Human Immunodeficiency Virus Type 1 Nef Does Not Alter T-Cell Sensitivity to Antigen-Specific Stimulation

KATHLEEN A. PAGE,^{1*} WIM C. A. VAN SCHOOTEN,² AND MARK B. FEINBERG^{1,3†}

Gladstone Institute of Virology and Immunology and Department of Microbiology and Immunology, University of California—San Francisco, San Francisco, California 94141-9100¹; Activated Cell Therapy, Inc., Mountain View, California 94043²; and Department of Medicine, University of California—San Francisco, San Francisco, California 94143³

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We have developed an in vitro model to study the influence that human immunodeficiency virus type 1 (HIV-1) may have on the ability of T cells to respond to antigenic challenge. We have examined consequences of HIV-1 gene expression on T-cell activation in antigen-dependent T cells that have stably integrated copies of replication-defective proviral HIV-1. Virus production by HIV-infected, antigen-dependent T cells was induced in response to antigenic stimulation and then decreased as infected cells returned to a state of quiescence. Contrary to the predictions of models proposing that Nef alters signal transduction pathways in T lymphocytes and thereby alters cellular activation, Nef expression in antigen-dependent T-cell clones did not influence their proliferative responses to low or intermediate concentrations of antigen and did not affect other measures of T-cell activation, such as induction of interleukin 2 receptor alpha-chain expression and cytokine production. In addition, we found no evidence for alteration of T-cell responsiveness to antigen by the *gag*, *pol*, *vif*, *tat*, or *rev* gene of HIV-1.

Certain human immunodeficiency virus (HIV) gene products have been proposed to modulate T-cell activation responses. In particular, Nef protein has variously been reported to increase or decrease T-cell responsiveness to signals emanating from the T-cell antigen receptor (TCR) (4, 9, 12, 14, 24, 33, 40, 41, 48, 55, 56). Nef's ability to trigger endocytosis and lysosomal degradation of cell surface CD4 places it close to an essential cofactor involved in T-cell activation (1, 3, 22). In addition, Nef has recently been found to associate with certain cellular serine/threonine and tyrosine kinases (32, 42, 50–52). These intriguing observations have fostered suggestions that Nef may facilitate the process of T-cell activation, perhaps as a means of augmenting the size of the pool of fully activated target cells competent to replicate HIV or to increase the likelihood that a latently infected T cell could be induced to produce virus following exposure to limiting concentrations of antigens or inductive lymphokines.

Earlier studies, using Jurkat T cells, indicated that Nef may inhibit T-cell activation by interfering with antigen receptor-mediated signaling or downstream responses, including NF- κ B activation and interleukin 2 (IL-2) production (33, 40, 41). Addition of recombinant Nef protein to peripheral blood mononuclear cell (PBMC) cultures has been reported to decrease T-cell surface IL-2 receptor alpha-chain expression and reduce proliferative responses to IL-2 (24). On the other hand, a variety of circumstantial evidence has accumulated to suggest that Nef may augment T-cell proliferative responses so as to facilitate virus replication. First, Nef is necessary for the establishment of high virus loads and disease induction in both simian immunodeficiency virus-infected macaques and HIV type 1 (HIV-1)-infected humans (13, 29, 30). Second, the abil-

ity of Nef to enhance HIV-1 and simian immunodeficiency virus replication in tissue culture infections is most evident upon infection of resting CD4⁺ T lymphocytes (38, 57). Third, Nef has been shown to interact with kinases similar to those known to operate in cellular signal transduction cascades (32, 42, 50–52).

We developed a tissue culture model system that allows evaluation of the effects that viral proteins have on the ability of primary T cells to respond to antigenic challenge. We examined consequences of T-cell activation in antigen-dependent T cells that harbor stably integrated copies of replication-defective proviral HIV-1. Following pseudotyping with the amphotropic murine leukemia virus envelope (Env) glycoprotein, replication-defective HIV-1 proviruses were introduced to an influenza virus hemagglutinin (HA)-specific human T-cell clone. These modified proviruses do not express cytopathic Env or cytostatic Vpr HIV proteins and thus permit quantitative evaluation of potential effects on T-cell responses to specific antigenic stimulation exerted by other HIV gene products. When the HIV⁺ T cells were stimulated with HA antigen on antigen-presenting cells, they showed a brisk increase in their level of HIV production that coincided with the initiation of cellular proliferation. After about a week, the cells produced progressively fewer HIV particles, decreased expression of markers of T-cell activation, including surface IL-2 receptor alpha chain, and returned to a resting state.

Using this cell culture system, we focused our inquiry on the role that HIV-1 Nef plays in altering CD4⁺ T-cell activation but also considered potential influences of the viral Gag, Pol, Vif, Tat, and Rev proteins. Our results do not support previous suggestions that Nef acts to either increase or decrease T-cell sensitivity to activation via the TCR. We found no evidence to support prior claims that other viral gene products, such as Tat, significantly alter T-cell proliferative responses or the pattern of cytokine production. When full-length *nef* (R7/3 allele) was expressed at stable, high levels in antigen-dependent T-cell clones, it did not alter their ability to be stimulated by low and intermediate concentrations of specific antigen presented on B

* Corresponding author. Present address: Department of Biology, Southern Oregon State College, 1250 Siskiyou Blvd., Ashland, OR 97520-5071. Phone: (541) 552-6788. Fax: (541) 552-6415. E-mail: page@wpo.sosc.osshe.edu.

† Present address: Office of AIDS Research, Office of the Director, National Institutes of Health, Bethesda, MD 20892.

cells. Further, in contrast to earlier suggestions, *tat* expression in antigen-specific T-cell clones at levels sufficient to support active HIV-1 production did not appreciably alter T-cell proliferative responses to antigen, the pattern of cytokines produced from infected cells, or the propensity of lymphocytes to die upon activation. We found no evidence that expression of the *gag*, *pol*, *vif*, *tat*, or *rev* gene of HIV-1 resulted in alteration of T-cell responsiveness to antigen or the pattern of cytokines produced from infected cells. Thus, although regulatory and accessory gene products of HIV-1 serve critical roles in the virus life cycle (60), they do not alter the ability of antigen-specific CD4⁺ T cells to become activated and proliferate upon exposure to antigenic stimulation.

