Clin Exp Immunol 1993; **91**:473–481

Inhibition of cellular activation of retroviral replication by CD8$^+$ T cells derived from non-human primates

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(Accepted for publication 10 November 1992)

SUMMARY

To test the hypothesis that CD8$^+$ T cells inhibit viral replication at the level of cellular activation, an Epstein–Barr virus (EBV)-transformed cell line (FEc1) from a simian immunodeficiency virus (SIV)-seropositive sooty mangabey monkey was transfected with a human CD4 gene and shown to be replication-competent for HIV-1, HIV-2 and SIV. Utilizing a dual-chamber culture system, it was found that inhibition of viral replication can be mediated by a soluble factor. The FEc1 cell line was transiently transfected with an LTR-driven CAT reporter gene. It was found that autologous CD8$^+$ T cells markedly inhibited CAT activity. Furthermore, co-transfection of the FEc1 cell line with an LTR-driven *tat* plasmid and LTR-CAT was able to quantitatively mitigate the suppressive effect. Thus, this inhibition appears to be directed at cellular mechanisms of viral transcription. Control transfections with an LTR-driven CAT plasmid with a mutation at the NFkB binding site yielded no CAT activity, suggesting that most viral replication as measured by CAT activity is dependent, to a large extent, upon cellularly derived NFkB binding proteins.

Keywords AIDS HIV immunoregulation SIV virus suppression

INTRODUCTION

Sooty mangabeys are among several species of African non-human primates which are naturally infected with the simian immunodeficiency virus (SIV) yet remain clinically asymptomatic [1]. When SIV is isolated from mangabeys and experimentally injected into rhesus and pigtailed macaques, these species develop an AIDS-like disease with the clinical symptoms of human HIV-1 infection [2]. The mechanism(s) by which the naturally infected African species remain clinically asymptomatic are currently unknown. Our laboratory has been studying the immune response of naturally infected sooty mangabeys in an effort to gain insight into the mechanisms of protective immunity in this species. Previously, we have shown that compared with the disease-susceptible macaques, mangabeys have a high frequency and absolute number of circulating CD8$^+$ T cells [3]. Furthermore, a great proportion of these cells appear to be activated, as they express HLA-DR on their cell surface. Finally, we have shown that CD8$^+$ T cells in the mangabeys, but not the macaques, appear to play an important role in the immunosurveillance system of this species [4].

CD8$^+$ T cells from HIV-1-infected humans and SIV-infected macaques have the ability to markedly inhibit HIV-1 and SIV replication, respectively, *in vitro* [5,6]. The precise pathway by which CD8$^+$ cells mediate this inhibition remains to be elucidated. Furthermore, the protective role this inhibition may play *in vivo* is unclear.

Since sooty mangabeys are naturally infected with SIV without any clinical sequela, we endeavored to investigate these questions, using the mangabey model of SIV infection. Toward this goal, we have developed a cell line derived from an SIV-infected mangabey which is replication-competent not only for SIV but also to a lower but reproducible level for HIV-1 and HIV-2. In this study we demonstrate the ability of autologous CD8$^+$ T cells to inhibit the replication of all three of these retroviruses in this cell line. In addition, by co-culturing SIV-infected cells with autologous lymphocytes, using a dual-chamber tissue culture vessel separated by a semipermeable membrane, we provide evidence that there is no obligate requirement for cell–cell contact to mediate suppression of viral replication and that such suppression can be mediated by a soluble factor. Furthermore, experiments performed using an LTR-driven CAT reporter gene indicate that CD8$^+$ T cells inhibit cellular activation of retroviral replication.

MATERIALS AND METHODS

Animals

All monkeys utilized in this study are housed at the Yerkes Regional Primate Research Centre (Atlanta, GA). They were

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maintained in accordance with the instructions of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and the PHS guidelines, *Guide for the Care and Use of Laboratory Animals*.

Viruses

The SIV used during this study was originally isolated from the peripheral blood mononuclear cells (PBMC) of a naturally infected sooty mangabey [1] by *in vitro* coculture with phytohaemagglutinin (PHA)-stimulated human PBMC (courtesy of Dr P. Fultz, YRPRC, Emory University, Atlanta, GA). A large pool of this SIVsmm-7 was prepared on contract withAdvanced Biotechnologies, Inc. (Silver Spring, MD). The virus was grown in the $CD4^+$ human cell line termed H-9 (courtesy of the NIH, AIDS Research and Reference Reagent Program), and the supernatant fluid was collected at a predetermined optimal time interval which showed the highest RT activity. The supernatant fluid was subjected to continuous-flow centrifugation at 3·5 *l*/h in a Beckman CF-32 rotor. The pelleted virus suspension was layered over a 20/40% (w/v) discontinuous sucrose gradient and then centrifuged at 100 000 ***g*** for 2 h in a Beckman SW27 rotor. The virus band was collected at the 20/40% sucrose interface and then centrifuged for 1 h at 100 000 ***g*** in a Beckman type-35 rotor. The virus pellet was resuspended in Tris 10 mM, sodium chloride 150 mM buffer, pH 7·5, so that 1 ml represented the yield from 1 *l* of the supernatant fluid ($\times 1000$). The virus particle count was determined to be $1{\cdot}33 \times 10^{10}$ virus particles/ml. The protein concentration was determined to be 0·87 mg/ml final concentration. The $TCID_{50}$ of this virus stock was determined by using the human $CD4^+$ H-9 cell line and was calculated to contain 10^5 $TCID_{50}$/ml of the virus stock. This value is lower than that obtained with other retroviruses and is reasoned to be most likely due to either the stripping of *env* proteins by the centrifugation procedures outlined above, or the use of the human H-9 cell line instead of non-human primate PBMC that were used to calculate the $TCID_{50}$.

