Downregulation of Cell-surface CD4 Expression by Simian Immunodeficiency Virus Nef Prevents Viral Super Infection

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Summary

The *nef* gene product encoded by the mac239 proviral clone of simian immunodeficiency virus (SIV) markedly enhances viral replication and pathogenesis in vivo. We have used this biologically active *nef* isolate to examine the phenotype of Nef in retrovirally transduced human T cells in culture. SIV Nef is shown to dramatically inhibit cell-surface expression of the CD4 glycoprotein without significantly affecting the total steady-state level of cellular CD4. This downregulation of the cell-surface CD4 receptor for human immunodeficiency virus type 1 (HIV-1) infection correlated with the acquisition of resistance to superinfection by HIV-1. However, SIV Nef did not affect the level of gene expression directed by the HIV-1 long terminal repeat. It is hypothesized that downregulation of cell-surface CD4 by Nef facilitates the efficient release of infectious progeny virions and, hence, viral spread in vivo.

The Nef protein of HIV-1 is an NH_2-terminally myristoylated protein of ~27 kD that associates with the cytoplasmic membranes of expressing cells (1–3). Nef is expressed early in the viral replication cycle from a set of multiply spliced mRNA species that also encodes the viral regulatory proteins Tat and Rev (4, 5). Unlike Tat and Rev, which are essential for viral replication in culture (4), the Nef gene product is clearly dispensable, although investigators differ on whether Nef has a slight inhibitory effect (6–8), no detectable phenotype (3, 9, 10), or a slight positive effect (11, 12) on the rate of replication. The difficulty of establishing a clear phenotype for HIV-1 Nef in culture, and of identifying fully active copies of *nef*, has been compounded by the high variability of the Nef open reading frame between different viral isolates (13, 14).

Recently, Kestler et al. (15) demonstrated that Nef is essential for both viral replication and pathogenesis in rhesus macaques infected with the mac239 proviral clone of simian immunodeficiency virus (SIV),[1] an animal model for HIV-1 infection that accurately reproduces much of the pathology seen in humans. This study both emphasized the critical importance of this protein in the viral life cycle and also identified, for the first time, a cloned *nef* gene that clearly retained full biological activity. Here, we demonstrate that stable expression of this SIV Nef protein in human $CD4^+$ T cells results in the downregulation of cell-surface CD4 expression and renders these cells resistant to infection by HIV-1.

Materials and Methods

Construction and Use of Retroviral Vectors. The *nef* gene was isolated from the SIV_{mac239} proviral clone (15) by the PCR (16) using primers that introduced unique EcoRI (5′) and XhoI (3′) restriction enzyme sites. The *nef* gene was then inserted into the polylinker present in the LXSN retroviral expression vector (17) where it would be transcribed under the control of the viral LTR promoter. Similar constructs containing the prokaryotic chloramphenicol acetyl transferase (*cat*) gene (18) or the mutant M10 form of the HIV-1 *rev* gene (19) were also prepared. The resultant plasmids were transfected into the packaging cell line GP+ENV-AM12 (20), and the released amphotropic retroviral particles used to infect the human $CD4^+$ T cell line CEM-SS (21). Infected CEM-SS cells were selected on the basis of acquisition of resistance to G418 and maintained as a pool (CEM-NEFP) or subjected to single-cell cloning by end-point dilution (all other cell lines).

Immunological Reagents. mAbs used in the flow cytometric analyses were obtained from Becton Dickinson & Co. (Mountain View, CA [Leu3A]), Ortho Pharmaceuticals, Raritan, NJ (OKT4, OKT4B, OKT4C, OKT4D), and Olympus Immunochemical (Lake Success, NY) (anti-HLA-ABC). Quantitative fluorescence standards (Quantum 27) were obtained from Flow Cytometry Standards Corp. (Research Triangle Park, NC). The rabbit polyclonal antiserum

[1] *Abbreviations used in this paper: cat,* chloroamphenicol acetyl transferase; CMV-IE, cytomegalovirus immediate early; SIV, simian immunodeficiency virus.

specific for human CD4 (22) was a gift of R. Sweet. The macaque anti-Nef antiserum (15) was a gift of R. Desrosiers (New England Regional Primate Center, Harvard Medical School, Southborough, MA).