Earlier concepts of the natural history of HIV-1 infection were predicated on the belief that very few cells that expressed HIV-1 were present in an infected individual during the clinically latent period of the disease. It was postulated that persistence of HIV-1 infection was maintained by a reservoir of latently infected cells that might escape immune clearance but that could be induced to produce infectious virus following T-cell activation. Imagining that very few cells were infected during much of the course of HIV-1 infection, numerous indirect mechanisms were advanced to explain the inexorable process of T-cell depletion characteristic of HIV disease. Within this paradigm, HIV accessory gene products were often speculated to be agents acting to establish, maintain, or allow resurgence from viral latency. However, recent studies of the magnitude and kinetics of HIV-1 replication in vivo have dramatically demonstrated that viral replication is active throughout the entire clinical course of HIV disease (27, 63). These studies have further demonstrated that HIV infection is sustained by continuous rounds of de novo infection of CD4⁺ T cells which, once infected, manifest an average life span of less than 2 days (27, 63, 45). These data suggest that T-cell depletion is a direct consequence of HIV infection of susceptible target cells. Virus infection is concentrated in T-cell populations that are proliferating in response to homeostatic mechanisms that replenish lost CD4⁺ T cells or in T cells activated by encounter with their specific antigens (58).

Recognition that the majority of virus expressed in an infected person at any time is produced from recently infected cells has prompted a reevaluation of the importance of chronically or latently infected cell populations in the persistence of HIV-1 infection. It is known that significantly more T cells in both peripheral blood and lymph nodes contain HIV-1 DNA sequences than actively synthesize detectable quantities of viral RNA (5, 7, 16, 34, 54, 59, 67). Although these cells that contain HIV proviral DNA but lack HIV RNA could represent latently infected cells, many have been found to harbor replication-defective proviruses (19). However, chronically or latently infected cells need not be numerous to contribute to the propagation of HIV-1 infection, as they might provide reservoirs of virus infection able to escape elimination by the immune response or antiviral therapies (16). Precise delineation of the relative proportions of populations of HIV-1-infected cells that are actively replicating virus, are latently infected, or contain replication-defective proviruses will be necessary to illuminate the process by which HIV-1 establishes and maintains persistent infection.

MATERIALS AND METHODS

Establishment and culture of HA-specific T cells. T-cell clones specific for the HA antigen of influenza virus were derived from an individual with the HLA class II phenotype DR4Dw4 DR7 DRw53 DQw8 DQw9. The method used for generation of CD4⁺, alpha-beta TCR-positive T cells has been previously described (25). In short, heparinized PBMC from an individual who had recently

received an influenza virus immunization booster were isolated by density gradient centrifugation on Ficoll-Isopaque (Pharmacia). The cells were then stimulated with a 1/100 dilution of influenza virus vaccine (Parke-Davis, Morris Plains, N.Y.) in RPMI medium (GIBCO) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 5% pooled human AB serum (Whittaker, Frederick, Md.). After incubation at 37°C in 5% CO₂ for 5 days, T-cell blasts were cloned by limiting dilution at a concentration of 1 cell per three wells in the presence of a feeder mixture consisting of 10⁶ allogeneic PBMC per ml (30 Gy irradiated), 10⁵ autologous Epstein-Barr virus (EBV)-transformed B cells (50 Gy irradiated), a 1/100 dilution of influenza virus vaccine, and 1 µg of Leucoagglutinin A (Pharmacia) per ml. Cultures with evidence of active cellular proliferation were transferred to 24-well tissue culture plates and restimulated with the same feeder mixture. Three days later, 10% natural IL-2 (Lymphocult T, Biotest; Serum Institute, Frankfurt, Germany) was added, and the cells were grown for an additional week before freezing in culture media containing 10% dimethyl sulfoxide. The clonality of T-cell line 103.13 was confirmed by Southern blot analysis of *Hind*III- and *Eco*RI-digested DNA with a TCR Cβ-specific probe (61). Specificity was determined with a peptide corresponding to amino acid residues 103 to 115 of the influenza virus HA protein (HA peptide 103-115) and autologous irradiated PBMC as antigen-presenting cells. The amino acid sequence of HA peptide 103-115 is PDYASLRSLVASS. The HA peptide was also effectively presented by CHO cells transfected with the HLA DRw4 alpha and beta chains (6), which confirmed the HLA restriction that had been established previously with a small panel of HLA-typed PBMC and EBV-transformed B cells.

Routine expansion of 103.13 T cells involved activation of the cells with HA peptide 103-115 presented on the surface of HLA DRw4 EBV-transformed B cells. B cells were cultured in RPMI medium supplemented with antibiotics and 10% fetal bovine serum (HyClone). Inactivation was carried out by treatment of the cells at a density of 2×10^7 per ml with 50 µg of mitomycin (Sigma) per ml in RPMI medium for 45 min at 37°C. The B cells were then washed four times by pelleting at $500 \times g$ for 5 min at 4°C and resuspension in ice-cold RPMI medium. After the last wash, the cells were resuspended at a concentration of 2×10^6 per ml in RPMI medium supplemented with 6% pooled human AB serum (Gemini BioProducts, Calabasas, Calif.), 10 mM HEPES (GIBCO), and 20 µM HA peptide 103-115 (synthesized by Biomolecular Resource Center, University of California, San Francisco). HA-specific T cells were pelleted at $500 \times g$ for 5 min at 4°C and resuspended in RPMI medium supplemented with 6% pooled human AB serum, antibiotics, and 10 mM HEPES (designated as T-cell growth medium) at a concentration of 10⁶ cells per ml. Equal volumes of T cells and inactivated antigen-presenting B cells were then mixed such that the T/B cell ratio was 1:2, and the cells were cultured at 37°C in 5% CO₂. Following activation, cells were maintained at a concentration of 0.5×10^6 to 2.0×10^6 per ml. The mitomycin-treated B cells disappeared from the cultures within 3 days as assessed by flow cytometric analysis for CD23⁺ cells. In order to maximize cell yields, 10 U of IL-2 (human, recombinant; GIBCO) per ml was added at either 3 or 4 days after antigenic stimulation. This stimulation protocol generally resulted in a 5- to 15-fold expansion of T-cell numbers over a period of approximately 12 days. T cells continued to divide after the first 12 days following antigenic stimulation, albeit more slowly, in the presence of exogenously added IL-2.

