JOURNAL OF VIROLOGY, Apr. 1997, p. 2740–2746 Vol. 71, No. 4
0022-538X/97/$04.00+0

Transduction of CD34$^+$ Hematopoietic Progenitor Cells with an *antitat* Gene Protects T-Cell and Macrophage Progeny from AIDS Virus Infection

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Received 23 October 1996/Accepted 10 January 1997

Transduction of hematopoietic stem cells with genes that inhibit human immunodeficiency virus (HIV) replication has the potential to reconstitute immune function in individuals with AIDS. We evaluated the ability of an autoregulated gene, *antitat*, to inhibit replication of simian immunodeficiency virus (SIV) and HIV type 1 (HIV-1) in hematopoietic cells derived from transduced progenitor cells. The *antitat* gene expresses an antiviral RNA encoding polymeric Tat activation response elements in combination with an antisense *tat* moiety under the control of the HIV-1 long terminal repeat. CD34$^+$ hematopoietic progenitor cells were transduced with a retroviral vector containing the *antitat* gene and then cultured under conditions that support in vitro differentiation of T cells or macrophage-like cells. Rhesus macaque CD4$^+$ T cells and macrophage-like cells derived from CD34$^+$ bone marrow cells transduced with the *antitat* gene were highly resistant to challenge with SIV, reflecting a 2- to 3-log reduction in peak SIV replication compared with controls. Similarly, human CD4$^+$ T cells derived from CD34$^+$ cord blood cells transduced with *antitat* were also resistant to infection with HIV-1. No evidence for toxicity of the *antitat* gene was observed in any of five different lineages derived from transduced hematopoietic cells. These results demonstrate that a candidate therapeutic gene introduced into hematopoietic progenitor cells can retain the ability to inhibit AIDS virus replication following T-cell differentiation and support the potential use of the *antitat* gene for stem cell gene therapy.

The limited success of existing therapies for human immunodeficiency virus (HIV) infection has prompted an intensive search for more effective modalities, including gene therapy. Although initial clinical trials of therapeutic genes in HIV-infected people have focused on transduction of mature CD4$^+$ T cells (6, 39), stem cell gene therapy for AIDS offers several potential advantages. Since HIV primarily infects hematopoietic cells, introduction of genes that induce resistance to HIV infection into hematopoietic stem cells (intracellular immunization [4]) is a particularly attractive strategy. The cardinal features of hematopoietic stem cells, self-renewal and pluripotency, represent distinct advantages of this approach. In light of the extensive proliferation of progeny from hematopoietic stem cells (28) and the apparently rapid turnover of CD4$^+$ T cells in HIV-infected individuals (13, 37), transduction of even a small fraction of hematopoietic stem cells with a protective gene might lead to expansion of a significant population of cells resistant to HIV infection. However, many challenges remain to reconstituting immunity in HIV-infected individuals by stem cell gene therapy, among them the low level of genetically modified cells observed in large animal models and human trials (10, 41), the potential for toxicity of constitutively expressed foreign genes in multiple hematopoietic lineages, and the lack of a suitable in vitro system to evaluate candidate gene therapy approaches.

Multiple genetic approaches have been devised to inhibit HIV replication. These approaches include use of RNA decoys, ribozymes, transdominant proteins, antisense molecules, inducible suicide genes, and intracellular antibodies (reviewed in references 6, 26, and 29). Of these strategies, an *antitat* gene consisting of a polymeric transactivation response (TAR) decoy in combination with an antisense-*tat* molecule has several features that make it a particularly attractive candidate for gene therapy (21). The *tat* gene product (Tat) is essential for HIV type 1 (HIV-1) replication, serving to transactivate transcription of all HIV-1 genes following binding of the Tat protein to the cognate TAR RNA element located in the HIV-1 long terminal repeat (LTR) (2, 32). Interactions between the Tat protein and the TAR sequence are conserved among primate lentiviruses (35), as reflected by the observation that genes containing an HIV-1 polymeric-TAR decoy are able to inhibit replication of simian immunodeficiency virus (SIV) (20), as well as diverse clinical isolates of HIV-1 (21). The ability of the HIV-1 polymeric-TAR decoy to inhibit both HIV-1 and SIV suggests that virus variants resistant to inhibition by a polymeric-TAR decoy are unlikely to arise and also permits in vivo studies of this approach in macaques infected with SIV. Expression of the *antitat* gene is regulated by a modified HIV-1 LTR, which is a weak promoter in the absence of Tat (2, 32). Thus, expression of *antitat* is likely to be low in uninfected cells and upregulated in HIV-1-infected cells (22), a feature which should reduce the potential problem of toxicity due to constitutive expression of a foreign gene in uninfected host cells. Finally, since it is an RNA-based approach, the *antitat* gene is unlikely to serve as a target for host immune responses that might serve to eliminate cells expressing a foreign protein (30).

