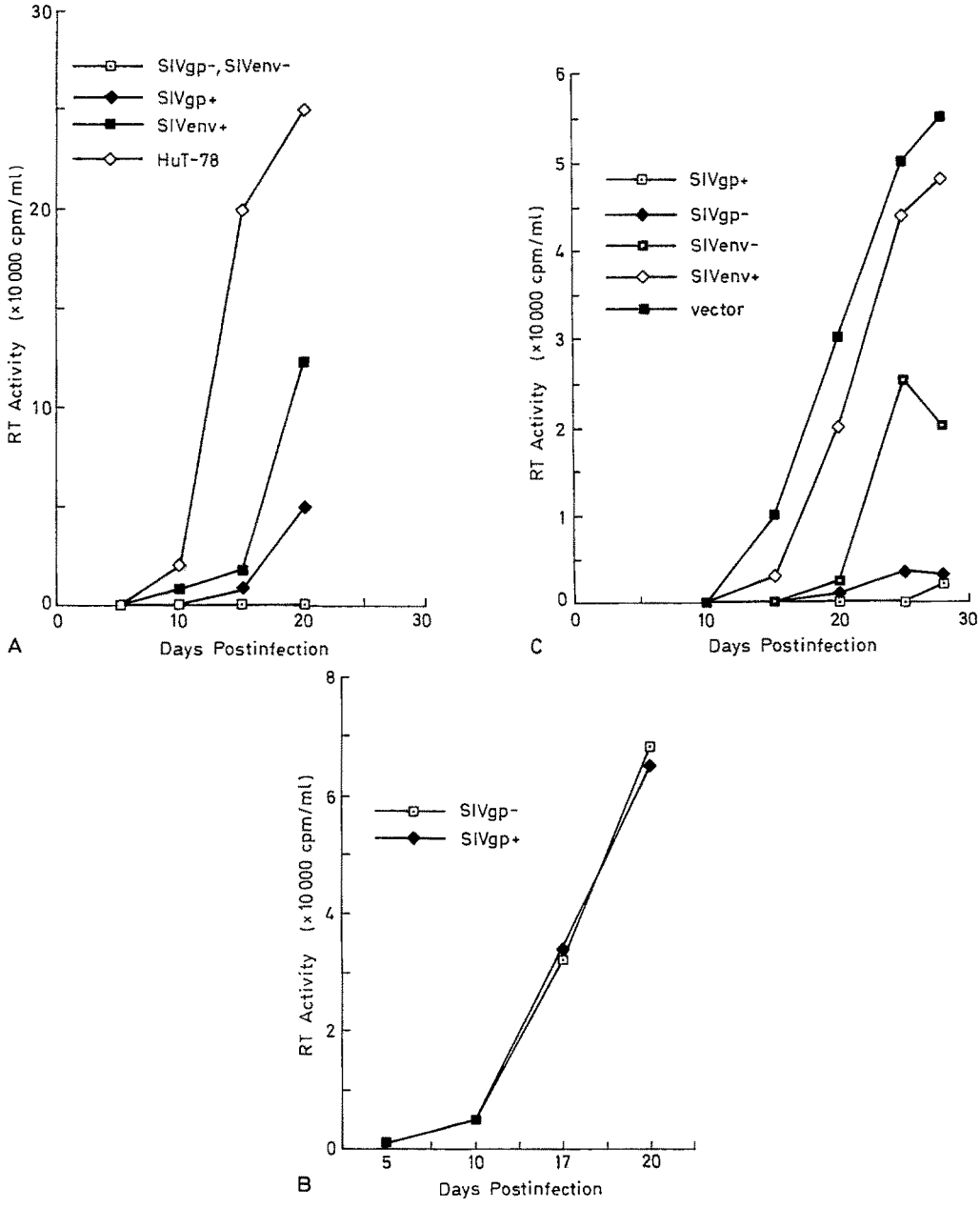

Fig. 5. Inhibition of SIVmac and HIV-1 replication in transduced HuT-78 cells. HuT-78 cells were transduced with recombinant viruses by co-culturing with producer lines as described in the method. About 10^6 viable cells grown in the absence (**A** and **C**) or presence (**B**) of G 418 were infected with 10 000 cpm reverse transcriptase activity (RT, approximately 400 $TCID_{50}$ on HuT-78) of uncloned SIVmac (**A** and **B**) or HIV-1 (**C**). *SIVgp*− Cells were transduced with antisense construct of *gag-pol* region. *SIVenv*− Cells were transduced with antisense construct of *env* region. *SIVenv*+ Cells were transduced with sense construct of *env* region. *SIVgp*+ Cells were transduced with sense construct of *gag-pol* region. In the parallel experiment, untransduced parental cells (*HuT-78*), without G418 selection, were infected with SIVmac as a control. Vector transduced cells were included as control for HIV infections (**C**)