Plasmids. The proviral constructs diagramed in Fig. 1 were derived from plasmid R7/3 (18). To generate plasmid HIV MC1 nef+, a 1,218-bp deletion was made in *env* from the *Nde*I site at HIV nucleotide 6402 to the *Bgl*II site at HIV nucleotide 7620 by restriction endonuclease digestion, followed by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase I. Inserted into this site was a blunted 1.1-kb *Xho*I-to-*Sal*I fragment from a version of the plasmid pMC1neo (Stratagene) that included a hybrid polyomavirus enhancer-herpes simplex virus thymidine kinase promoter and the coding sequences for the *neo* gene but that lacked a polyadenylation signal. The *nef* gene of HIV MC1 nef+ was exchanged with the nonfunctional *nef* gene of HXB Δstart (38) by transfer of the *Bam*HI-to-*Xba*I fragment of HXB Δstart to *Bam*HI- and *Xba*I-cut HIV MC1 nef+, creating HIV MC1 nef-. To generate HIV A nef+, a blunted 0.9-kb *Mlu*I-to-*Hinc*II fragment from the plasmid pMC1neo that includes only the coding sequences of the *neo* gene was inserted into the *env* gene of R7/3, previously deleted from the *Nde*I site at HIV nucleotide 6402 to the *Bgl*II site at 7620. HIV A nef- was generated from plasmid HIV A nef+ by exchange with the nonfunctional *nef* gene of HXB Δstart in the same manner as for HIV MC1 nef-. The *neo* gene of HIV A nef+/- was expressed from the HIV long terminal repeat (LTR), presumably from a singly spliced mRNA equivalent to the *env* mRNA. The HIV provirus R7 *Bgl*II del was prepared by deletion of *env* sequences from the *Bgl*II site at HIV nucleotide 7040 to the *Bgl*II site at HIV nucleotide 7620 and deletion of the *nef* gene from the start codon of *nef* at HIV nucleotide 8796 to the *Bgl*II site at HIV nucleotide 9050. *neo* gene coding sequences were inserted at the site of *nef* deletion in plasmid R7 *Bgl*II del, and as such, this provirus also relies on *neo* expression from the HIV LTR, in this case from a transcript equivalent to the multiply spliced *nef* transcript. To prepare the plasmid R7ΔB nef+, the 1.1-kb *Xho*I-to-*Sal*I fragment from plasmid pMC1neo was blunted, ligated to *Bgl*II linkers, and then inserted into plasmid R7/3 at the site of a *Bgl*II fragment deletion from HIV nucleotides 7040 to 7620. R7ΔB nef- was generated from R7ΔB nef+ by transfer of the *Bam*HI-to-*Xba*I fragment of HXB Δstart to *Bam*HI- and *Xba*I-cut R7ΔB nef+. The amphotropic

FIG. 1. HIV proviral constructs.

murine leukemia virus envelope expression plasmid pSV-A-MLVenV has previously been described (44).

Transfections for virus production and generation of stable T-cell lines. Amphotropic-envelope-pseudotyped virus was prepared by transfection of plasmid DNA by the calcium phosphate procedure, with the following modifications. Fifteen micrograms each of an HIV proviral construct and pSV-A-MLVenV per T75 tissue culture flask of COS-7 or 293T cells was used, and 25 μ M chloroquine was added to enhance transfection efficiency. One day after transfection, the culture medium was replaced with fresh Dulbecco modified Eagle medium (GIBCO) containing 10% fetal bovine serum and antibiotics. At approximately 48 h posttransfection, the medium was replaced again with T-cell growth medium. Between 65 and 72 h posttransfection, the virus-containing culture supernatants were harvested, filtered through a 0.45- μ m-pore-size membrane, and stored at -70°C . Virus supernatants were assessed for p24 content by an HIV-1 p24 antigen-capture enzyme-linked immunosorbent assay (ELISA) (Coulter), and they were assessed for infectivity on human osteosarcoma cells (HOS; American Type Culture Collection) by enumeration of G418-resistant HOS colonies 12 days postinfection. The T-cell clone 103.13 was infected with amphotropic-envelope-pseudotyped HIV by addition of virus supernatants to the

cells 3 or 4 days after antigenic stimulation. The next day, the cells were pelleted and resuspended in fresh T-cell growth medium containing 1 mg of G418 (Geneticin; GIBCO) per ml and 10 U of IL-2 per ml. T-cell cultures were maintained in G418 selection for 10 to 12 days before antigenic restimulation. Three days after antigenic restimulation, G418 selection was reinstated and maintained for the duration of the culture. Healthy cultures of G418-resistant T cells were obtained after two to four cycles of stimulation.

Flow cytometry. The following antibodies were obtained (Becton Dickinson): leu20 (anti-CD23) for detection of B cells, leu3a phycoerythrin conjugate (anti-CD4-PE), leu4 fluorescein conjugate (anti-CD3-FITC), anti-IL-2R fluorescein conjugate (anti-CD25-FITC), and goat anti-mouse immunoglobulin fluorescein conjugate (GAM-FITC). Negative control antibodies (Becton Dickinson) were monoclonal mouse immunoglobulin G1 antibodies against irrelevant antigens that were either unconjugated or phycoerythrin or fluorescein conjugates. Cells were incubated with antibodies according to the manufacturer's specifications and analyzed on a Becton Dickinson FACScan flow cytometer. Dead cells were gated out of analysis by staining with 0.02 μ g of propidium iodide per ml.