Two other lentiviruses were utilized. These included the HIV-1 pool termed *LAV*, which was grown in A3.01 cell line that was prepared by our laboratory by using a protocol similar to the protocol used in experiments with SIVsmm. The virus stock contained 2×10^5 $TCID_{50}$/ml. The other lentivirus used was HIV-2 *rod* subclone SR-3 [7], which was prepared by our laboratory (with techniques similar to those used with the HIV-1) by using the SupT1 cell line. This virus stock contained 1×10^5 $TCID_{50}$/ml.

Media

Media used throughout this study was RPMI 1640 supplemented with 100 U/ml of penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Gibco, Grand Island, NY) and 10% heat-inactivated fetal bovine serum (FBS; Hyclone Corp., Logan, UT). Where indicated, these media were supplemented with 10 U/ml of rIL-2 (Hoffman LaRoche, Nutley, NJ).

Isolation of lymphocytes and CD8 depletion

PBMC were isolated using a 60% Percoll gradient as previously described [3]. They were washed twice in media, counted, and adjusted to a concentration of 2×10^6/ml. PHA-P (Gibco) at a final concentration of 0·2% was added to cell cultures which were incubated at 37°C in a 7% CO_2 humidified atmosphere for 3 days. The cells were then washed twice, and aliquots of the cells were depleted of $CD8^+$ T cells, using a biomagnetic separation system [8]. In brief, 10^7 lymphocytes were incubated with 100 μl of Leu-2a (anti-CD8; Becton Dickinson, Mountain View, CA) on ice for 40 min. Excess antibody was removed by washing the cells in media. The media were then aspirated, and the cells were next incubated with 2 ml of goat anti-mouse immunoglobulin-conjugated beads (Collaborative Research, Bedford, MA) for 40 min on ice. $CD8^+$ cells were depleted by placing the lymphocytes in a magnetic field. $CD8^+$ cells were recovered from the beads by allowing them to incubate overnight at 37°C in a 7% CO_2 humidified atmosphere.

FEc1 cell line

The FEc1 cells are an Epstein–Barr virus (EBV)-transformed B cell line isolated from the PBMC of an SIV^+ sooty mangabey (FEc) as described elsewhere [8]. Briefly, PBMC from this monkey FEc were incubated overnight at 37°C in a 7% CO_2-humidified incubator with supernatant fluid from the culture of the B95–8 marmoset cell line (ATCC, Rockville, MD). The B95–8 cell line sheds the EBV in the supernatant fluid. After overnight incubation, the FEc cells were washed in medium and resuspended in the same medium supplemented with 20% heat-inactivated (56°C, 30 min) fetal calf serum (FCS). The cells were cultured in 24-well tissue culture plates at 37°C in the 7% CO_2-humidified incubator, and medium was replaced whenever required. By 6–8 weeks, transformed cells were readily apparent; these cells were expanded in 250-ml flasks and were carried as a cell line in the laboratory. The FEc1 cell line consists of large EBV-transformed cells that are readily distinguishable from lymphocytes morphologically. For the first 10 tissue culture passages, these EBV-transformed mangabey cells produced anti-SIV antibodies. However, antibody production by these cells has since ceased. The cells at about the twelfth passage were CD4 transfected with the gene that codes for the human CD4 molecule and with a neomycin resistance gene, using a Moloney-derived virus vector kindly provided by Dr R. Morgan (NHLBI, NIH). Selection for cells in which the CD4 gene was integrated was accomplished by growing the cells in the presence of 500 μg/ml of neomycin. Expression of CD4 was confirmed by flow microfluorometric (FMF) analysis, using fluorescein-conjugated MoAb Leu-3a. This cell line (which is oligoclonal) has been stable for the last 2 years and has been shown consistently to express CD4.

N939 cell line

The N939 transformed cell line was derived from a rhesus macaque (named *N939*) by using herpes virus saimiri in a protocol essentially similar to the protocol used for preparing the EBV-transformed FEc1 cell line as described above. The PBMC used were obtained from monkey *N939* before experimental inoculation with 10^4 $TCID_{50}$ of SIVsmm-9.

CD8 suppression of reverse transcriptase activity

The standard assay consisted of culturing PBMC from the monkey to be tested with 0·1% PHA-P for 3 days *in vitro* at a concentration of 2×10^6 cells/ml. The PHA-P blasts were then layered over a Ficoll–Hypaque gradient and centrifuged at 450 ***g***, and the cells at the interface were collected. These cells were washed, and an aliquot was depleted of $CD8^+$ cells as described above. Unfractionated PHA blasts were adjusted to 2×10^6

cells/ml, and the CD8-depleted PHA blasts were adjusted to contain approximately the same number of $CD4^+$ cells contained in the unfractionated PBMC. To each culture was added 20 μl of the $\times$ 1000 SIVsmm stock to yield about 2×10^3 $TCID_{50}$ per culture as an exogenous source of virus. The cultures were performed in media containing 10 U/ml of rIL-2 and were incubated at 37°C in a 7% CO_2-humidified atmosphere. Supernatant fluids from the cultures were collected every fourth day and assayed for reverse transcriptase (RT) activity. In efforts to establish a standard assay, the CD4-expressing FEc1 cell line was used as a constant target cell line. In this case, 5×10^5 of the FEc1 cell line were cultured with SIVsmm alone (control) or co-cultured with 1×10^6 day-3 $CD8^+$ PHA blasts and the same dose of virus (2×10^3 $TCID_{50}$) in media containing 10 U/ml of rIL-2. In select experiments, instead of SIVsmm, an approximately similar dose of HIV-1 or HIV-2 (courtesy of the NIH, AIDS Research and Reference Reagent Program) was added to the cultures. Supernatant fluids from these cultures were collected and assayed for RT activity. In addition, some of the experiments used a dual-chamber culture assay [10], in which FEc1 cell line was cultured in the bottom chamber, and the autologous $CD8^+$ PHA blasts were placed in the top chamber in the same numbers as described above, and RT activity in the supernatant fluids was measured.