Analysis of Protein Expression. Expression of the 34-kD SIV Nef protein was confirmed by incubation of 10^6 cells derived from each CEM-SS subclone with [^{35}S]methionine for 2 h as previously described (23). After cell lysis, radiolabeled proteins were subjected to immunoprecipitation (23) using an antiserum obtained from an SIV-infected rhesus macaque (15). Precipitated proteins were resolved by electrophoresis through a 10% SDS-acrylamide gel and visualized by autoradiography.

The level of expression of CD4 in the various transduced CEM-SS T cell lines was quantitated by Western blot analysis. Cells (10^7) derived from the CEM-SS subclones and from the cell lines HeLa and HeLa/CD4 clone 1022 (24) were lysed in 1 ml of TNE (50 mM Tris-HCl, pH 8.0, 1% NP-40, 2 mM EDTA, 100 μM Na_3VO_4, 20 μg/ml leupeptin) and immunoprecipitated overnight (23) at 4°C with 200 μl of OKT4 prebound to 10% protein A–Sepharose (Pharmacia Inc., Piscataway, NJ). The beads were extensively washed before adherent proteins were solubilized by heating at 100°C in Laemmli sample buffer. Precipitated proteins were separated by electrophoresis through an 8% SDS-polyacrylamide gel before transfer to nitrocellulose (Scheicher & Schuell, Inc., Keene, NH). The membrane was preincubated in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20, 5% nonfat milk, and then hybridized overnight at room temperature using a 1:1000 dilution of a rabbit anti-CD4 polyclonal antibody (22). Anti-CD4 antibody binding was visualized using a goat anti–rabbit antiserum conjugated to alkaline phosphatase (GIBCO-BRL, Gaithersburg, MD). The quantitative recovery of the ~55-kD CD4 protein was confirmed by the demonstration that immunoprecipitation of the supernatant for a second time brought down minor and equal amounts of CD4 for all the CEM-SS cell lines tested.

Cell Culture and Viral Infection. All CEM-SS cell lines were maintained in RPMI-1640 medium supplemented with 10% FCS and gentamycin. Transduced cells were selected and maintained in 0.8 mg/ml G418. Viral replication was analyzed by infecting cells (5×10^5) derived from each of the various CEM-SS subclones with 0.5 ml of a viral stock of the HIV-1 isolate IIIB (19). Titration by end-point dilution demonstrated that this stock contained ~4×10^3 median tissue culture infectious doses per ml for CEM-SS cells. At 3 d after infection, the cells were pelleted, washed, and resuspended at 10^6 cells per ml in fresh medium. This process was repeated at 2-d intervals in conjunction with sampling of the media for analysis of viral replication rate. The level of p24 Gag secreted into the supernatant media over each 48-h period was determined using a quantitative ELISA (DuPont Biotechnology, Wilmington, DE).

Transfection Assays. Cells (2×10^6) derived from the CEM-SS subclones were transfected (19) using plasmids (4 μg) containing the *cat* gene under the control of the HIV-1 LTR promoter (BC12/HIV/CAT) or the cytomegalovirus immediate early (CMV-IE) promoter (BC12/CMV/CAT) (25). The HIV/CAT plasmid was cotransfected with 2 μg of a plasmid (pcTat) encoding the HIV-1 Tat *trans*-activator while the CMV/CAT plasmid was cotransfected with an equal level of a negative control plasmid (pBC12/CMV) (25). At 48 h after transfection, the cultures were harvested, and relative levels of CAT enzyme activity, in counts per minute, determined by the diffusion method (18). The presented values were adjusted for minor variability in the level of total protein in each extract, as determined by the method of Bradford (26).