Proliferation assays. T-cell proliferation in response to HA peptide was measured by [^3H]thymidine uptake into cellular DNA. B cells were inactivated with

mitomycin and washed as described above. Inactivated B cells were resuspended at a concentration of 10^6 per ml in RPMI medium containing 10% fetal bovine serum, antibiotics, and HA peptide at various concentrations from 0 to 10 μ M for 3 hours at 37°C in 5% CO₂. The cells were then pelleted at $500 \times g$ for 5 min and resuspended in fresh T-cell growth medium lacking G418. T cells stimulated with antigen 10 to 14 days previously were pelleted at $500 \times g$ for 5 min and resuspended in fresh T-cell growth medium. T cells (2×10^4) were added to the wells of U-bottom 96-well plates, followed by the addition of 4×10^4 B cells. The volume of culture medium was 150 μ l per well. Control wells included no cells, B cells only, or T cells only. Plates were organized such that duplicate or triplicate determinations were made at each dose of antigen. Cell mixtures were incubated undisturbed for 2 days, at which time [³H]thymidine (NEN) was added at a final concentration of 0.04 mCi/ml. The cultures were then returned to the incubator for 18 to 24 h of culture. An automated 96-well plate harvester was used to harvest cells and isolate cellular DNA containing incorporated [³H]thymidine onto filters. [³H]thymidine incorporation was measured after solubilizing radioactivity with Biofluor (New England Nuclear) and analysis with a Beckman LS6000 scintillation counter. Purified, sodium azide-free monoclonal antibody leu3a (Becton Dickinson) or YTA-312 (Herman Waldmann, Oxford, United Kingdom) was added to HA-specific T cells at a concentration of 4 μ g/ml during T-cell proliferation assays in order to verify CD4 dependence of the proliferative response to antigen.

Western immunoblot analysis. T cells (2×10^6) stimulated 9 days previously with antigen were pelleted at $500 \times g$ for 5 min at 4°C. The cells were resuspended in 5 ml of ice-cold phosphate-buffered saline (PBS) and pelleted again at $500 \times g$ for 5 min at 4°C. The cells were resuspended in 100 μ l of ice-cold lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40) and incubated on ice for 5 min before clarification of the extract by centrifugation at $12,000 \times g$ for 5 min at 4°C. The lysate supernatant was stored at -20°C. A chronically infected T-cell line was prepared by infection of H9 cells with the HIV-1 molecular clone R7/3 (18, 38) (also referred to as HIV *nef*⁺). After the initial die-off of cells due to viral cytopathic effect, a chronically infected cell line that remained viable and produced 326 ng of p24 per ml was maintained. A cellular lysate of these cells was made at 22 days after HIV infection. Samples were denatured in sample loading buffer, electrophoresed on a sodium dodecyl sulfate-12% polyacrylamide gel, and electrophoretically transferred to a nitrocellulose membrane (Hybond-ECL; Amersham). The blot was blocked overnight at 4°C in PBS containing 5% nonfat milk and 0.5% Tween 20. The blot was then incubated with a rabbit anti-Nef antiserum (reference no. 2121; National Institutes of Health AIDS Research and Reference Reagent Program) diluted 1:2,000 in PBS containing 2% bovine serum albumin, 0.1% Tween 20, and 0.02% thimerosal for 1.5 h at room temperature. After being extensively washed in PBS containing 0.5% Tween 20, the blot was incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibody (Amersham) diluted 1:2,000 in PBS containing 5% nonfat milk and 0.5% Tween 20. The blot was again washed extensively in PBS containing 0.5% Tween 20, and enhanced chemiluminescence detection reagents were used to visualize Nef protein (Amersham).

RESULTS

Isolation of an HA-dependent T-cell clone and derivation of stable HIV⁺ T-cell lines. To study the influence of HIV on T-cell activation, we sought to develop an in vitro system that would accurately reflect the physiology of infected T cells activated by antigenic stimulation in vivo. We isolated and cloned CD4⁺, influenza virus-specific T cells from the PBMC of an HIV-seronegative individual who had recently received an influenza virus vaccination booster. These primary human cells are dependent on periodic stimulation with a peptide antigen from the HA protein of influenza virus to proliferate. One clone, 103.13, was chosen as the parental cell line for generation of HIV⁺ lines. 103.13 cells are able to undergo repeated rounds of antigenic stimulation leading to cellular proliferation and, as such, behave as functional memory cells. Antigenic challenge of 103.13 cells leads to transient cellular activation events, including TCR down-modulation, IL-2 receptor induction, induction of cytokine secretion, and cellular proliferation. The use of cloned human T cells circumvents many problems associated with experiments in bulk cultures of primary T cells. The cells are homogeneous with respect to their TCR and so can be reliably and reproducibly activated with a peptide antigen. Mitogens are not required for propagation of the cells. Cells can be synchronously activated by antigen and then brought back to a resting state by withdrawal of exogenous IL-2. We introduced noncytopathic, replication-defective HIVs

TABLE 1. Nomenclature of cells and viruses

Cell line	Provirus
Parent	None
A <i>nef</i> ⁺	HIV A <i>nef</i> ⁺
A <i>nef</i> ⁻	HIV A <i>nef</i> ⁻
M <i>nef</i> ⁺ , M <i>nef</i> ⁺ 2	HIV MC1 <i>nef</i> ⁺
M <i>nef</i> ⁻	HIV MC1 <i>nef</i> ⁻
TATA del	HIV MC1 <i>nef</i> ⁺ TATA del
R7 BglII del	R7 BglII del, <i>nef</i> ⁻

to T-cell clone 103.13. In doing so, we generated a model for HIV-infected T cells in which virus expression is induced by antigenic stimulation and diminishes as cells return to a state of quiescence.