Transfection and CAT assay

The plasmids used were as follows: pU3RIII [11]; pIIIextatIII [11]; NFkB mutant pU3RIII (Kb-) with a mutation at the NFkB binding sites [12]; pCH110, an SV_2-β galactosidase reporter plasmid [13], and a pSV_2-CAT plasmid. FEc1 cells or the N939 cells (10^7) were washed twice with PBS and resuspended in 1 ml of serum-free OPTIMEM (Gibco). To this suspension, plasmid amounts of 5 μg/10^6 cells (with the exception of pIIIextatIII, for which only 2·5 μg/10^6 cells were used) and 25 μg of salmon sperm DNA (Sigma Chemical Co., St Louis, MO) were added. Transfection was achieved by electroporation using a BioRad gene pulser apparatus, at 500 μF, 400 Ω, and 450 V with the time-constant automatically adjusted by the pulser for these settings. After transfection, the cells were washed and aliquoted at 2×10^6 cells/well in a 24-well plate. To these plasmid-transfected FEc1 or N939 cell cultures varying numbers of effector cells at ratios of 1:1, 0·5:1 and 0·25:1 (effector:target) were added. The cultures were incubated for 3 days. On day 3, 60 μl of cell extract was prepared, and CAT activity was assayed as previously described [14] or by an ELISA for CAT (Boehringer Mannheim, Indianapolis, IN). It is important to note that comparisons of CAT activity were made among samples which were transfected at the same time, thus eliminating the possibility that the differences were due to transfection efficiency. The relative density measurements of the x-ray films which show CAT activity, as illustrated in the data shown in Figs 1, 2 and 3, were also quantified, and changes were calculated as a percentage of the values obtained with the cell line that was cultured with media alone. In addition, phenotypic frequency and total number of viable cells in each culture were also determined.

Reproducibility of assay

Inhibition of CAT activity by $CD8^+$ T cells has been demonstrated consistently in our laboratory. All comparisons of CAT activity were made among samples that were transfected at the same time and then aliquoted. Thus, differences in CAT activity cannot be due to differences in transfection efficiency. Furthermore, for the experiment described in Fig. 3, duplicate 5-μl aliquots from each culture extract were assayed for β galactosidase activity to determine transfection efficiency as previously described [13]. Before the preparation of cell extracts, cell counts were performed using trypan blue exclusion to ensure that differences in CAT activity were not secondary to cell death.

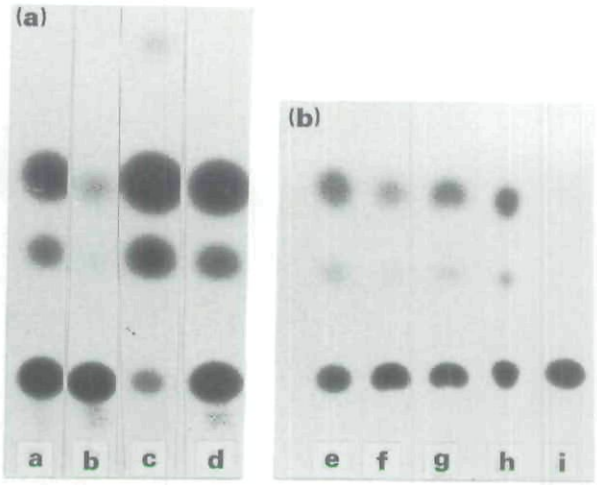

Fig. 1. (a) Experiment 1: FEc1 cells were transfected with pU3RIII alone (lanes a, b) or pU3RIII + pIIIextatIII (lanes c, d). The cells were cultured at 2×10^6/well for 3 days with (lanes b, d) or without (lanes a, c) 2×10^6 activated autologous lymphocytes. (b) Experiment 2: FEc1 cells were transfected with pU3RIII and cultured as described above. Lane e, cells alone; lane f, cells + 10^6 whole activated lymphocytes; lane g, cells + 10^6 CD8-depleted activated lymphocytes; lane h, cells alone; lane i, cells + 10^6 CD8-enriched activated lymphocytes. (These are the same $CD8^+$ T cells which were depleted from the culture shown in lane g.)

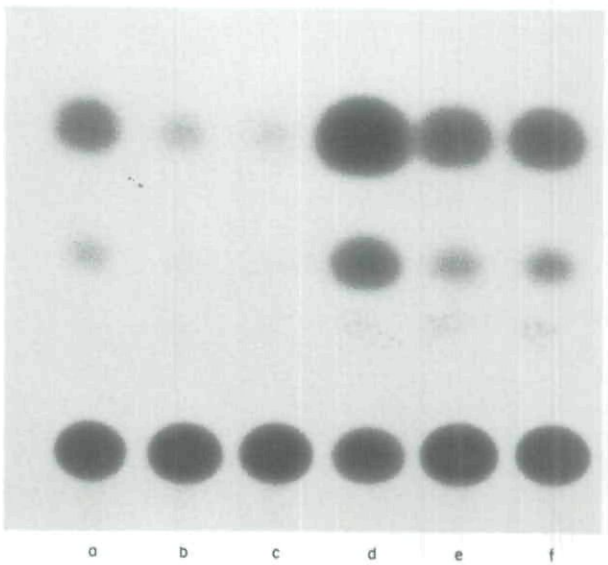

Fig. 2. FEc1 cells were transfected with pU3RIII (lanes a, b, c) or pU3RIII + pIIIextatIII (lanes d, e, f). These cells were cultured at 2×10^6 cells/well alone (lanes a, d), with 2×10^6 activated lymphocytes (lanes b, e), with 2×10^6 activated lymphocytes in an upper chamber separated by a semipermeable membrane (lanes c, f).