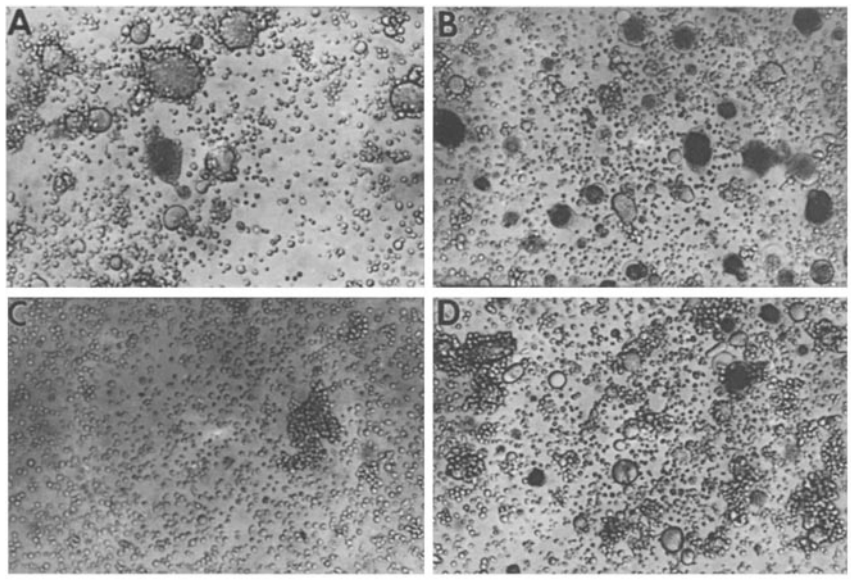

Fig. 6. Syncytium formation of SIVmac infected HuT-78 cells. Cells transduced with recombinant viruses, expressing sense RNA (**A** and **B**) or antisense RNA (**C** and **D**) of *gag-pol* region and grown in the absence (**A** and **C**) or presence (**B** and **D**) of G418 as described in Fig. 5. The giant cell fusion was easily seen in unprotected cells (**A, B, D**). The photographs were taken 20 days postinfection

be noted that significant cytopathic effect (approximately 90%) was observed in unprotected cells at 25 days postinfection. Therefore, the RT assay was terminated after 25 days. To study the mechanism of nearly complete protection of HIV-1 infection, the cellular DNA was extracted from the cells harvested at 25 days postinfection and subjected to PCR analysis. In agreement with the RT results, no HIV-1 proviral *pol* sequence and little HIV-1 proviral *pol* sequence can be detected in the SIVgp+ and SIVgp− challenged cells, respectively (Fig. 7). The sensitivity of this PCR analysis is less than 5 copies per sample (data not shown). The inability to detect HIV-1 proviral sequence in SIVgp+ transduced cells could be due to the cell death of small number of infected cells in which complete viral replication is blocked but expression of HIV-1 *env* gene is normal. When HIV-2 was used as the challenging virus [4], similar results were obtained (data not shown). All of these challenging experiments were repeated at least twice. The results shown were the mean of two initial experiments. Subsequent experiments performed several months later also demonstrated similar results. These data suggested that there might be different mechanisms by which transduced cells were protected from SIVmac and HIV-1 (or HIV-2) infections.