Evaluation of candidate genes for stem cell gene therapy for

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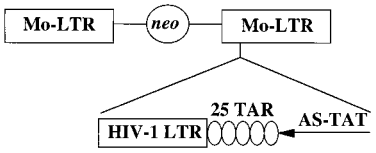

FIG. 1. Structure of the retroviral vector containing the *antitat* gene. The *antitat* gene inhibits viral replication by two mechanisms: sequestration of the Tat protein by the polymeric-TAR decoy and blocking of translation of the *tat* RNA by the antisense component. Expression of *antitat* is autoregulated by the HIV-1 LTR, which in the presence of the Tat protein activates expression of *antitat*. Insertion of the *antitat* gene into the U3 region of a double-copy murine retroviral vector results in integration of two copies of the *antitat* gene in transduced cells (11). Mo-LTR, Moloney murine leukemia virus LTR; AS-TAT, antisense *tat.*

AIDS has been hindered by the difficulty of supporting in vitro T-cell differentiation of hematopoietic stem cells. Although inhibition of HIV replication in myelomonocytic cells derived from transduced CD34$^+$ hematopoietic progenitor cells has been demonstrated (3, 42), no data are currently available on whether any approach is able to inhibit AIDS virus replication in T cells derived from transduced CD34$^+$ cells. We therefore established an in vitro system that could be used to identify genes that are able to protect CD4$^+$ T cells derived from transduced CD34$^+$ cells from HIV or SIV infection. We recently demonstrated that thymic stromal cultures from rhesus monkeys can support T-cell differentiation of rhesus and human CD34$^+$ cells (31). Using rhesus thymic stroma cultures, we evaluated the ability of the *antitat* gene to inhibit SIV and HIV-1 replication in CD4$^+$ T cells derived from transduced CD34$^+$ progenitor cells. Both rhesus and human CD4$^+$ T cells derived from transduced CD34$^+$ cells were highly resistant to SIV and HIV-1, respectively, providing the first evidence that introduction of therapeutic genes into hematopoietic progenitor cells can result in T-cell progeny resistant to AIDS virus infection.

MATERIALS AND METHODS

Bone marrow harvest and immunomagnetic bead CD34 cell isolation. Rhesus monkeys (*Macaca mulatta*) used in this study were normal, colony-born animals maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and *Guide for the Care and Use of Laboratory Animals* (35a). Heparinized bone marrow was harvested from rhesus macaques, and CD34$^+$ cells were purified by using immunomagnetic beads (Dynal, Lake Success, N.Y.) as previously described (31). Rhesus CD34$^+$ cells obtained in this fashion were >90% CD34$^+$ and contained <1% residual CD3$^+$ T cells. Purified CD34$^+$ cells were subsequently further depleted of CD3$^+$ cells by incubation with a CD3-specific antibody 6G12 (14) and sheep anti-mouse immunoglobulin magnetic beads (Dynal). Human cord blood was obtained from Advanced Biotechnologies (Columbia, Md.) under human study protocols approved by the local institutional review board, and CD34$^+$ cells were isolated as described above, using a human CD3-specific monoclonal antibody (12F6 [40]).

Flow cytometry analysis. Antibodies used for immunophenotyping of rhesus cells included anti-CD3 (6G12), anti-CD4 (OKT4; Ortho Diagnostics, Raritan, N.J.), anti-CD8 (Leu-2a; Becton Dickinson, San Jose, Calif.), anti-CD68 (Dako, Carpinteria, Calif.), and anti-CD34 (QBend-10; Immunotech, Westbrook, Maine). Anti-CD3 (Leu4; Becton Dickinson) was used to detect human CD3. All cells were stained in the presence of phosphate-buffered saline with 2% mouse serum. Three-color analytic flow cytometric (fluorescence-activated cell sorting [FACS]) analysis was performed on a Becton Dickinson FACScan. Cell sorting was carried out on a Becton Dickinson FACS Vantage.

Retroviral transduction. CD34$^+$ bone marrow (rhesus)- or cord blood (human)-derived cells were transduced for 3 days with either a retroviral vector containing the *antitat* gene inserted in the 3′ LTR of a double-copy retroviral vector (Fig. 1) and produced in the PA317 amphotropic packaging cell line (21) or the LN retroviral vector produced in the PG13 packaging cell line (PG13/LN c8, from D. Miller, Fred Hutchinson Cancer Research Center; obtained through the American Type Tissue Collection [CRL 10685]) (23). Both the *antitat* producer cell line and the control retroviral vector producer cell line (PG13/LN) were grown in an artificial capillary system (Cellco, Germantown, Md.). Transductions were performed in the presence of irradiated (15 Gy) allogeneic rhesus or human bone marrow stroma. Nonadherent cells were removed after 3 days of transduction and used to establish in vitro T lymphopoiesis or macrophage cultures as described below.

T-cell differentiation of CD34$^+$ bone marrow cells. To support differentiation into T cells, transduced CD34$^+$ cells were cultured on a thymic stromal monolayer as previously described (31). Briefly, thymic stromal monolayers were established in 24-well plates from cryopreserved fetal rhesus thymuses, and nonadherent cells were vigorously washed away after 3 days. After 7 days, thymic stromal monolayers were inoculated with 2×10^5 CD34$^+$ cells/well and cultured in the presence of RPMI 1640 with 10% fetal calf serum (FCS). After 14 days, samples were removed for flow cytometry and PCR analysis, and remaining cells were expanded by stimulation with concanavalin A (ConA; 5 μg/ml) for rhesus cells or phytohemagglutinin (0.25 μg/ml) for human cells in the presence of recombinant interleukin-2 (IL-2; provided by M. Gately, Hoffman-La Roche) and irradiated human peripheral blood mononuclear cells (PBMC). CD3$^+$ CD4$^+$ CD8$^-$ and CD3$^+$ CD4$^-$ CD8$^+$ cells were obtained by cell sorting on a FACS Vantage. Purified T cells were then restimulated as described above and selected in G418 (200 μg/ml, active concentration; Gibco BRL, Gaithersburg, Md.) for 7 days. Following another round of restimulation, T cells were challenged with SIVmac239.