Proviruses that were introduced to 103.13 cells are shown in Fig. 1. These proviruses were constructed with sequences from the HIV molecular clone R7/3, which was derived from the HIV-1 LAI isolate (18, 38). The *vpr* and *vpu* genes in these constructs were defective. The absence of a functional *vpr* gene was an important aspect of our experimental design, because *vpr* has been shown to cause a block to cell cycle progression in T cells (39, 49). To avoid cytopathic consequences of HIV Env (gp120/gp41) expression, the *env* gene was inactivated by a deletion of either 580 bp (R7 BglII del and R7ΔB *nef*⁺/-) or 1,218 bp (HIV A *nef*⁺/- and HIV MC1 *nef*⁺/-). *nef* was either left intact or exchanged for a *nef* double mutant containing a start codon deletion and premature termination codon midway through the coding sequence. The *nef* gene of R7/3 has previously been shown to be functional in assays for CD4 down-modulation and *nef*-mediated viral infectivity enhancement (23, 36, 38). A *neo* gene was inserted into the proviral sequences so that infected T cells could be selected for by G418 resistance. We prepared and tested the different constructs shown in Fig. 1 to compare levels of HIV production that would result from stable integration of these proviruses. We also prepared a construct, HIV MC1 *nef*-TATA del, that lacks a functional TATA box in the viral U3 region and is hence unable to initiate transcription from the HIV promoter resident in the LTR. This construct expresses *neo* from the MC1 promoter-enhancer sequences and was used to control for any alterations that might occur in T cells that have undergone infection and G418 selection. In order to generate virus infectious for only a single round of replication, we complemented the *env*-HIV proviral constructs with the amphotropic murine leukemia virus *env* during transfection of 293T cells. Pseudotyped, replication-defective virus was produced by the 293T cells in titers ranging from 8×10^3 to 3×10^4 infectious units per ml. We used this virus to infect the T-cell clone 103.13. Two days after infection, cells were cultured in the antibiotic G418 to select for infected cells. We obtained several HA-dependent T-cell lines that had stably integrated copies of HIV proviruses (Table 1). These lines were maintained as bulk cultures of G418-selected cells and therefore represented populations of cells with many distinct integration sites. The infectious titer of the pseudotyped virus used to prepare the cell lines was high enough to generate significant numbers of G418-resistant cells within two to four rounds of cellular stimulation with HA peptide antigen. HIV *nef*⁺ and HIV *nef*⁻ cell lines were obtained within similar time frames. There was no apparent deleterious or enhancing effect of *nef* on our ability to obtain stable, G418-resistant cell lines.

Effect of T-cell activation on HIV gene expression. HIV gene expression has been shown to be activated in vitro by stimuli that lead to activation of T cells. Induction of HIV gene ex-

FIG. 2. Induction of virus production by antigenic stimulation. T cells were activated by peptide antigen on day 0, and levels of HIV particles released into the culture supernatant were then monitored with a quantitative p24 antigen capture ELISA (Coulter). The cells were maintained at a density of 0.5×10^6 to 1.0×10^6 per ml, and the media were changed at least every 2 days.

pression and virus production in response to antigen, a physiologically relevant stimulus, has not been reported previously. Virus production was measured in M nef⁺ and M nef⁻ cells by quantitation of HIV p24 core antigen released into the culture supernatant. Augmentation of virus production by antigenic stimulation was reproducibly observed in all of the HIV⁺ T-cell lines tested. Virus production was increased about fivefold within 3 days of activation and then decreased over a period of about a week (Fig. 2). Addition of IL-2 to cultures of antigen-dependent T cells allowed continued cellular proliferation for several weeks in the absence of antigenic stimulation but did not result in further increases in HIV production. Withdrawal of IL-2 in the absence of antigenic restimulation resulted in cessation of cellular proliferation and, eventually, cell death.

Cell lines M nef⁺ and M nef⁻ produced relatively low levels of virus both constitutively and upon cellular activation (Fig. 2A and B). Cell lines A nef⁺ and A nef⁻ also produced relatively low levels of HIV particles (data not shown). When 103.13 T cells were infected with provirus R7 BglII del and selected for G418 resistance, a cell line that expressed more than 100-fold-greater levels of p24 and whose HIV production was also increased after exposure to antigen was obtained (Fig. 2C). R7 BglII del provirus has a 580-bp deletion in the *env* gene, whereas HIV MC1 nef and HIV A nef proviruses have a 1,218-bp deletion. Northern blot analysis of cells transduced with these proviral constructs showed that relative to T cells with integrated R7 BglII del, T cells harboring HIV MC1 nef⁺ and HIV A nef⁺ expressed reduced levels of genomic and singly spliced viral RNAs (data not shown). We speculate that the additional *env* sequences present in R7 BglII del are necessary for stability of *env*-containing mRNAs and thereby allow for efficient expression of unspliced *gag-pol-vif* transcripts and singly spliced *env* transcripts. However, expres-

sion of fully spliced *tat*, *rev*, and *nef* transcripts may not be appreciably altered.

Evaluation of *nef* expression. It is apparent that HIV A nef⁺ and HIV MC1 nef⁺ proviruses express relatively low levels of *gag*, *pol*, and *vif* gene products, as well as having deletions in *env*, *vpr*, and *vpu*. To verify that Nef was both intact and expressed at physiologically normal levels in the cell lines A nef⁺, M nef⁺, and M nef⁺2, the level of Nef protein was measured by immunoblot analysis. It is difficult to predict the level at which Nef would be expressed in vivo in acutely or chronically infected T cells. In an attempt to approximate what that level might be, we analyzed the level of Nef expression in H9 cells chronically infected with HIV-1 R7/3 and producing high levels of virus. Cellular extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with an anti-Nef antiserum. Results shown in Fig. 3 indicate that cell lines A nef⁺, M nef⁺, and M nef⁺2 all express Nef to a similar degree. The level of Nef expression in these cells is less than that observed in chronically infected H9 cells, but not dramatically so. Whereas others have observed truncations in the *nef* gene product after culture of *nef*-transfected immortalized T cells (4), we observed stable expression of the full-length gene product. Nef expressed in HA-specific T cells also retained functional activity, as assessed by the down-modulation of CD4 from the surface of the *nef*⁺ cells (see below).