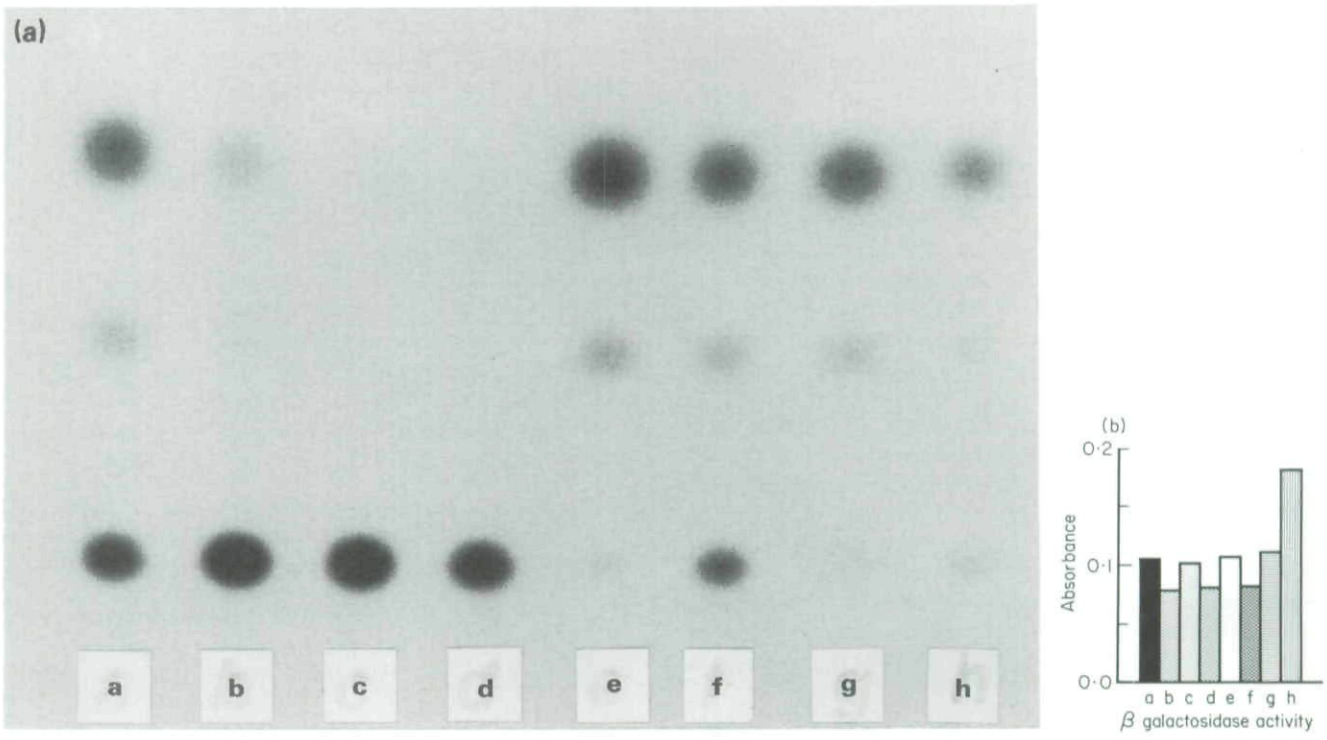

Fig. 3. FEc1 cells were co-transfected with SV_2 β galactosidase and the following: pU3RIII (lanes a, b), pU3RIII Kb^- (lanes c, d), pU3RIII + pIIIextatIII (lanes e, f), pU3RIII Kb^- + pIIIextatIII (lanes g, h). These cells were cultured at 2×10^6 cells/well either alone (lanes a, c, e, g) or with 2×10^6 activated lymphocytes (lanes b, d, f, h). On day 3 the cells were harvested, and cell lysates were assayed for both CAT activity (a) and β galactosidase activity (b).

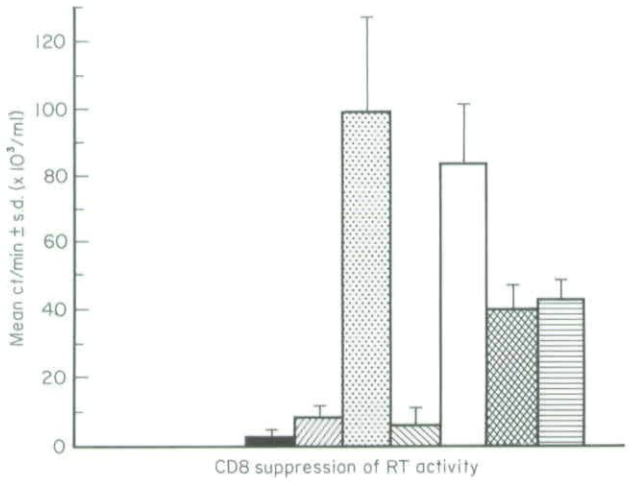

Fig. 4. Exogenous simian immunodeficiency virus (SIV) was added to cultures containing unfractionated peripheral blood mononuclear cells (PBMC) or CD8-depleted PBMC, and the supernatant fluids were harvested every 4 days and assayed for reverse transcriptase (RT) activity. The data represent the mean RT activity of supernatant fluid from triplicate cultures harvested on day 12 when peak RT activity was noted. ■, Background; ▩, mangabey (Mn) (+) PBMC; ▨, Mn (+) CD8-depleted; ▧, Mn (−) PBMC; □, Mn (−) CD8-depleted; ▩, rhesus macaque (Rh) (−) PBMC; ▤, Rh (−) CD8-depleted.