Colony-forming assays and differentiation of macrophage-like cells from CD34$^+$ cells. CFU were derived from transduced CD34$^+$ cells by culturing at a concentration of 10^4 cells per ml in complete methylcellulose media (Stem Cell Technologies) containing 3 U of erythropoietin (10 ng/ml; Stem Cell Technologies) per ml, human granulocyte-macrophage colony-stimulating factor (GM-CSF; 30 ng/ml; Genzyme, Cambridge, Mass.), human IL-3 (20 ng/ml; Genzyme), and human stem cell factor (50 ng/ml; Genzyme) in the presence and absence of G418 (400 μg/ml, active concentration; Gibco BRL). Clonogenic progenitors were counted at 14 and 21 days, based on standard criteria (34). Transduction efficiency was calculated by dividing the number of G418-resistant CFU by the total number of CFU for each culture condition examined. DNA PCR performed on G418-resistant CFU confirmed the presence of the *neo* gene in >95% of colonies examined ($n > 100$). To induce differentiation of macrophage-like cells from myelomonocytic cells, individual myelomonocytic colonies were pooled and cultured in 24-well plates in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, GM-CSF (20 ng/ml), macrophage colony-stimulating factor (M-CSF; 40 U/ml; Genzyme), and G418 (400 μg/ml) (42). After 7 days of selection, cells were challenged with SIVmac316.

Proliferation assays. T cells derived from CD34$^+$ cells were washed and resuspended in RPMI 1640 with 10% FCS at a concentration of 10^6 cells/ml. One hundred microliters (10^5 cells) was added to each of six replicate wells of a 96-well plate. Cells were stimulated with ConA (5 μg/ml) in the presence of IL-2 (20 U/ml) and irradiated human PBMC (10^5 cells/well in 100 μl of RPMI 1640 with 10% FCS). After 7 days in culture at 37°C, each well was pulsed with 1 μCi of [*methyl*-^{3}H]thymidine for 16 h and harvested onto a glass fiber filter, and incorporation of [^{3}H]thymidine was determined with a liquid scintillation counter.

Viral infection of T cells and macrophage-like cells. Cells derived from CD34$^+$ cells transduced with *antitat* or the control vectors were infected with SIVmac239 or SHIV-4 (T cells) or SIVmac316 (macrophage-like cells) for 4 h at 37°C. Human CD4$^+$ T cells containing the *antitat* gene or the control vector were infected with HIV-1 NL4-3 (1). Cell cultures were infected in duplicate, using a multiplicity of infection (MOI) of 0.01 and 0.001 50% tissue culture infective doses ($TCID_{50}$)/cell. Viral replication was assessed by enzyme-linked immunosorbent assay determination of viral antigens (SIV p27 antigen [Coulter, Hialeah, Fla.]; HIV-1 p24 antigen [DuPont, Wilmington, Del.]) according to the manufacturers' protocols. The lower limit detection for SIV p27 is 50 pg/ml; that for HIV p24 is 5 pg/ml.

RESULTS

Inhibition of SIV replication in a transformed T-cell line by the *antitat* gene. Previous studies demonstrated that a RNA decoy consisting of 50 tandem repeats of the HIV-1 TAR element in combination with a *gag*-specific ribozyme inhibited SIV replication by approximately 90% in stably transduced T-cell lines (20). The *antitat* gene encodes a polymeric TAR decoy containing 25 copies of the HIV-1 TAR but contains an antisense-*tat* moiety in place of the *gag*-specific ribozyme, resulting in greater inhibition of HIV-1 replication than observed with the polymeric TAR decoy alone (21). We wished to confirm that the *antitat* gene was also able to inhibit SIV replication. Molt 3 cells were transduced with a retroviral vector containing the *antitat* gene and neomycin phosphostransferase gene (*neo*) or with a control vector containing *neo* alone. Transduced cells were grown in the presence of the neomycin analog G418, and the resulting cell lines were challenged with SIVmac251. Molt 3 cells containing the *antitat* gene were highly resistant to productive SIV infection, whereas Molt 3 cells containing *neo* alone supported vigorous SIV replication,

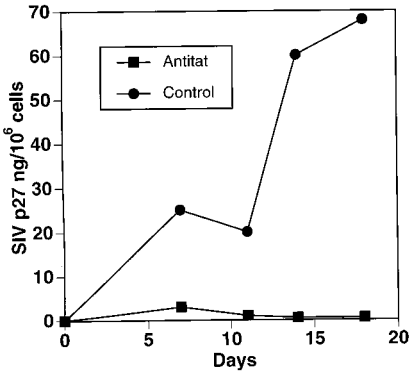

FIG. 2. Inhibition of SIV replication in Molt 3 cells transduced with the *antitat* gene. Molt 3 cells transduced with the *antitat* and control retroviral vectors (21) were infected with SIV, and viral replication was assessed by SIV p27 antigen production.