Effect of HIV-1 gene expression on surface markers involved in T-cell activation. T-cell clone 103.13 and five HIV⁺ cell lines derived from it were analyzed for surface expression of the TCR CD3, the alpha chain of the IL-2 receptor, and the signaling accessory protein CD4. None of the *nef*⁻ cell lines differed from the parent cell line in their levels of these T-cell surface proteins. Although the HIV Tat protein has been reported to inhibit the expression of the major histocompatibility

FIG. 3. Western blot for Nef expression. Cell extracts of the indicated cell lines were prepared and analyzed by immunoblotting with a rabbit anti-Nef antiserum as described in Materials and Methods. Extracts equivalent to 2×10^5 cells were loaded per lane, except for lane H9-R7/3, which contains the equivalent of 1×10^5 chronically infected H9 cells. Molecular mass markers (in kilodaltons) are indicated.

complex class I promoter in transient transfection studies (28), all HIV⁺ T-cell lines expressed the same level of cell surface major histocompatibility complex class I antigen as the parental T-cell clone, despite their production of active Tat protein (data not shown). The three *nef*⁺ cell lines demonstrated a three- to fivefold-reduced level of CD4 surface expression (Fig. 4A). This level of CD4 down-modulation is similar to that observed in Nef-transgenic mice and some *nef*-transfected immortalized T-cell lines (3, 56). Surface levels of CD3 and the IL-2 receptor alpha chain were similar on all cell lines. It has been proposed that Nef inhibits expression of activation-induced IL-2 receptor alpha chain (21). To investigate this possibility, we measured the surface expression of the IL-2 receptor alpha chain before and after stimulation of cells with antigen (Fig. 4B). All of the cell lines induced IL-2 receptor alpha chain to a similar degree. We also measured the down-modulation and reappearance of the TCR following antigenic stimulation in the parent, A *nef*⁺, and M *nef*⁺ cell lines. Cells were stained with a fluorescently labeled anti-CD3 antibody at various times after stimulation with antigen, and their mean cellular fluorescence was measured by flow cytometry (Fig. 5). *nef*⁺ cells responded normally to antigen with respect to modulation of the TCR. These results indicate that signaling pathways in T-cell clone 103.13 are not fundamentally altered by expression of HIV-1 *gag*, *pol*, *vif*, *tat*, *rev*, and *nef* genes.

Cytokine secretion by *nef*⁺ and *nef*⁻ T cells. Induction of secretion of specific cytokines contributes to functional properties of activated helper T cells. Interestingly, the HIV-1 LTR is responsive to activation by several cytokines produced by helper T cells, including granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF- α), and IL-6 (15, 17, 20, 21, 26, 43, 46). HIV-1 gene expression may be augmented by cytokine activation of viral gene expression as well as by direct induction of viral transcription by factors such as NF- κ B that are activated during T-cell signaling responses to antigen. To determine if introduction of HIV-1 genes to T cells might directly alter their cytokine production profile, we measured release of various cytokines before and after antigenic stimulation. Parental 103.13, M *nef*⁺, and TATA del cell lines all produced similar levels of TNF- α , GM-CSF, gamma interferon (IFN- γ), and IL-6 before and 2

days after antigenic challenge (Fig. 6). This pattern of induction of cytokines by antigenic stimulation characterizes the 103.13 T-cell clone as a Th₀ type of helper T cell. Although M *nef*⁺ cells contain a *vpr*⁻ *vpu*⁻ *env*⁻ HIV-1 provirus that produces replication-defective viral particles, their pattern of cytokine production in response to antigenic challenge was not appreciably different than those of the parental T-cell clone 103.13 or the control cell line TATA del. We also compared the induction of cytokine secretion in response to antigenic challenge by the cell line harboring the *nef*⁻ R7 BglII del provirus, which produces high levels of HIV upon antigenic stimulation, and the non-virus-producing, G418-resistant control cell line TATA del (Table 2). We noted only a small relative increase (approximately twofold) in the induction of TNF- α after antigen activation in the cell line R7 BglII del compared with that of the control; no significant differences in the induction of the other cytokines tested were found. Increased secretion of TNF- α as a result of HIV infection has previously been reported by others (8, 35) and might, if of sufficient magnitude, contribute to increased HIV production from infected cells. In contrast to previous reports, production of quantities of Tat protein by resident proviruses sufficient to support high-level virus production has no significant effect on T-cell production of IL-2 or IL-6 and, at most, only a small effect on TNF- α production. Thus, we have no evidence for significant direct alterations of cytokine secretion by introduction of HIV-1 proviral sequences to T cells.

Proliferative responses to antigen by *nef*⁺ and *nef*⁻ T cells.

To assess whether Nef expression either increases or decreases the sensitivity of antigen-specific T cells to antigenic stimulation, we quantitated the proliferation of several cell lines in response to various concentrations of their cognate antigen. If Nef acts to enhance sensitivity of T cells to activation by antigen, then a shift in the antigen dose-response curve would be expected to demonstrate increased proliferation at lower antigen concentrations. The proliferative response of cells to various concentrations of antigen presented on autologous B cells was measured by [³H]thymidine incorporation into DNA at 48 to 72 h poststimulation (Fig. 7). Cellular proliferation is a late event in T-cell activation and the result of several biochemical events that act collectively to propel cells through the cell cycle. The dependence of 103.13 cells on antigenic stimulation is demonstrated by the lack of proliferation when no antigen is present in the culture and by the graded response to increasing concentrations of antigen. The threshold level of antigen required to elicit a proliferative response was similar for all cell lines tested. As shown in Fig. 7A and B, the M *nef*⁺ and A *nef*⁺ cell lines gave a slightly lower proliferative response than the parental cells when tested at the highest antigen doses. We speculate that this may be due to a limiting amount of CD4 present on the surface of *nef*⁺ cell lines. The CD4 dependence of the proliferative response is shown in Fig. 7C. As with most CD4⁺ T-cell clones, 103.13 cells require engagement of CD4 as a costimulatory surface receptor during antigen-induced activation. We were able to completely block the proliferation of both the parent and M *nef*⁺ cell lines with the anti-CD4 antibody leu3a. In this experiment, no inhibitory effect of Nef on T-cell proliferation was observed at any antigen concentration. As a control, we also tested the effect of the anti-mouse CD4 antibody YTA-312 on the ability of parent and M *nef*⁺ cells to respond to antigen. This irrelevant antibody had no effect on proliferation of the cells (data not shown). These experiments indicate that reduction of surface CD4 in cell lines M *nef*⁺ and A *nef*⁺ is not sufficient to significantly alter their antigen-dependent responses, except at the highest antigen doses in some experiments.