RESULTS

Inhibition of viral replication by CD8⁺ T cells from naturally SIV-infected disease-resistant sooty mangabeys

$CD8^+$ T cells from naturally infected seropositive sooty mangabeys and, for control purposes, $CD8^+$ T cells from (i) disease-susceptible rhesus macaques experimentally infected with 10^4 $TCID_{50}$ of SIVsmm; (ii) uninfected rhesus macaques; and (iii) seronegative sooty mangabeys were assayed for their ability to inhibit the replication of SIVsmm in equivalent numbers of autologous CD8-depleted $CD4^+$-enriched populations of mononuclear cells *in vitro*. $CD8^+$ T cells from the uninfected macaques did not demonstrate measurable inhibitory activity, consistent with previously published data [5,6]. Of great importance was the observation that $CD8^+$ T cells from not only seropositive but also seronegative mangabeys mediated marked inhibition of SIV replication (Fig. 4). The experiments have been repeated at least 10 times with different seropositive and seronegative mangabeys and experimentally infected and non-infected rhesus macaques, and have given essentially similar results. These results were not secondary to differences in the total number of $CD4^+$ cells that remained in the culture, since cultures of unfractionated and CD8-depleted PBMC from the same sample of blood, upon examination, contained a similar number of total $CD4^+$ cells as determined by FACS analysis. In addition, the differences noted between macaques and mangabeys were not secondary to any major difference (± 10%) in the

Table 1. CD8-mediated suppression of lentivirus replication*

Culture constituents	RT activity (ct/min per ml)	Per cent inhibition
FEc1 cells + SIV	111 183	—
FEc1 cells + SIV + autologous $CD8^+$ T cells	16 398	85
FEc1 cells + HIV-2	7188	—
FEc1 cells + HIV-2 + autologous $CD8^+$ T cells	1680	77
FEc1 cells + HIV-1	7700	—
FEc1 cells + HIV-1 + autologous $CD8^+$ T cells	1730	74

* FEc1 cells (5×10^5) were infected with simian immunodeficiency virus (SIV), HIV-2, or HIV-1 and cultured for 7 days in the presence or absence of 2×10^6 phytohaemagglutinin (PHA)-activated autologous $CD8^+$ T cells. Reverse transcriptase (RT) activity was determined on day 7 as described in Materials and Methods. Background RT activity for this assay gave a value of 667 ct/min.

frequency or total number of $CD4^+$ cells utilized, since the cultures were performed by using an essentially similar total number of $CD4^+$ cells.

$CD8^+$ T cells from an SIV-infected sooty mangabey have the ability to inhibit HIV-1 and HIV-2

In an effort to examine the mechanisms contributing to the ability of $CD8^+$ T cells to inhibit SIV replication, B cells from an SIV-infected seropositive sooty mangabey (*FEc*) were transformed with EBV (see Materials and Methods) and transfected with a human CD4 gene. This cell line, termed FEc1, stably expresses CD4 as determined by FACS analysis and is replication-competent for SIV. As shown in Table 1, autologous $CD8^+$ T cells are able to inhibit SIV replication in this cell line. Furthermore, this cell line is replication-competent for HIV-1 and HIV-2. The data in Table 1 demonstrate the ability of $CD8^+$ T cells from the mangabey FEc to inhibit the replication of these lentiviruses as well and suggest that the target of inhibition is most likely a common pathway used by this group of lentiviruses for viral replication. Other lentiviruses such as EIVA, visna, and CAEV, however, may use a different pathway. It is also not clear whether $CD8^+$ cells from an SIV-infected monkey can inhibit the replication of a non-'lenti' retrovirus such as simian retrovirus D.

$CD8^+$ T cells can inhibit LTR-driven viral replication

Replication of HIV-1 in infected lymphocytes is linked to lymphocyte activation [15]. Mitogens, anti-CD3, and anti-CD28—all of which mediate T cell activation—have also been shown to up-regulate virus replication in infected cells. There is a duplicate NFkB binding site in the LTR of HIV-1, and the binding of cellular factors to these sites has been demonstrated [12,16]. With this in mind, we sought to determine whether $CD8^+$ T cells could inhibit cellular activation of viral replication.

FEc1 cells were transiently transfected with pU3RIII CAT plasmid, which contains the CAT gene driven by the HIV-1 LTR. These cells were cultured for 3 days in the presence or absence of autologous activated lymphocytes. This experimental strategy was employed to determine if autologous lymphocytes had the functional capability to inhibit CAT activity, providing a model that would permit us to investigate the replication stage of lentivirus replication which was the target of inhibition mediated by the $CD8^+$ T cells. Figure 1 shows the data obtained from two separate experiments designed to address this issue. In experiment 1 (Fig. 1), comparison of the data obtained (lanes a and b) clearly demonstrates that the presence of autologous lymphocytes inhibits the transactivation of the pU3RIII plasmid (>90% inhibition). Lanes c and d show data from cultures in which the FEc1 cells were co-transfected with pU3RIII and pIIIextatIII, a plasmid which has the HIV-1-LTR driving the *tat* gene and can efficiently produce *tat* in the absence of cellular binding factors. This plasmid not only transactivates itself but will also transactivate pU3RIII. Once again there is inhibition, although it is quantitatively less (<20%), suggesting that the trans *tat* is able to overcome some of the suppression. Since *tat* is produced by a separate plasmid and stimulates the LTR independently of any cellularly derived factors, the data suggest that the suppressive effect is directed at cellular activation of viral replication. Although it is clear that lane c may not be in the linear range of this assay, it is shown here only to demonstrate that the *tat* is able to transactivate and produce CAT activity.

In experiment 2 we sought to demonstrate further the ability of an enriched population of $CD8^+$ T cells (as opposed to unfractionated activated lymphocytes) to inhibit viral replication at the level of transcription. Lane e shows CAT activity derived from FEc1 cells cultured alone. Lane f shows CAT activity derived from FEc1 cells cultured with unfractionated activated PBMC, while lane g shows CAT activity derived from FEc1 cells cultured with CD8-depleted activated PBMC. Once again, as was seen in experiment 1, unfractionated PBMC inhibited CAT activity (lane f, >90% inhibition). On the other hand, the FEc1 cell line co-cultured with CD8-depleted PBMC showed no significant inhibition (lane g, <10% inhibition). Addition of these highly enriched populations of $CD8^+$ T cells recovered from the depletion experiment to the FEc cell line markedly inhibited CAT activity (lane i, >95% inhibition) when compared with CAT activity in FEc1 cells cultured as described above (lane h). These data support the concept that $CD8^+$ T cells can inhibit viral replication at the level of LTR-mediated viral activation.