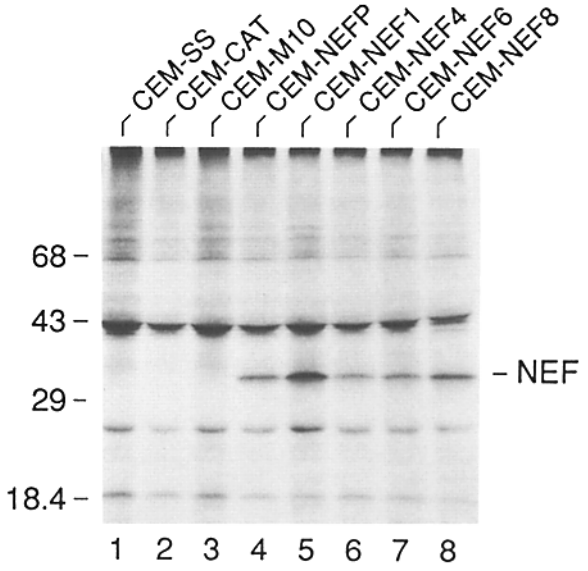

Figure 1. Expression of SIV Nef in human T cells. Expression of the Nef protein of SIV_{mac239} was examined by immunoprecipitation analysis (23) of radiolabeled cultures of the indicated CEM-SS–derived cell cultures using an antiserum obtained from an SIV-infected rhesus macaque (15). A specific protein band that migrated with the mobility predicted for SIV Nef (~37 kD) was detected in *nef*-transduced cells exclusively. (*Left*) Relative migration of protein molecular weight markers.

Results

To examine the properties of the SIV_{mac239} *nef* gene product in culture, we stably transduced the HIV-1 permissive human CD4$^+$ T cell line CEM-SS (21) with amphotropic retroviral vectors designed to express either Nef, the prokaryotic *cat* gene or the *trans*-dominant negative M10 mutant of HIV-1 Rev. (19) (Fig. 1). Transduced cells were maintained either as heterogeneous pools (CEM-NEFP) or as homogeneous subclones (CEM-NEF1, 4, 6, and 8; CEM-M10; CEM-CAT and -CAT2). Immunoprecipitation analysis using antiserum derived from an SIV-infected macaque (15) confirmed expression of the ~34-kD SIV Nef protein in CEM-SS cells transduced with the retroviral Nef expression construct (Fig. 1, lanes *4–8*).

Although it has been argued that expression of HIV-1 *nef* results in a significant downregulation of cell-surface CD4 expression (27), others have been unable to reproduce this effect (7, 8, 10). We examined the effect of SIV Nef on the cell-surface expression of CD4 and other antigens using flow cytometric analysis (Fig. 2 *A*). Expression of cell-surface CD4 was uniformly high (≥94% positive) on the Nef$^-$ T cells (Fig. 2 *B*). In contrast, Nef-expressing T cells displayed low levels of CD4 positivity ranging from a high of ~28% in the uncloned CEM-NEFP cells to a low of ~3% in the CEM-NEF1 subclone. This reduction proved stable over several months in culture and was not due to a conformational change in the CD4 epitope or masking, as essentially identical data were obtained with a range of anti-CD4 mAbs (Leu3A, OKT4, OKT4B, OKT4C, and OKT4D) specific for different CD4 epitopes, as well as with a rabbit polyclonal anti-CD4