Cell associated transmission also inhibited in the transduced cells expressing antisense RNA

HIV spreads by cell-free, as well as cell-associated transmission. The above data suggested that HuT-78 cells transduced with antiviral genes could prevent cell-

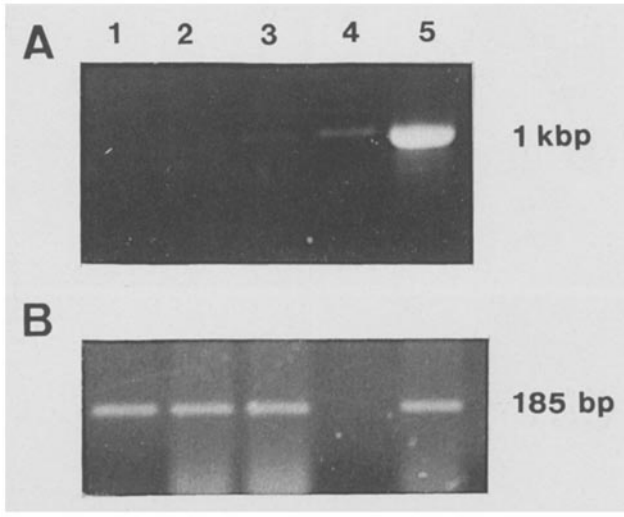

Fig. 7. PCR analysis of HIV-1 sequence. The cellular DNA was extracted from SIVgp− and SIVgp+ transduced HuT-78 cells after infecting with HIV-1 for 25 days and subjected to PCR analysis for HIV-1 *pol* (**A**) and human β-globin gene (**B**) as described in Materials and methods. *1* Uninfected HuT-78 cells; *2* and *3* SIVgp+ and SIVgp− transduced HuT-78 cells, respectively; *4* 10 fg of plasmid DNA pHXB 2; *5* HIV-1 persistently infected HuT-78 cells. The HIV-1 *pol* (*1 kbp*) gene and human b-globin gene (*185 bp*) are shown

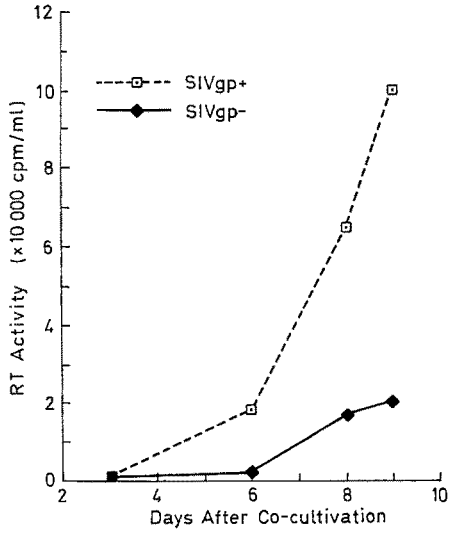

Fig. 8. Viral activities of transduced HuT-78 cells after co-cultivation. Approximately 10^6 transduced HuT-78 cells were co-cultured with SIVmac persistently infected HuT-78 cells at a ratio of 100 : 1. The reverse transcriptase activities (*RT*) were monitored at 3, 6, 8, and 9 days after co-cultivation by removing 2 ml of cell-free supernatant each time. *SIVgp*− SIVmac infected HuT-78 cells co-cultured with HuT-78 cells transduced with antisense construct of *gag-pol* region. *SIVgp*+ SIVmac infected HuT-78 co-cultured with HuT-78 cells transduced with sense construct of *gag-pol* region of SIVmac

free transmission. To investigate whether transduced cells could also be protected from cell-associated transmission, HuT-78 cells transduced with the antisense or sense construct of *gag-pol* region were co-cultured at a 100 : 1 ratio with HuT-78 cells persistently infected with SIVmac. It is believed that the ratio of uninfected to infected cells is much greater in infected individuals [31]. After nine days of co-cultivation (Fig. 8), the viral activities of unprotected cell pop-

ulations (transduced with sense construct) were five times greater than those in protected cell populations (transduced with antisense construct). The viral activities in protected cell populations were mostly derived from persistently infected HuT-78 cells. Furthermore, significant syncytium formation (90–95%) was observed in unprotected cell populations compared to low levels of syncytium formation (5–10%) in protected cell populations after 9 days of cocultivation. These data suggested that cell-associated viral transmission was also inhibited in the protected cell populations.

Inhibition of viral replication in transduced cells is virus specific

In order to demonstrate that inhibition of viral replication is virus specific, we used the same constructs, pSIVgp+ and pSIVgp− DNA, to transfect the ST cell line. The transfected cells were selected with G418 (1.5 mg/ml, active) then challenged with TGEV at an M.O.I. of 0.5. The results suggested that there was no protection against viral infection in pSIVgp+ and pSIVgp− transfected ST cells, compared to ST cells transfected with an antisense copy of the peplomer gene of TGEV (Table 1). This data indicates that the inhibition of SIVmac and HIV replication in the transduced HuT-78 cells is virus specific.