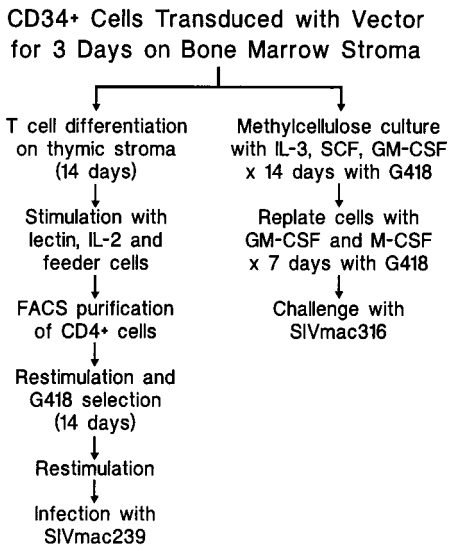

FIG. 3. Outline of the techniques used to obtain $CD4^+$ T lymphocytes and macrophage-like cells from transduced $CD34^+$ cells. Feeder cells consisted of irradiated human PBMC. For rhesus cells, ConA was used for lectin stimulation; for human cells, phytohemagglutinin was used. Infections were carried out at MOIs of 10^{-2} and 10^{-3} $TCID_{50}$/cell for 4 h at 37°C. SCF, stem cell factor.

with peak viral replication of over 60 ng of p27 antigen per ml (Fig. 2). Similar results were obtained for cells infected with SIVmac239 (data not shown). Thus, the *antitat* gene induces high level resistance to SIV infection as well as HIV-1 infection.

Retroviral transduction of $CD34^+$ hematopoietic cells with the *antitat* gene. We next evaluated transduction efficiency of $CD34^+$ bone marrow-derived cells by retroviral vectors containing either the *antitat* gene and the selectable marker *neo* or, as a control, a vector containing *neo* alone. To enhance transduction efficiency, transductions were performed in the presence of bone marrow stroma (24, 27). No exogenous cytokines were added, since stimulation of $CD34^+$ cells with cytokines used to enhance transduction efficiency (IL-3, IL-6, and stem cell factor) significantly impairs the ability of $CD34^+$ cells to subsequently differentiate into T cells (30a). As assessed by outgrowth of CFU in the presence of the neomycin analog G418, transduction of $CD34^+$ cells by the *antitat* gene was relatively efficient, resulting in 14 to 30% G418-resistant colonies (Table 1). Similar values were obtained with the control *neo* vector. No difference in the relative frequency of myelomonocytic or erythroid colonies derived from $CD34^+$ cells transduced with the *antitat* vector was observed compared with the control vector, suggesting that the *antitat* gene was not toxic to the development of these hematopoietic lineages.

T-cell differentiation of rhesus $CD34^+$ cells transduced with *antitat*. Because differentiation of T cells is generally restricted to the unique microenvironment of the thymus (36), in vitro analysis of T cells derived from genetically modified progenitor cells has been difficult. We used rhesus thymic stromal cultures to support T-cell differentiation of rhesus $CD34^+$ cells transduced with a retroviral vector containing *antitat*. In this system, T-cell differentiation proceeds in a manner that recapitulates normal thymic ontogeny and results in the production of a polyclonal population of $CD4^+$ and $CD8^+$ T cells (31). Rhesus $CD34^+$ cells were transduced with retroviral vectors as described above and then cultured on rhesus thymic stroma to support T-cell differentiation. Untransduced $CD34^+$ cells were processed in parallel as controls. After 14 days of culture on thymic stroma, phenotypic analysis of transduced and untransduced $CD34^+$ cells revealed $CD3^+$ cells in all cases, with no differences observed between the *antitat* and control vectors (percentages of $CD3^+$ cells ± standard deviations [SD] were as follows: untransduced, 89 ± 3; control vector, 77 ± 10; and *antitat*, 76 ± 6). Rhesus T cells obtained from thymic stromal cultures were then expanded by stimulation with the lectin ConA in the presence of IL-2 and irradiated human PBMC as outlined in Fig. 3. Purified $CD4^+$ T cells were then obtained by cell sorting and then reexpanded in the presence of the neomycin analog G418. PCR analysis of G418-selected $CD4^+$ T cells derived from $CD34^+$ cells transduced with the control or *antitat* vector demonstrated the presence of *neo* DNA (data not shown). Compared with either untransduced $CD34^+$ cells or $CD34^+$ cells transduced with the control vector containing *neo* alone, no difference was noted in the phenotype of T cells derived from $CD34^+$ cells transduced with *antitat*, suggesting that the *antitat* gene did not interfere with T-cell differentiation (Table 2).

TABLE 1. Transduction efficiency of rhesus $CD34^+$ cells by a retroviral vector encoding the *antitat* gene

Expt	Untransduced			Control vector			*antitat* vector		
	$G418^r$ CFU[a]	% $G418^r$	M/E[b]	$G418^r$ CFU	% $G418^r$	M/E[c]	$G418^r$ CFU	% $G418^r$	M/E
1	0/140	0	2.4:1	58/149	39	2.9:1	49/164	30	2.4:1
2	3/161	2	2.2:1	52/170	30	2.1:1	64/234	27	2.2:1
3	2/108	2	3:1	22/111	20	3:1	12/87	14	2.8:1

[a] G418-resistant ($G418^r$) CFU were determined following culture in methylcellulose with 400 μg of G418 per ml.
[b] M/E, myelomonocytic/erythroid cell ratio.
[c] M/E ratios for CFU derived from transduced $CD34^+$ cells (LN control and *antitat* vectors) were calculated for $G418^r$ colonies.