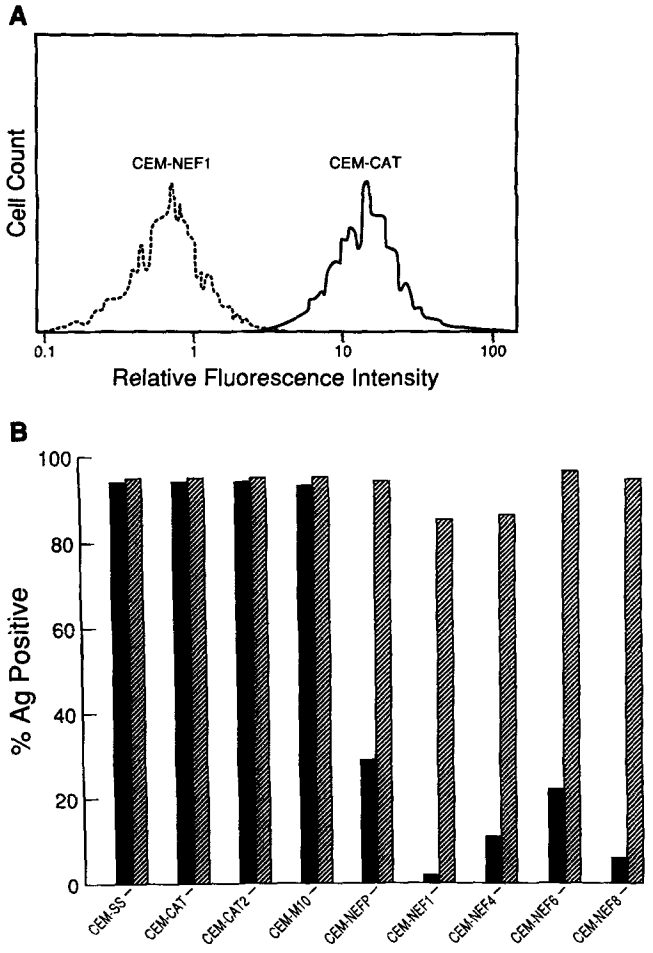

Figure 2. Expression of SIV *nef* inhibits cell-surface expression of CD4. The various CEM-SS subclones were analyzed by flow cytometry using mAbs specific for CD4, HLA-ABC, and other lymphocyte cell-surface markers. (*A*) Representative fluorescence intensity profiles obtained using R-PE–conjugated LEU3A anti-CD4 mAb are shown for the CEM-SS subclones CEM-NEF1 and CEM-CAT. In this experiment, the mean fluorescence intensity was 0.846 for the CEM-NEF1 subclone, and 18.55 for the CEM-CAT subclone whereas the background fluorescence level, obtained using an R-PE conjugated negative control mAb, was 0.261. By comparison to quantitative fluorescence standards, these data indicate a drop from a mean of ~4.8 × 10^5 cell-surface CD4 receptors per CEM-CAT cell to ~1.5 × 10^4 CD4 receptors per CEM-NEF1 cell. (*B*) This graph compares the percentage of cells in each subclone positive for cell-surface CD4 expression (*solid bars*) or HLA-ABC expression (*hatched bars*). Positivity is here arbitrarily defined as a fluorescence intensity greater than 1.96 on the scale given in *A*.

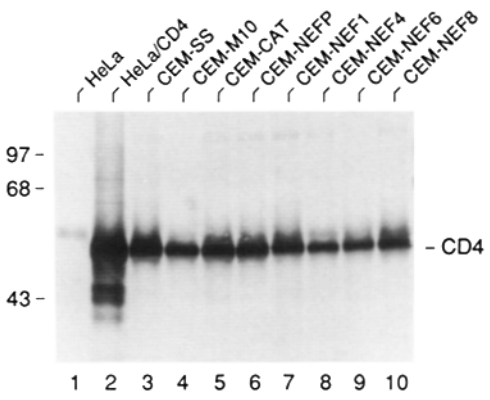

Figure 3. Expression of SIV Nef does not affect the steady-state level of CD4 protein expression. Western blot analysis was used to compare the relative level of the ~55-kD CD4 protein expression in the indicated CEM-SS subclones and in the cell lines HeLa (negative control) and HeLa/CD4 clone 1022 (24) (positive control). (*Left*) Relative migration of protein molecular weight markers.

antiserum (22). This effect was also specific, in that no significant reduction in the cell-surface expression of HLA-ABC, HLA-DR, TCR, intercellular adhesion molecule 1 (ICAM-1), CD38, CD45, or CD69 was observed (Fig. 2 *B* and data not shown). Quantitative flow cytometric analysis of the CEM-SS subclones CEM-NEF1 and CEM-CAT confirmed a 97% reduction in the average number of cell-surface CD4 molecules on the surface of these Nef-expressing T cells (Fig. 2 *A*). Of interest, the two subclones that displayed the greatest reduction in cell-surface CD4 expression, i.e., CEM-NEF1 and CEM-NEF8, also expressed the highest level of Nef (Fig. 1, lanes *5* and *8*).