Table 1. Cytopathic effect of TGEV infection on transfected ST cells

Cells	CPE (%)
pMNC/ST	90
pMNCTGP+/ST	80
pMNCTGP−/ST	10
pSIVgp−/ST	80
pSIVgp+/ST	80
pZiPNeoSV/ST	80

ST cells were transfected with plasmid DNA and selected with 1.5 mg/ml of active G418. The pool of G418 resistant cells was infected with TGEV at M.O.I. of 0.5 for 2 h. After aspirating the viral supernatant, DMEM supplemented with 10% FBS was added and incubated at 37 °C and 5% CO_2 for 30 h. The CPE was scored at 30 h postinfection by judging the percentage of cells lysed. *pMNC/ST* ST cells transfected with MNC vector; *pMNCTGP+/ST* and *pMNCTGP−/ST* cells transfected with vector encoding sense and antisense copy of peplomer gene of TGEV, respectively [35]; *pSIVgp−/ST*, *pSIVgp+/ST*, and *pZiPNeoSV/ST* cells transfected with DNA as described previously

Discussion

Biological heterogeneity of HIV or SIV is reflected by differences in host range, replicative properties, and cytopathic effect in infected cells [7, 12, 28, 32]. Restriction endonuclease mapping and sequence analyses of HIV [24] and SIV [3] isolates, as well as the patterns of neutralization of different isolates by antibodies, also demonstrate genetic diversity, particularly in the envelope glycoprotein [6]. The genetic variation is a major obstacle to the design of vaccines or therapeutic agents effective to different variants. SIV macaque isolate shares about 55% amino acid sequence identity with HIV-1 in the *gag* and *pol* genes and less than 31% in the *env* gene [25]. Our studies have demonstrated that an antiviral gene (antisense RNA or viral gene) can be delivered to CD4+ cells by means of a retroviral-mediated gene transfer technique. The transduced cells were not only protected from parental viral infection (SIV, macaque and sooty mangabey isolates), but also from other related viral infections (HIV-1 and HIV-2). The mechanisms by which the retroviral vectors inhibited the replication of immunodeficiency viruses is not clear. One possible mechanism is that the antisense RNA binds to the viral RNA before reverse transcription occurs or arrests translation of targeted mRNA. In the transduced cells expressing SIVmac associated *gag* and *pol* polypeptides, the SIVmac polypeptides may function as mutant peptides, which may interact with HIV polypeptides, and inhibit HIV replication by interfering virus assembly [33].

Three lines of evidence indicated that the effective inhibition of SIVmac and HIV replication was unlikely due to clonal variation. First, the pooled transduced HuT-78 cells were used for challenge experiments. In fact, the fraction of HuT-78 cells transduced with the antisense construct of *env* region effectively inhibits SIVmac but not HIV-1 and HIV-2. Furthermore, fractions of HuT-78 transduced with sense construct of *gag-pol* region did not support HIV-1 replication but support a certain degree of SIVmac and SIVsm (sooty mangabey isolate, data not shown). Second, height level of gene transduction efficiency ($> 60\%$) by co-culturing HuT-78 cells with producer lines was employed in this experiment. Third, no specific pattern of integrated provirus was shown by Southern hybridization (data not shown).

A major advantage of this approach is the potential of in vivo applicability. Antiviral genes can theoretically be introduced into bone marrow stem cells from AIDS patients through the use of retroviral vectors. The manipulated marrow cells could then be infused back to the patients where progeny derived from the bone marrow stem cells carrying the antiviral gene would be resistant to viral infection. The feasibility and safety of using retroviral gene transfer for human gene therapy has been demonstrated recently [17, 23, 27]. The approaches described in this report could be tested in SIV infected monkeys.