TABLE 2. Immunophenotyping of T cells derived from CD34$^+$ cells transduced with the *antitat* or control retroviral vectors[a]

Day	Stage[b]	Untransduced			Control vector			*antitat* vector		
		4$^+$ 8$^+$	4$^+$	8$^+$	4$^+$ 8$^+$	4$^+$	8$^+$	4$^+$ 8$^+$	4$^+$	8$^+$
14[c]	Expansion	54 ± 5	23 ± 7	14 ± 2	56 ± 6	24 ± 5	17 ± 4	58 ± 6	20 ± 8	14 ± 1
28	FACS	44 ± 6	26 ± 6	23 ± 2	42 ± 2	28 ± 8	20 ± 11	40 ± 3	32 ± 4	22 ± 5
42	Expansion	ND[d]	94 ± 2	94 ± 3	ND	92 ± 4	93 ± 2	ND	92 ± 3	95 ± 2
56	G418	ND	ND	ND	ND	93 ± 2	93 ± 4	ND	94 ± 3	91 ± 2
70	SIV	ND	ND	ND	ND	92 ± 5	ND	ND	93 ± 2	ND
84		ND	ND	ND	ND	ND[e]	ND	ND	93 ± 3	ND

[a] Data represent the means ± SD of two independent experiments for the indicated subsets of T cells.
[b] Stage in the experimental protocol outlined in Fig. 3.
[c] Phenotyping of T cells derived from thymic stromal cultures of CD34$^+$ cells on day 14 was performed prior to expansion with ConA, IL-2, and irradiated PBMC. T cells were sorted by FACS into CD4$^+$ and CD8$^+$ subsets at day 28 and placed in G418 selection at day 56, as outlined in Fig. 3. Cells were challenged with SIV at approximately day 72.
[d] ND, not done.
[e] Poor cell viability prevented flow cytometric analysis.

Rhesus CD4$^+$ T cells derived from CD34$^+$ cells transduced with *antitat* are protected from SIV infection. To evaluate whether the *antitat* gene was able to inhibit SIV replication in primary T cells derived from transduced CD34$^+$ cells, G418-selected CD4$^+$ T cells were stimulated with ConA and then infected at an MOI of 10^{-2} $TCID_{50}$/cell with a SIV strain that replicates well in T cells (SIVmac239 [15]). SIV replication was markedly inhibited in cultures of CD4$^+$ T cells derived from *antitat*-transduced CD34$^+$ progenitor cells, while vigorous viral replication was observed in T cells derived from control-transduced cells (Fig. 4A). Twenty-one days after infection, there was no detectable SIV replication (<50 pg of SIV p27/ml) in CD4$^+$ T cells containing the *antitat* gene. In contrast, SIV p27 exceeded 20 ng/ml in cultures of control T cells containing *neo* alone, reflecting over a 400-fold inhibition of viral replication. Similar levels of inhibition were observed when an MOI of 10^{-3} $TCID_{50}$/cell was used (data not shown). The *antitat* gene also blocked cytopathicity induced by SIV in CD4$^+$ T cells. At the end of a 21-day culture, T cells derived from CD34$^+$ cells transduced with *antitat* maintained viability (76% ± 4%) (mean ± SD, $n = 3$), whereas T cells derived from CD34$^+$ cells transduced with the control vector exhibited considerable cytopathic effect (27% viability ± 17%). Inhibition of SIV replication in T cells containing *antitat* was observed for up to 40 days after infection, even following two cycles of restimulation of cells with ConA and irradiated feeder cells (Fig. 4A). In addition, CD4$^+$ T cells containing *antitat* could be maintained in culture by periodic restimulations with ConA in the presence of irradiated PBMC and IL-2 for over 16 weeks. Challenge of CD4$^+$ T cells containing *antitat* after 16 weeks of culture and reselection with G418 revealed equivalent levels of resistance to SIV infection (Fig. 4B), suggesting that inactivation of the *antitat* gene did not occur over this period.

To examine the ability of *antitat* to inhibit transactivation of viral replication by the HIV-1 *tat* gene in rhesus CD4$^+$ T cells, we also infected cells with a chimeric virus containing an HIV-1 *tat* gene. HIV-1 isolates will not replicate in rhesus cells, but by maintaining key portions of the SIV genome, replication-competent chimeric viruses (referred to as SHIV) containing HIV-1 genes have been created (17). We used the SHIV-4 isolate, which contains the HIV-1 IIIB *tat*, envelope, and *rev* genes in place of the corresponding SIV genes (17). Rhesus CD4$^+$ T cells derived from CD34$^+$ cells transduced with *antitat* were also resistant to productive infection with SHIV-4, with up to 2.5 logs of inhibition observed on day 14 of culture (Fig. 4C). Comparable levels of inhibition were also observed when an MOI of 10^{-3} $TCID_{50}$/cell was used (data not shown).