To identify the level at which Nef affected cell-surface expression of CD4, we next measured the steady-state level of CD4 protein expression using Western blot analysis (Fig. 3). These data revealed that the level of expression of CD4 in the Nef^+ T cells was unchanged relative to that observed in the Nef^- cells. Immunoprecipitation analysis of pulse-labeled cells demonstrated that the rate of CD4 protein synthesis was also unaffected by coexpression of Nef (data not shown). We therefore conclude that downregulation of cell-surface CD4 expression by Nef results from the sequestration of CD4 in a currently unidentified intracellular compartment. However, the observation that the relative mobility of the CD4 glycoprotein in the CEM-Nef cells was indistinguishable from that observed in the parental CEM-SS cells suggests that Nef does not affect the normal posttranslation processing of CD4.

The CD4 molecule is the cell-surface receptor for both HIV-1 and SIV (4). Inhibition of cell-surface CD4 expression might therefore be predicted to reduce the efficiency of viral infection of T cells in culture. To test this possibility, we infected CEM-SS cells, three transduced Nef^- cell lines (CEM-CAT, -CAT2, and -M10) and five Nef^+ cell lines (NEFP, 1, 4, 6, and 8) with a constant level of the HIV-1 isolate IIIB. The rate of spread of the virus was then monitored by measurement of supernatant p24 Gag protein. As shown in Fig. 4, both the parental CEM-SS T cells and the two subclones transduced with the *cat* gene were highly permissive for HIV-1 replication. The cell line CEM-M10 expresses the *trans*-dominant negative M10 mutant of HIV-1 Rev previously shown to confer significant resistance to HIV-1 replication in culture (19). The data presented in Fig. 4 confirm this earlier finding by demonstrating a ≥100-fold inhibition in the rate of viral replication in the CEM-M10 cells when compared with the positive controls. Remarkably, analysis

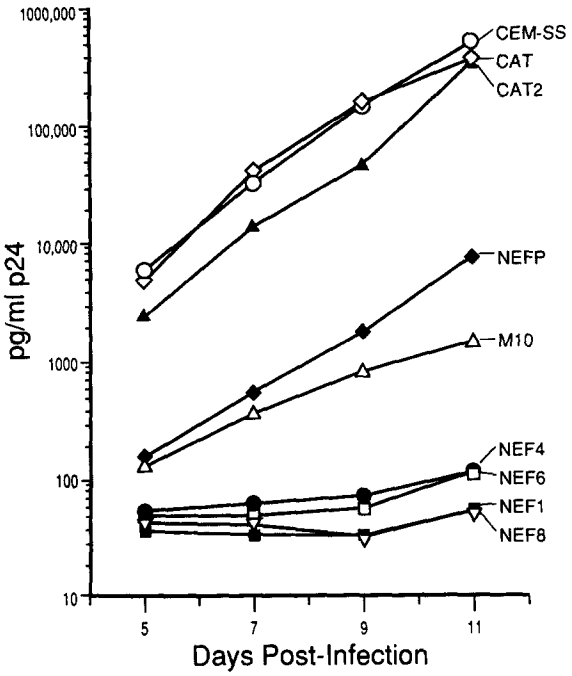

Figure 4. Expression of SIV Nef in human T cells inhibits infection by HIV-1. Cultures of the indicated CEM-SS-derived subclones were infected (19) with equal amounts of a titered HIV-1 virus stock and the rate of viral replication monitored at 2-d intervals by quantitation of the level of released $p24^{GAG}$ protein.