FIG. 4. Cell surface expression of CD4 and the IL-2 receptor alpha chain. (A) Cells were stained with the anti-CD4 antibody leu3a conjugated to phycoerythrin (solid lines) or a negative control antibody conjugated to phycoerythrin (dashed lines) 10 days after antigenic stimulation. (B) Cells were stained with the anti-IL-2 receptor antibody anti-CD25 conjugated to fluorescein (solid lines) or to a negative control antibody conjugated to fluorescein (dashed lines) preactivation (10 days since prior stimulation) and 4 days postactivation.

The influence of high levels of virus production on the ability of T cells to respond to antigen was assessed in an antigen dose-response assay with parental 103.13 cells and *nef*⁻ R7 BglII del cells. As shown in Fig. 7D, parent and R7 BglII del cells proliferated similarly in response to antigen. Over time, however, the level of virus expression from cell line R7 BglII del decreased significantly, suggesting that high levels of virus production may confer a selective disadvantage to cells during repeated rounds of stimulation (see below).

Activation of HIV production by antigenic stimulation of nonselected cells. The methods we used to generate stable, G418-resistant cell lines necessarily favored cells able to maintain proliferative responses to antigen. Cells that may have suffered toxic effects or signaling defects due to the introduction of HIV gene products would rapidly disappear from culture. Any such effects would also be expected to be operative in vivo and influence the potential to generate chronically or latently infected cells. The fact that we obtained *nef*⁺ and *nef*⁻ cell lines with equal efficiency argues against strong toxic or growth inhibitory effects of *nef* in the T cells used in this study. As previously discussed, the cell line R7 BglII del (a *nef*⁻ cell line) expressed very high levels of virus but grew out in G418 selection more slowly than the other HIV⁺ T cells and decreased production of p24 over time. This suggests that high levels of HIV *gag*, *pol*, and *vif* expression confer a selective disadvantage to infected cells. In order to examine the effect that *nef* might have in the context of high virus production, we prepared the constructs R7ΔB *nef*⁺ and R7ΔB *nef*⁻. The R7ΔB constructs have the same 580-bp *env* deletion as the construct R7 BglII del but differ from R7 BglII del in that the *neo* gene was inserted at the site of *env* deletion, leaving the *nef* sequences intact (Fig. 1). We were able to infect and select an adherent cell line, 293, with pseudotyped R7ΔB^{nef} viruses and obtained several G418-resistant clones that produced relatively high levels of HIV particles (2 to 250 ng of p24/ml). Thus, it appears that the sequences in envelope retained in

FIG. 5. TCR down-modulation. Cells were stained for surface expression of TCR with the antibody leu4 conjugated to fluorescein at the times indicated after stimulation with HA peptide antigen. The mean cellular fluorescence at each time point was determined by flow cytometry.

constructs R7 BglIII del and R7ΔB nef+/- but absent in constructs HIV A nef+/- and HIV MC1 nef+/- (HIV nucleotides 6402 to 7040) are indeed necessary for stable, high-level expression of *gag*, *pol*, and *vif*. 103.13 T cells were infected with pseudotyped virus generated with R7ΔB nef+/- and subjected to G418 selection. We were unable to obtain any stable G418-resistant T-cell lines with the same methods that were successful for generating A nef+/-, M nef+/-, and R7 BglIII del cell lines. We speculate that this may be due to inhibitory effects of high levels of HIV *gag*, *pol*, and/or *vif* expression in nontransformed T cells. In the absence of G418 selection, 103.13 T cells did show evidence of infection with R7ΔB nef+/- and were able to respond to antigenic stimulation, as evidenced by enhancement of virus production after antigenic challenge. In the experiment whose results are shown in Fig. 8, T cells infected with R7ΔB nef+ and R7ΔB nef- were stimulated repeatedly with antigen, and virus production was measured by p24 output. This experiment was done in the absence of G418 selection in order to analyze enhancement of HIV production by T-cell activation in the absence of selective pressure for infected cells. Antigenic stimulation of T cells resulted in significant increases in virus production in nef+ and nef- infected cells. In nonselected cells, p24 production progressively decreased with each cycle of activation until, ultimately, no p24 antigen could be detected in the transduced cultures (Fig. 8). These results suggest that the HIV+ cells within the culture were at a selective disadvantage such that only the uninfected

cells eventually grew out. Interestingly, as with the stable cell lines, no significant differences in behavior in response to antigenic stimulation were noted between unselected nef+ and nef- cells.

DISCUSSION

We investigated the influence of Nef on the responses of a primary, HA-specific T-cell clone to antigenic stimulation. We observed a significant decrease in CD4 expression on the surface of HA-specific T cells that expressed nef+ proviruses. nef+ and nef- T cells responded similarly to antigenic stimulation, as measured by down-modulation of the TCR, induction of the alpha chain of the IL-2 receptor, and induction of various cytokines. The magnitude of induction of virion production after antigenic stimulation was equivalent in nef+ and nef- T cells. We observed a slight reduction in the level of antigen-induced T-cell proliferation in nef+ T cells, but this effect was observed only at high concentrations of antigen. Our results indicate that HIV-1 nef does not significantly alter T-cell responses to antigen.

We also measured the influence that expression of other HIV gene products had on several parameters of T-cell activation. We compared T-cell responses to antigen in uninfected cells and in HA-specific T cells that produce Env-deficient viral particles. Expression of HIV-1 *gag*, *pol*, *vif*, *tat*, and *rev* genes did not influence the ability of HA-specific T cells to transiently down-modulate surface TCR, induce IL-2 receptors, produce a variety of cytokines, or proliferate in response to antigen.