Inhibition of SIV replication in T cells containing the *antitat* gene was not due to downregulation of CD4, as demonstrated by flow cytometric analysis of G418-selected cells (Table 2), nor was it due to inhibition of cellular proliferation, as shown

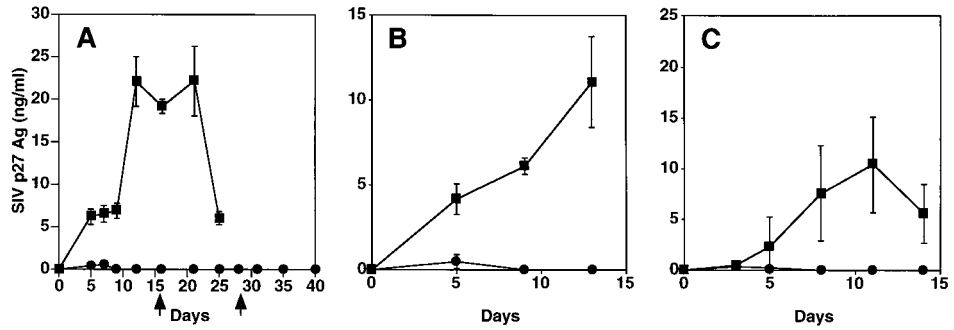

FIG. 4. CD4$^+$ T cells derived from CD34$^+$ cells transduced with the *antitat* gene are resistant to productive infection with SIV and SHIV. Rhesus CD4$^+$ T cells containing the *antitat* gene were derived from transduced progenitor cells as outlined in Fig. 3. (A) Inhibition of SIV replication by *antitat* in CD4$^+$ T cells. CD4$^+$ T cells were infected with SIVmac239, and SIV infection was assessed by determination of SIV p27 antigen. T cells were restimulated with ConA and irradiated PBMC on days 16 and 28 (indicated by arrows). Data are representative of three independent experiments and are presented as the means ± SD of duplicate wells. (B) Continued inhibition of SIV replication by *antitat* following long-term culture of CD4$^+$ T cells. CD4$^+$ T cells derived from CD34$^+$ T cells transduced with either the *antitat* or a control retroviral vector were maintained in culture for 16 weeks, cultured in the presence of G418, restimulated, and challenged with SIV. Data represent the means of two duplicate cultures ± SD. (C) Inhibition of SHIV replication by *antitat* in CD4$^+$ T cells. Expanded CD4$^+$ T cells were infected with SHIV-4 at an MOI of 10^{-2} $TCID_{50}$/cell, and viral replication was assessed by SIV p27 antigen determination. ●, *antitat* vector; ■, control vector.

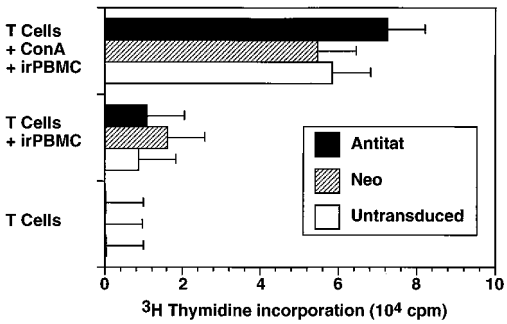

FIG. 5. Proliferative response of rhesus $CD4^+$ T cells derived from transduced $CD34^+$ cells. $CD4^+$ T cells were derived from transduced or untransduced $CD34^+$ cells as outlined in Fig. 3. T cells were stimulated with lectin (ConA; 5 μg/ml) and irradiated PBMC (irPBMC) in the presence of IL-2 (20 U/ml). Data are plotted as the means of six replicate wells ± SD and are representative of two independent experiments.

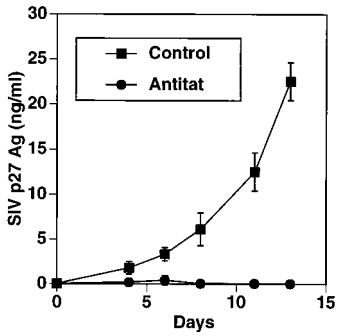

FIG. 6. Inhibition of SIV replication in macrophage-like cells derived from $CD34^+$ cells transduced with the *antitat* gene. $CD34^+$ cells transduced with *antitat* or the control *neo* vector were cultured in the presence of cytokines GM-CSF and M-CSF in order to support differentiation of macrophage-like cells. Cells were then infected with SIVmac316 at an MOI of 10^{-3} $TCID_{50}$/cell, and viral replication was assessed by SIV p27 antigen production. Data are representative of two independent experiments.

by equivalent levels of [^{3}H]thymidine uptake in T cells containing *antitat* or *neo* alone (Fig. 5). In addition, reverse transcription-PCR analysis of T-cell receptor Vβ chain heterogeneity (8, 31) following G418 selection, restimulation, and SIV infection revealed a polyclonal population of T cells (15 of 25 Vβ chains [data not shown]). This level of heterogeneity is comparable to that observed in T-cell lines derived from untransduced $CD34^+$ cells and cultured in a similar fashion (31).

Taken together, these data demonstrate that introduction of the *antitat* gene into rhesus hematopoietic progenitor cells results in a polyclonal population of T cells that are highly resistant to productive infection with both SIV and SHIV and that the ability of T cells containing *antitat* to resist SIV infection is maintained following prolonged periods of in vitro culture.

Macrophage-like cells derived from *antitat*-transduced $CD34^+$ cells are resistant to SIV infection. A distinct advantage of stem cell gene therapy for HIV disease is the potential to induce resistance to infection in both macrophages and $CD4^+$ T cells derived from transduced hematopoietic stem cells. To evaluate whether *antitat* was able to inhibit SIV replication in macrophage-like cells derived from transduced rhesus $CD34^+$ cells, myelomonocytic colonies obtained from transduced cells were cultured in the presence of the cytokines M-CSF and GM-CSF (Fig. 3). Adherent cells cultured under these conditions expressed the cell surface molecules CD14 and CD68, characteristic of monocyte/macrophage lineage cells (data not shown). Macrophage-like cells derived in this manner were then challenged with SIVmac316, a strain of SIV which replicates efficiently in macrophages (25). Macrophage-like cells containing *antitat* were resistant to challenge with SIVmac316, whereas cells containing *neo* were easily infected (Fig. 6). Thus, transduction of $CD34^+$ cells with the *antitat* gene confers resistance to productive infection in multiple lineages of the hematopoietic system.