In previous reports [15, 26, 29], partial and transient inhibition of HIV was achieved in cell culture by antisense RNA. In these three cases, only small size antisense RNA was used (less than 1 kbp) for inhibition of HIV replication. In

our study, a 1.5 kbp antisense construct of SIVmac *gag* region (including LTR, primer binding site and the majority of *gag*) did not effectively inhibit SIVmac replication (data not shown). Antisense RNA of SIVmac *env* region (3.5 kb) can effectively inhibit SIVmac replication and partially inhibit HIV replication. The antisense RNA of SIVmac *gag-pol* region (5.6 kb) can effectively inhibit both SIVmac and HIV replication. From our Northern analysis, there is no significant difference in RNA levels in those transduced cells expressing antisense and sense RNA. These data suggest that the effectiveness of the antisense RNA inhibition could be related to the size of antisense sequences. Since the viral titers used for the challenge in the previous experiments were not clearly defined, a correlation can not be drawn between these experiments. Recently, Sczakiel et al. [30] have shown effective long-term inhibition of HIV-1 by antisense RNA when a low M.O.I. of challenging virus was used. The effective inhibition of SIV and HIV replication in this report may also be due to low M.O.I. used.

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References

1. Baltimore D (1988) Intracellular immunization. Nature 335: 395–396
2. Cepko C, Roberts BE, Mulligan RC (1984) Construction and application of a highly transmissible murine retrovirus shuttle vector. Cell 37: 1053–1062
3. Chakrabarti L, Guyader M, Alizon M, Daniel MD, Desrosier RC, Tiollais P, Sonigo P (1987) Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature 328: 543–547
4. Clavel F, Guetard D, Brun-Vezinet F, Chamaret S, Rey M, Santos-Ferreira MO, Laurent AG, Dauguet C, Katlama C, Rouzioux C, Klatzmann D, Champalimaud JL, Montagnier L (1986) Isolation of a new human retrovirus from West African patients with AIDS. Science 233: 343–346
5. Daniel MD, Letvin NL, King NW, Kannagi M, Sehgal PK, Hunt RD, Kanki PJ, Essex M, Desrosiers RC (1985) Isolation of T-cell tropic HTLV-III-like retrovirus from macques. Science 228: 1201–1204
6. Daniel MD, Letvin NL, Segal PK, Schmidt DK, Silvia DP, Solomon KR, Hodi FS, Ringler DJ, Hunt RD, King NW, Desrosiers RC (1988) Prevalence of antibodies to three retroviruses in a capative colony of macaque antibodies to three retroviruses in a capative colony of macaque monkeys. Int J Cancer 41: 601–608
7. Daniel MD, Li Y, Naidu YM, Durda MJ, Schmidt DK, Troup CD, Silva DP, Mackey JJ, Kestler HW III, Sehgal PK, King NW, Ohta Y, Hayami M, Desrosiers RC (1988) Simian immunodeficiency virus from African Green monkeys. J Virol 63: 4123–4128
8. Desrosiers RC (1988) Simian immunodeficiency viruses. Annu Rev Microbiol 42: 607–625
9. Desrosiers RC, Wyand MS, Kodama T, Ringler DJ, Arthur LO, Sehgal PK, Letvin NL, King NW, Daniel MD (1989) Vaccine protection against simian immunodeficiency virus infection. Proc Natl Acad Sci USA 86: 6353–6357