Our finding that HIV-1 Nef and Tat do not modify the ability of antigen-specific T-cell clones to become activated and proliferate in response to specific antigen is in contrast to a number of reports in which the impact of specific HIV gene products on T-cell activation was studied either by using immortalized T-cell lines or by treating cultures of PBMC with bacterially produced recombinant viral proteins. In the majority of these previous studies, T-cell activation was accomplished with agents such as phorbol esters and lectins to mimic antigenic stimulation. These approaches are subject to potentially serious artifacts. Accurate delineation of the effects that HIV gene products exert on T-cell signaling may be difficult in cell lines whose ability to respond appropriately to immune stimulation has been fundamentally disregulated by leukemic transformation. Experimental approaches that use addition of recombinant Nef or Tat to T cells also have important limitations. In these experiments, recombinant viral proteins are often added at much higher concentrations than are likely to be found in vivo, and inadvertent contaminants such as endotoxins may alter normal T-cell proliferative responses. Finally, the accuracy and specificity with which phorbol esters and lectins recapitulate the signals transmitted via the TCR upon encounter with an actual antigen are not known.

Although experimental observations that suggest a role for

TABLE 2. Fold induction upon antigenic stimulation

Cytokine	Fold induction for cell line:	
	TATA del	R7 BglIII del
GM-CSF	11.5	11.0
IFN-γ	4.0	3.6
IL-6	8.0	8.0
IL-4	30	26
TNF-α	7.5	17

FIG. 6. Cytokine production in response to antigenic stimulation. Cytokine accumulation in culture supernatants was measured by ELISA (R & D Systems, Minneapolis, Minn.) before and 2 days after stimulation with the HA peptide antigen. Solid bars, parent; dashed bars, TATA del; open bars, M nef+.

Nef in modulating T-cell sensitivity to activation are intriguing, resolution of this issue has been problematic. Nef-transgenic mice have significantly decreased levels of CD4⁺ T cells in the periphery and, in some cases, within the thymus as well (56). The observed decrease in mature CD4⁺ T-cell numbers was attributed to aberrant thymocyte differentiation resulting from Nef-induced heightened sensitivity to T-cell activation (56). In a tissue culture model, expression of a chimeric CD8-Nef fusion protein in Jurkat T cells induced two opposite phenotypes, depending on the intracellular location of the hybrid molecule (4). In cells where the chimera accumulated in the cytoplasm, inhibition of early events of TCR-mediated signaling was seen.

In contrast, cells selected for cell surface expression of CD8-Nef appeared to undergo spontaneous activation and apoptosis. It is not known how faithfully the chimeric CD8-Nef protein mimics the function(s) of native Nef protein, and the demonstrated toxicity of the CD8-Nef chimera complicates interpretation of these results (4).

HIV *nef* and SIV *nef* have been reported to associate with a serine/threonine kinase capable of phosphorylating Nef in vitro (32, 42, 51). Nef also contains proline-rich motifs (PxxP) that mediate binding to the *src* tyrosine kinase family members Hck and Lyn (50). Interaction of Nef with specific cellular kinases has led to suggestions that Nef may alter T-cell signal-

FIG. 7. Proliferative responses of T cells to HA peptide antigen. T cells were stimulated to proliferate by various doses of HA peptide antigen presented by autologous EBV-transformed B cells. Proliferation was quantitated by measurement of [3 H]thymidine uptake as described in Materials and Methods. For panel C, leu3a (4 μ g/ml) was added to the cells as indicated above to demonstrate CD4 dependence of the T-cell proliferative response. Ab, antibody.

ing pathways that promote T-cell activation. However, the relevant targets of these kinases may be viral rather than cellular proteins, as the primary function of Nef may be to enhance virus infectivity rather than T-cell activation (2, 11, 37). Rather than influence the susceptibility of T cells to activation by antigens, Nef's specific association with cellular kinases may permit their recruitment to serve in enhancing virus assembly or infectivity. Indeed, the PxxP motifs have been shown to be required for enhanced growth of *nef*⁺ viruses in PBMC (50).

In addition to its essential role in the HIV-1 life cycle, Tat has been reported to exert a variety of effects on CD4⁺ T lymphocytes (10, 28, 31, 47, 53, 62, 64–66). Tat has been found to induce apoptosis during T-cell activation (31, 66) and to diminish IL-2 receptor alpha-chain expression (47). Other researchers report no effect of Tat on IL-2 receptor alpha-chain expression (11). Some experimenters have observed increases in IL-2, IL-6, and TNF- α due to Tat expression (53, 64, 65), whereas others found no effect of Tat on cytokine production (8, 47). In all, these conflicting reports do not permit synthesis of a coherent picture concerning the effect of Tat on the modulation of T-cell activation and cytokine production. Our experiments demonstrated no effect of the HIV-1 *gag*, *pol*, *vif*, *tat*, or *rev* gene on production of cytokines GM-CSF, IFN- γ , IL-6, and IL-4. TNF- α production induced by antigenic stimulation of the HIV⁺ cell line R7 BgIII del was about twice the amount induced in its parental cell line, but the HIV gene product(s) responsible for this small increase is not known.

In summary, we have developed a useful model system for analysis of the effects of T-cell activation on HIV production and the effects of HIV gene expression on T-cell activation. We have shown that virus production by HIV-infected cells is directly increased by antigenic stimulation. We have also shown that expression of HIV *gag*, *pol*, *vif*, *tat*, *rev*, and *nef* genes does not significantly alter T-cell responses to antigen. High levels of HIV Gag, Pol, and Vif appear to be selected against, indicating a cytotoxic effect of long-term, high-level particle production. Antigen-dependent T cells are experimentally tractable primary cells that will continue to be useful for further analysis of the dynamic interactions between HIV and T cells.

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FIG. 8. Sequential activation of virus production by antigenic stimulation of cells infected with R7 Δ B *nef*^{+/−}. Three days after stimulation of 103.13 cells with HA peptide antigen, cells were infected with R7 Δ B *nef*⁺ or R7 Δ B *nef*[−] pseudotyped with an amphotropic envelope. The next day, the cells were washed extensively to remove inoculating virus and returned to culture. The cells were subject to restimulation with HA peptide antigen on days 12, 25, and 38 (arrows). Cell density was maintained at 0.5×10^6 to 2.0×10^6 cells per ml, and media were changed every 2 to 3 days. The quantity of HIV-1 p24 antigen present in culture supernatants was measured with a p24 ELISA (Coulter).

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