Table 2. CD8-mediated suppression of viral replication by a soluble factor*

Culture	RT activity (ct/min per ml)	Per cent inhibition
SIV	23245	—
SIV + autologous lymphocytes	5170	77
SIV + autologous lymphocytes separated by a membrane	13330	43

* FEc1 cells (5×10^5) were infected with simian immunodeficiency virus (SIV) and cultured (i) alone, (ii) with 2×10^6 phytohaemagglutinin (PHA)-activated autologous lymphocytes, and (iii) with 2×10^6 PHA-activated autologous lymphocytes separated by a semipermeable membrane.

RT, Reverse transcriptase.

Inhibition of viral replication by a soluble factor released by CD8⁺ T cells

Experiments were designed to determine whether a soluble factor was responsible for the inhibition of viral replication. Two-chamber culture conditions were set up as previously described [10]. The data in Table 2 demonstrate that autologous lymphocytes are able to inhibit viral replication in FEc1 cells even when physically separated by a semipermeable membrane. However, the inhibition is not as potent as when a similar number of lymphocytes and FEc1 cells are co-cultured in the same chamber (43% *versus* 77%). In these experiments, viral replication was measured by assaying for RT activity. The total number of FEc1 cells in each of these cultures at the end of the incubation period was essentially similar. Thus, the inhibition was not secondary to differences in the growth of the FEc1 cells in the various assay conditions. The protocol for the experiment shown in Fig. 2 was essentially similar to that described above, except that in the Fig. 2 protocol, viral replication was quantified by the measurement of CAT activity. As such, transwell cultures were set up with the transfected cells in the bottom well and with activated lymphocytes in the top. As seen in Fig. 2, the presence of the activated lymphocytes separated by a semipermeable membrane (lane c, >95% inhibition) was just as potent as co-culture of unfractionated activated autologous lymphocytes (lane b, >90% inhibition) in inhibiting CAT activity when compared with FEc1 cells cultured in media alone (lane a). Likewise, there is inhibition of CAT activity in the cells which were co-transfected with the pU3RIII and the pIIIextatIII, although once again the inhibition is less than that demonstrated in the cells transfected with pU3RIII alone (15–25%, lanes d–f). These data, taken together, suggest that CD8-mediated suppression occurs at the level of viral activation of replication, that a soluble factor is involved, and that such inhibition of virus replication may be mediated by a mechanism other than cytolytic destruction of virus-infected target cells. The concept of a soluble factor involved in inhibition of virus replication is supported by similar data from other laboratories [10,17,18]. Furthermore, such findings suggest that this inhibition is non-MHC-restricted and not due to conventional cytotoxic T cell function, and the data argue against the concept forwarded by Tsubota *et al.* [19] that phenotypically CD8⁺ T cells, which require cell–cell contact and are MHC-restricted, are the sole pathway involved in inhibition of virus replication.

CD8⁺ T cells inhibit NFkB-mediated activation of viral replication

Several groups have demonstrated the ability of cellularly derived nuclear factors to bind to the NFkB element of the LTR and activate virus replication [12,15]. Thus, it was of interest to determine whether cellularly derived NFkB binding proteins were responsible for the activation of the pU3RIII in the FEc1 cells. In efforts to address this issue, a pU3RIII plasmid with a mutation in the NFkB binding motif (termed Kb⁻) was utilized. The FEc1 cells were also transfected with an SV_2 'cis'-activated β galactosidase plasmid. This approach permitted the analysis of the efficiency of the transient transfection, which could be measured independently of CAT activity. Figure 3a, lane b, demonstrates the inhibition (>95%) of CAT activity derived from FEc1-transfected cells by co-culture with activated lymphocytes as compared with FEc1 cells cultured with media alone (lane a). As seen in Fig. 3b, there is also some inhibition (10%) of the SV_2 β galactosidase activity by these lymphocytes; as the SV_2 promoter also contains an NFkB-like binding region, this slight inhibition may be caused by inhibition at this site. Lanes c and d show that there is virtually no CAT activity when the FEc1 cells are transfected with the Kb⁻ CAT plasmid. On the other hand, these cells show substantial β galactosidase activity, indicating that the lack of CAT activity is not secondary to an unsuccessful transfection. Lanes e, f show once again that cells which are co-transfected with pU3RIII and pIIIextatIII are less susceptible to CD8-mediated suppression (lane f showing 22% inhibition when compared with lane e). That is, the *tat* provided *in trans* is able, to a certain degree, to mitigate this suppression. Finally, lanes g and h show CAT activity from cells which were co-transfected with the pU3RIII (Kb⁻) and the pIIIextatIII. Each culture demonstrated substantial CAT activity, with about 10% lower CAT activity in lane h as compared with lane g. These data suggest that in the absence of cellular NFkB binding factors, activation of the Kb⁻ plasmid can be driven *in trans* by *tat*. Since the Kb⁻ pU3RIII can be driven in the presence of *tat*, the 'lack' of activity demonstrated in lanes c and d is not due to a defective plasmid, but rather indicative of the fact that cellular activation of the LTR is dependent on the NFkB binding sites.