Human $CD4^+$ T cells derived from $CD34^+$ cells transduced with *antitat* are resistant to HIV infection. To evaluate the efficacy of *antitat* in inhibiting HIV-1 replication in T-cell progeny of transduced human progenitor cells, we used $CD34^+$ cells isolated from human cord blood. We have previously shown that rhesus thymic stroma supports the in vitro differentiation of human $CD34^+$ cells, resulting in a polyclonal population of $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ T lymphocytes (31). $CD34^+$ cord blood-derived progenitor cells were transduced with *antitat* or the control vector as described above, and purified $CD4^+$ T cells were obtained via cell sorting. Following selection in the presence of G418, $CD4^+$ T cells were infected with a stock of HIV-1 derived from the NL4-3 molecular clone (1). Human $CD4^+$ T cells containing the *antitat* gene were highly resistant to productive HIV infection, with no detectable viral replication 14 days after infection (Fig. 7). Thus, the *antitat* gene also confers resistance to HIV-1 infection in T cells derived from transduced human hematopoietic progenitor cells.

DISCUSSION

The development of stem cell gene therapy for AIDS has been limited by the lack of an appropriate in vitro model that would facilitate evaluation of the efficacy and toxicity of candidate genes to inhibit HIV replication. The major barrier to such a model has been the fact that T-cell differentiation is generally dependent on an intact thymus and therefore difficult to support in vitro. We have recently described a system that uses primary thymic stromal cultures from fetal rhesus macaques to support the in vitro T-cell differentiation of both rhesus and human $CD34^+$ bone marrow-derived cells (31). Using this culture system, we have shown marked inhibition of SIV and HIV-1 replication in T cells derived from rhesus and human $CD34^+$ progenitor cells transduced with a retroviral vector containing the *antitat* gene. Moreover, macrophage-like

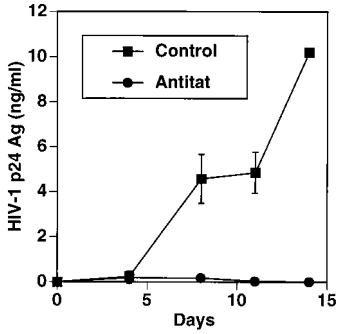

FIG. 7. Inhibition of HIV-1 replication in $CD4^+$ T cells derived from human $CD34^+$ progenitors transduced with the *antitat* gene. Human $CD4^+$ T cells were derived from transduced $CD34^+$ cord blood-derived cells as outlined in Fig. 3. Expanded $CD4^+$ T cells were infected with HIV-1 NL4-3, and viral replication was assessed by determination of HIV-1 p24 antigen levels in culture supernatants. The data are representative of experiments performed in duplicate, using $CD34^+$ cells from two different donors.

cells derived from rhesus CD34$^+$ cells transduced with *antitat* were also highly resistant to infection with SIV. We observed over 2 logs of inhibition of viral replication in cells containing *antitat*, generally reflecting suppression of SIV and HIV replication below the level of detection, despite vigorous viral replication in control cells. Similar levels of suppression of viral replication by *antitat* were observed with 4 different SIV or HIV-1 strains, both in CD4$^+$ T cells and macrophage-like cells and in rhesus and human cells. This broad range of inhibition of viral isolates in different hematopoietic cell lineages provides strong evidence for the suitability of the *antitat* gene for stem cell gene therapy for AIDS.

In addition to the potent inhibition of viral replication by the *antitat* gene, we noted no evidence for toxicity of *antitat* in any of the hematopoietic lineages analyzed. Because of the differentiation of stem cells into multiple lineages and the complex intermediate stages involved in lineage-specific differentiation, introduction of foreign genes into stem cells carries with it the potential for interference with normal hematopoiesis. This is particularly true for T cells, where the development of mature CD4$^+$ and CD8$^+$ T cells from hematopoietic stem cells involves a number of intermediate progenitor cells and expression of multiple genes (33). Under a variety of culture conditions, no overt evidence for toxicity of the *antitat* gene was observed in any of five different hematopoietic cell types analyzed. CD34$^+$ progenitor cells transduced with the *antitat* gene were able to differentiate into CD4$^+$ and CD8$^+$ T cells, macrophage-like cells, and erythroid and myelomonocytic colonies. No difference in the differentiation of *antitat*- and control vector-transduced cells into these different lineages was observed. T cells containing the *antitat* gene exhibit a normal phenotype, had normal proliferative responses to mitogenic stimuli, and could be maintained for prolonged periods in culture without loss of function of the foreign gene. Although a more definitive assessment of the potential for toxicity will require in vivo studies, these results suggest that the *antitat* gene does not interfere with hematopoietic and lymphoid differentiation or function.