10. Eglitis MA, Kantoff PW, Gilboa E, Anderson WF (1985) Gene expression in mice after high efficiency retroviral-mediated gene transfer. Science 230: 1395–1398
11. Eglitis MA, Kantoff PW, Jolly JD, Jones JB, Anderson WF, Lothrop CD Jr (1988) Gene transfer into hematopoietic progenitor cells from normal and cyclic hematopoietic dogs using retroviral vectors. Blood 71: 717–722
12. Evan LA, McHugh TM, Stites DP, Levy JA (1987) Differential ability of human immunodeficiency virus isolates to productively infect human cells. J Immunol 138: 3415–3418
13. Guild BC, Finer MH, Housman DE, Mulligan R (1988) Development of retrovirus vectors useful for expressing genes in cultured murine embryonal cells and hematopoietic cells in vivo. J Virol 62: 3795–3801
14. Gross-Bellard M, Oudet P, Chambon P (1972) Isolation of high-molecular weight DNA from mammalian cells. Eur J Biochem 36: 32
15. Joshi S, Brunschot AV, Asad S, Elst IVD, Read SE, Bernstein A (1991) Inhibition of human immunodeficiency virus type I multiplication by antisense and sense RNA expression. J Virol 65: 5524–5530
16. Kitagawa M, Lackner AA, Martfeld DJ, Gardner MB, Dandekar S (1991) Simian immunodeficiency virus infection of macaque bone marrow macrophages correlates with disease progression in vivo. Am J Pathol 138: 921–930
17. Laneuville P, Chang W, Kamel-Reid S, Fauser AA, Dick JE (1988) High-efficiency gene transfer and expression in normal human hematopoietic cells with retrovirus vectors. Blood 71: 811–814
18. Letvin NL, Daniel MD, Sehgal PK, Desrosiers RC, Hunt RD, Waldron LM, Mackey JJ, Schmidt DK, Chalifoux LV, King NW (1985) Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. Science 230: 71–73
19. Markowitz D, Goff S, Bank A (1988) Construction and use of a safe and efficient amphotropic packaging cell line. Virology 167: 400–406
20. Miller AD, Rosman GJ (1989) Improved retroviral vectors for gene transfer and expression. Biotechniques 7: 980–990
21. Murphy-Corb M, Martin LM, Davison-Fairburn B, Montelaro RC, Miller M, West M, Ohkawa S, Baskin GB, Zhang JY, Putney SD, Allison AC, Eppstein DA (1989) A formalin-inactivated whole SIV vaccine confers protection in macaques. Science 246: 1293–1297
22. Naidu YM, Kestler HW III, Li Y, Butler CV, Silva DP, Schmidt DK, Troup CD, Sehgal PK, Sonigo P, Daniel MD, Desrosiers RC (1988) Characterization of infectious molecular clones of Simian immunodeficiency virus (SIVmac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecular cloned SIVmac. J Virol 62: 4091–4096
23. Palmer TD, Hock RA, Osborne WRA, Miller AD (1987) Efficient retrovirus-mediated transfer and expression of a human adenosine deaminase gene in diploid skin fibroblasts from an adenosine deaminase-deficient human. Proc Natl Acad Sci USA 84: 1055–1059
24. Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalski JA, Whitehorn EA, Baumeister K, Ivanoff L, Petteway SR Jr, Pearson ML, Lautenberger JA, Papas TS, Ghrayeb J, Chang NT, Gallo R, Wong-Staal F (1985) Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313: 277–284
25. Regier DA, Desrosiers RC (1990) The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res Hum Retroviruses 6: 1221–1231
26. Rhodes A, James W (1990) Inhibition of human immunodeficiency virus replication in cell culture by endogenously synthesized antisense RNA. J Gen Virol 71: 1965–1974
27. Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson

EM, Lotze MT, Yang JC, Topalian SL, Merina MJ, Culver K, Miller AD, Blaese RM, Anderson WF (1990) Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumour-infiltrating lymphocytes modified by retroviral gene transduction. New infiltrating lymphocytes modified by retroviral gene transduction. N Engl J Med 323: 570–578

28. Saag MS, Hahn BH, Gibbons J, Li Y, Parks ES, Parks WP, Shaw GM (1988) Extensive variation of human immunodeficiency virus type 1 in vivo. Nature 334: 440–447
29. Sczakiel G, Pawlita M (1991) Inhibition of human immunodeficiency virus type 1 replication in human T cells stably expressing antisense RNA. J Virol 65: 468–472
30. Sczakiel G, Oppenlander M, Rittner K, Pawlita M (1992) Tat- and rev-directed antisense RNA expression inhibits and abolishes replication of human immunodeficiency virus type 1: a temporal analysis. J Virol 66: 5576–5581
31. Simmonds P, Balfe P, Peutherer JF, Ludlam CA, Bishop JO, Brown AJL (1990) Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J Virol 64: 864–872
32. Tersmette M, Goede R, Al BJ, Winkel IN, Gruters RA, Cuypers HT, Huisman HG, Miedema F (1988) Differential syncytium-inducing capacity of human immunodeficiency virus isolate: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. J Virol 62: 2026–2032
33. Trono DM, Feinberg B, Baltimore D (1989) HIV-1 gag mutant can dominantly interfere with the replication of the wild-type virus. Cell 59: 113–120
34. Tung FYT, Abraham S, Sethna M, Hung SL, Sethna P, Hogue BG, Brian DA (1992) The 9-kDa hydrobphobic protein encoded at the 3′ end of the porcine transmissible gastroenteritis coronavirus genome is membrane-associated. Virology 186: 676–683
35. Tung FYT (1993) A 3.5 kbp cDNA encoding the peplomer gene of TGEV was subcloned into MNC vector under the control of cytomegalovirus early promoter (unpublished)

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