Although the above data provide relative values for the degree of inhibition of CAT activity in the various experiments (numbers in parentheses in the text above are calculated from density analysis), the recent availability of an ELISA that

Table 3. Specificity of the *in vitro* suppression of LTR-driven CAT activity by $CD8^+$ T cells*

Effector cells	Target cells	E:T ratio	OD (mean ± s.d.) Target cells transfected with pSV$_2$-CAT	pU3RIII-LTR-CAT	pKb$^-$-LTR-CAT	pU3RIII-LTR-CAT plus pIIIextatIII
—	FEc1	—	0·378 ± 0·029	1·672 ± 0·121	0·076 ± 0·009	2·639 ± 0·175
Unf. PBMC	FEc1	1:1	0·384 ± 0·046	0·778 ± 0·085‡	0·084 ± 0·012	2·737 ± 0·196
$CD8^-$ PBMC	FEc1	1:1	0·396 ± 0·051	1·894 ± 0·201	0·096 ± 0·014	2·845 ± 0·274
Unf. PBMC	FEc1	0·5:1	0·397 ± 0·034	0·945 ± 0·086‡	0·077 ± 0·009	2·798 ± 0·216
$CD8^-$ PBMC	FEc1	0·5:1	0·374 ± 0·041	1·902 ± 0·097	0·084 ± 0·004	2·884 ± 0·111
Unf. PBMC	FEc1	0·25:1	0·368 ± 0·061	1·245 ± 0·104‡	0·081 ± 0·031	2·767 ± 0·123
$CD8^-$ PBMC	FEc1	0·25:1	0·379 ± 0·054	1·846 ± 0·091	0·075 ± 0·021	2·674 ± 0·127
—	N939	—	0·409 ± 0·073	1·537 ± 0·082	0·094 ± 0·012	2·874 ± 0·241
Unf. PBMC	N939	1:1 (+)†	0·398 ± 0·042	0·667 ± 0·062‡	0·098 ± 0·017	2·795 ± 0·156
$CD8^-$ PBMC	N939	1:1 (+)	0·456 ± 0·045	1·789 ± 0·094	0·087 ± 0·026	2·996 ± 0·148
Unf. PBMC	N939	1:1 (−)†	0·374 ± 0·031	1·438 ± 0·116	0·088 ± 0·027	2·798 ± 0·147
$CD8^-$ PBMC	N939	1:1 (−)	0·396 ± 0·037	1·815 ± 0·089	0·096 ± 0·021	2·814 ± 0·159

* Epstein–Barr virus (EBV)-transformed cells from a mangabey (FEc) and from a rhesus macaque (N939) were each transfected with the individual plasmids pSV$_2$-CAT, pU3RIII-LTR-CAT, pKb$^-$-LTR-CAT or were co-transfected with the plasmids pU3RIII-LTR-CAT plus pIIIextatIII and then cultured in media alone, autologous unfractionated (Unf.) peripheral blood mononuclear cells (PBMC), or $CD8^+$ T cell-depleted PBMC at ratios of 1:1, 0·5:1 and 0·25:1. CAT activity was measured by using ELISA purchased commercially.

† +, −, PBMC and CD8-depleted PBMC from monkey N939 before experimental infection with SIVsmm-9 (−) and > 2 years after experimental infection with SIVsmm-9 (+).

‡ Values statistically significant ($P < 0·001$) when compared with respective control values of 1·672 ± 0·021 for FEc1 and 1·537 ± 0·082 for N939.

measures CAT prompted us to repeat the above experiments to derive more precise data. Again, the FEc1 cell line or a herpes transformed cell line from a rhesus macaque before experimental infection with SIVsmm-9 (termed *N939*) was each transiently transfected with pU3RIII-LTR-CAT, the mutant pKb$^-$-LTR-CAT, the pU3RIII-LTR-CAT plus pIIIextatIII, and, for purposes of control, the plasmid SV$_2$-CAT. After transfection, 5×10^5 of each of these cells were co-cultured in triplicate wells with autologous unfractionated or CD8-depleted PHA-activated PBMC for 3 days. In the case of N939, cultures consisted of the N939 herpes transformed cells, aliquots of which were transfected with each of the above plasmids and then co-cultured for 3 days with PHA-activated unfractionated or CD8-depleted PBMC from the same monkey (*N939*) before and after experimental infection with SIVsmm-9. As seen in Table 3, ELISA results basically confirmed the results obtained using the TLC method for measuring CAT activity. It is important to note that the cells transfected with the pSV$_2$-CAT plasmid did not appear to be influenced by unfractionated or CD8-depleted effector cells. However, FEc1 or the N939 cells that were transfected with pU3RIII showed marked inhibition of CAT activity ($P < 0·001$) when co-cultured with unfractionated FEc PBMC or N939 PBMC after SIV infection but not before SIV infection, respectively. The inhibition appeared to be related to the dose of unfractionated PBMC. Furthermore, no CAT was measurable in the cells transfected with pKb$^-$ plasmid, and the inhibition that was mediated by $CD8^+$ cells in cells that were transfected with pU3RIII alone was overcome, to a large extent, by co-transfection with pIIIextatIII. Total cell counts and viability cell counts were performed in each of the cell cultures. These data showed that all cultures were > 95% viable, with the same total number of cells (range $5·75 \times 10^5$ to 6×10^5) at the end of the culture period, suggesting that the degree of inhibition of CAT activity could not be secondary to cytotoxic mechanisms. Studies performed by using ^{51}Cr-labelled FEc1 target cells that were transfected with each of the above plasmids and co-incubated with unfractionated or CD8-enriched FEc1 cells failed to demonstrate significant cytolytic activity (< 5% net cytotoxicity, data not shown). Appropriate negative and positive controls were included in each experiment. One should also note that the experiment as shown in Table 3 has been repeated three times, with each experiment yielding basically identical data.

Taken together, we believe these data demonstrate that (i) most viral activation in this model is mediated by NFkB binding proteins; (ii) the ability of $CD8^+$ T cells to inhibit viral replication in this model is directed at these cellularly derived factors.