The *antitat* gene has a number of advantages for stem cell gene therapy for AIDS. Since Tat is essential for replication of HIV (9), blocking the ability of Tat to transactivate viral replication should lead to an almost complete inhibition of viral replication. Moreover, due to the conservation of Tat-TAR interactions, mutant viruses resistant to inhibition by the *antitat* gene are unlikely to arise. Autoregulated expression of the *antitat* gene by the HIV-1 LTR is also an attractive feature for stem cell gene therapy. Preliminary analysis in CEM×174 cells transduced with the *antitat* retroviral vector has confirmed that transcription of *antitat* is low in uninfected cells and increases significantly 24 h after SIV infection (30b). Finally, T cells containing *antitat* are resistant to HIV- and SIV-induced cytopathicity, allowing expansion of CD4$^+$ T cells from HIV-infected subjects in vitro (19). The ability of the *antitat* gene to confer a survival advantage on T cells and macrophages containing *antitat* may result in in vivo expansion of a even a small number of transduced cells in an HIV-infected individual.

The use of rhesus thymic stromal cultures to support T-cell differentiation of CD34$^+$ progenitor cells is a particularly valuable in vitro model for the development of stem cell gene therapy for AIDS (31). T-cell differentiation of CD34$^+$ cells cultured on thymic stroma mirrors that of normal T lymphopoiesis. T cells derived from these cultures are polyclonal, respond to normal mitogenic stimuli, are susceptible to SIV and HIV infection, and can be expanded and maintained in culture for extended periods of time. In addition, rhesus thymic stromal cultures can be established from cryopreserved cells, providing a convenient and easily reproducible system for supporting T-cell differentiation. These characteristics should facilitate definition of the optimal transduction conditions and vectors for stable integration and expression of genes in T cells derived from transduced progenitor cells. For the analysis of candidate genes for the treatment of AIDS, T cells derived from thymic stromal cultures can be examined in detail with respect to gene expression and the ability to induce resistance to AIDS virus infection with a variety of different viral isolates.

Multiple lines of evidence support the conclusion that T cells and macrophage-like cells containing the *antitat* gene are derived from in vitro differentiation of transduced progenitor cells. CD34$^+$ cells used for these studies were >90% pure and had been previously depleted of both T cells and macrophages. Previous experiments have shown that T cells derived from rhesus thymic stromal cultures of CD34$^+$ cells are not derived from either the thymic stroma or from T cells representing minor contaminants of the CD34$^+$ cell population (31). Moreover, mature T cells do not proliferate in the presence of thymic stroma. In the absence of such a mitogenic effect, expansion and retroviral transduction of a minor contaminating population of T cells is unlikely. In addition, we have also isolated and expanded G418-resistant CD4$^+$ CD8$^+$ T cells from thymic stromal cultures of *antitat*-transduced CD34$^+$ cells and shown that SIV replication is significantly inhibited in these cells as well (unpublished observation). Detection of the *antitat* gene in CD4$^+$ CD8$^+$ T cells, which are the dominant phenotype of developing immature T cells and rare in peripheral blood (5, 33), provides additional evidence that the T cells containing *antitat* analyzed in this report are the result of in vitro differentiation of transduced CD34$^+$ cells.

These results provide strong support for the potential use of the *antitat* gene for stem cell gene therapy for AIDS. However, in spite of these encouraging results, many barriers to the implementation of stem cell gene therapy in HIV-infected individuals remain. Although retroviral transduction of murine hematopoietic stem cells can be accomplished fairly efficiently (18), most trials in large animal models and humans have achieved relatively low levels of foreign genes in hematopoietic cells, generally less than 1% (10, 41). These disappointing results probably reflect inadequate transduction of hematopoietic stem cells and the difficulties of introducing genes into a relatively quiescent cell population by using retroviral vectors that are dependent on cell division for integration. Other potential barriers to the success of stem cell gene therapy for AIDS include dysregulation of hematopoietic cell differentiation in HIV-infected individuals (12) and abnormalities in thymic function that might impair differentiation of transduced progenitor cells (38).

The challenges to the development of stem cell gene therapy for AIDS strongly support preclinical studies in nonhuman primates. Infection of rhesus macaques with SIV is widely considered to be the leading animal model for the study of AIDS (16). Moreover, the close phylogenetic relationship of macaques with humans and their use as a preclinical model for stem cell gene therapy (41) also represent significant advantages. Issues that can be more appropriately addressed in nonhuman primates rather than SCID-hu mice include engraftment of transduced hematopoietic stem cells in a more physiologic environment, the effects of SIV infection on differentiation, circulation and longevity of transduced hematopoietic cells, and the ability of stem cell gene therapy to restore immune function. Finally, because the *antitat* gene is a potent inhibitor of both HIV-1 and SIV replication, analysis in the SIV-macaque model should provide efficacy and toxicity data to facilitate clinical trials in humans. Taken together, these

considerations strongly support the utility of the rhesus macaque model for the preclinical analysis of stem cell gene therapy. Rigorous evaluation of stem cell gene therapy in the rhesus macaque model should facilitate development of this approach to treat HIV-1-infected individuals.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants RR-00055, RR-00168, and AI-36550.

We thank Ron Desrosiers and Bruce Walker for critical review of the manuscript, Ron Desrosiers for providing SIVmac239 and SIVmac316, Joseph Sodroski for SHIV-4, David Scadden for human bone marrow stroma, Maurice Gately for recombinant IL-2, Johnson Wong for rhesus and human CD3-specific monoclonal antibodies, and Carolyn A. O'Toole for manuscript preparation.

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