DISCUSSION

Using the mangabey model of SIV infection, we have sought to examine the mechanism(s) behind CD8-mediated suppression of viral replication. Mangabeys are naturally infected with SIV yet display none of the clinical sequelae of such infection, therefore providing a unique model to study potential protective

immune effector mechanisms to retroviral infection in the absence of concomitant opportunistic infection. It is intriguing that not only do sooty mangabeys possess $CD8^+$ T cells which inhibit retroviral replication, but seronegative sooty mangabeys also possess such effector cells, in some cases many years before they seroconvert. Such findings raise the possibility that perhaps the early development of this lineage of $CD8^+$ T cells in mangabeys before frank seroconversion may contribute in some way to their relative resistance to SIV infection. In fact, our laboratory has preliminary data (unpublished) that seronegative infant mangabeys (< 2 years old) possess such $CD8^+$ T cells.

The ability of $CD8^+$ T cells from an SIV-infected sooty mangabey to inhibit HIV-1 and HIV-2 infection was addressed using a $CD4^+$ EBV-transformed mangabey B cell line. It was found that the $CD8^+$ T cells from the SIV-infected mangabey could, in fact, inhibit HIV-1 and HIV-2 replication *in vitro*. Since all three of these viruses are lentiviruses, it suggests that the pathway of inhibition by $CD8^+$ T cells is common to lentiviruses and not strictly antigen-specific. However, it is important to realize that since we are using PHA-activated $CD8^+$ T cells, we are examining the effector step of this inhibition and not the antigen recognition step. Presumably, $CD8^+$ T cells recognize SIV *in vivo* in an antigen-specific fashion which leads to the expansion of these cells. The expansion of such cells may indeed be SIV-specific, although the effect of such cells is not SIV-specific. The antigen responsible for this expansion has yet to be elucidated. However, the data in Fig. 3 show only minimal inhibition of β galactosidase driven by the simian virus 40 promoter, suggesting that this mechanism has some degree of specificity. This specificity is further exemplified by the lack of inhibition of CAT by $CD8^+$ cells in cultures transfected with the pSV_2 plasmid (see Table 3). In this regard, it would be interesting to determine if $CD8^+$ T cells from an SIV-infected mangabey have the ability to inhibit non-lentivirus retroviruses such as the simian retrovirus D.

Using dual-chamber culture conditions, we demonstrated that CD8-mediated suppression of both SIV replication and LTR-driven CAT activity can be mediated by a soluble factor. Such data are in agreement with the findings of Walker & Levy [10] and Brinchmann *et al.* [18], both of whom were measuring suppression of HIV-1 replication by human $CD8^+$ T cells. These transwell experiments suggest that CD8-mediated inhibition is not simply CTL activity against virally infected cells. Such data, however, conflict with the views of Tsubota *et al.* [19], who provide phenotypic evidence that CD8 suppression of viral replication is mediated by cytotoxic T cells by virtue of the cell surface markers expressed by such cells. Our laboratory has been unable to correlate CD8-mediated suppression with cytolysis of infected cells (unpublished). In this regard, it is important to note that Walker *et al.* [17] and Wiviott *et al.* [20] have reported the isolation of $CD8^+$ T cell clones which inhibit viral replication yet lack CTL activity.

It is now apparent that the interaction of HIV-1 with T cells and monocytes exceeds the mere tropism of the virus for these cell types. Antigen, anti-CD3, mitogens, and various cytokines—all of which are able to stimulate T cell activation—also activate virus in latently infected cells [21]. At the molecular level, several groups have demonstrated the ability of NFkB binding proteins to enhance viral replication in both T cell and monocyte cell lines [12,22]. Using an NFkB mutant pU3RIII reporter gene, we have demonstrated that most viral transcription, as reflected by CAT activity in the FEc1 cells, is positively influenced by NFkB binding proteins, and this activity can be markedly inhibited by $CD8^+$ T cells. Cells which were co-transfected with the pU3RIII and pIIIextatIII plasmids generated higher CAT activity, as expected. The co-transfected cells were inhibited by $CD8^+$ cells to a much lesser extent than when *tat* was absent. *In vivo*, CD8-mediated suppression may therefore be overcome if there is an abundance of *tat* within an infected cell. Reports concerning the ability of cis-acting viral factors to induce replication in NFkB viral mutants have recently been presented [23].

Since viral replication is so intimately linked to factors responsible for T cell activation, it is unclear whether $CD8^+$ T cell-mediated inhibition is primarily an antiviral response or an immunosuppressive response. There is a definite down-regulation of T-helper cell function in HIV-1 infection [24]. Shearer *et al.* [25] have shown that asymptomatic HIV-1-infected individuals have suppressed T helper cell function to certain nominal antigens. Via *et al.* [26] have proposed that this represents active immunosuppression and may be protective by preventing the activation and subsequent infection of these cells. We hypothesize, therefore, that the observed suppression may be due to the ability of $CD8^+$ T cells to down-regulate NFkB-mediated activation of infected cells. In the future we hope to use this model to characterize further the role of the NFkB protein family on cellular activation and the potential role of $CD8^+$ cells in regulating this axis.

ACKNOWLEDGMENTS

The authors are grateful to Dr H. M. McClure, Ms Ellen Lockwood, Professor Thomas Gordon, and associated scientists at the Yerkes Regional Primate Centre who facilitated the collection of pertinent laboratory data and blood specimens. We thank Ann Mayne and Matthew Vuchetich for their technical expertise and Patricia Guenthner for her assistance in preparing plasmids. This work was supported by grant AI-27057-03 from the National Institutes of Health and by NIH grant DRR-00165 from the National Centre for Research Resources to the Yerkes Regional Primate Research Centre. The Yerkes Centre is fully accredited by the American Association of Laboratory Animal Care. F. V. is a Fellow of the Swiss National Fund for the Advancement of Science (823A-030700).

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