of the four Nef-expressing CEM-SS subclones demonstrated an even more dramatic inhibition in the rate of viral replication, although a low level of virion production did remain detectable throughout the experimental time course. The pool of G418-resistant T cells (CEM-NEFP) obtained after the initial infection of CEM-SS cells with the retroviral Nef expression vector proved somewhat more permissive for the spread of HIV-1 (Fig. 4). We hypothesize that this intermediate phenotype reflects the heterogeneous nature, in terms of proviral integration site and Nef expression, of this transduced T cell population when compared with the Nef-expressing subclones, a hypothesis also consistent with the broad range of cell-surface CD4 expression observed on these cells by flow cytometry (data not shown).

The data presented in Fig. 4 demonstrate that expression of Nef can render T cells resistant to HIV-1 infection. As Nef expression inhibits cell-surface expression of CD4, it seemed likely that this resistance resulted from a block to viral penetration. Previous studies demonstrating that cultures displaying low levels of cell-surface CD4 are less susceptible to infection by HIV-1 are consistent with this interpretation (28). However, it has also been proposed that Nef might inhibit transcription directed by the HIV-1 LTR (29, 30). To test whether the inhibition of HIV-1 replication in these Nef-expressing CEM-SS cells resulted from an inhibition of HIV-1 LTR-specific gene expression, we transfected Nef-expressing and control T cells with plasmids containing the *cat* indicator gene under the control of the HIV-1 LTR (HIV/CAT) or under the control of a human cytomegalovirus promoter (CMV/CAT) (25). In the former case, we also cotransfected an expression vector encoding the HIV-1 Tat *trans*-activator (25). If Nef were a specific inhibitor of LTR-specific gene expression, we would predict that the level of CAT expression induced by the HIV/CAT vector would be markedly reduced in cells that express Nef, when compared to the level seen with the CMV/CAT plasmid. However, these studies failed to demonstrate any inhibitory effect of SIV Nef on HIV-1–specific gene expression (Table 1). These observations are therefore consistent with earlier reports that have failed to detect an inhibitory effect of either SIV Nef or HIV-1 Nef on viral gene expression in culture (3, 9, 10, 15).

Discussion

The *nef* gene of the mac239 proviral clone of SIV is unique in that the full biological activity of the encoded protein has been validated in vivo (15). In this report, we have used human $CD4^+$ T cells transduced with a retroviral *nef* expression vector to examine the in vitro properties of this viral gene product. We demonstrate that expression of SIV Nef dramatically and specifically reduces cell-surface expression of the CD4 glycoprotein receptor but has no detectable effect on the steady-state level of CD4 protein expression. These observations therefore serve to validate the similar, yet controversial (7, 8, 10), data of Garcia and Miller (27) obtained using the *nef* gene of HIV-1 strain SF2. Most importantly,

Table 1. *SIV Nef Does Not Inhibit HIV-1 LTR-driven Gene Expression*

Cell line	CAT activity		Ratio (HIV/CMV)
	HIV/CAT plus Tat	CMV/CAT	
CEM-SS	11,463	9,534	1.20
CEM-M10	10,633	13,380	0.79
CEM-NEFP	6,228	12,470	0.50
CEM-NEF1	32,498	27,647	1.18
CEM-NEF4	26,019	15,300	1.70
CEM-NEF6	13,124	14,905	0.88
CEM-NEF8	15,857	16,477	0.96

The effect of Nef on HIV-1 LTR–specific gene expression was examined by transfection (19) of the indicated CEM-SS subclones with plasmids containing the indicator gene *cat* under the control of either the HIV-1 LTR or the CMV-IE promoter (25). In the former case, a plasmid that directs expression of the HIV-1 Tat *trans*-activator was also cotransfected. A comparison of the level of CAT enzyme activity (18) induced by the HIV-1 LTR construct with that induced by the CMV promoter construct demonstrates that these promoters give comparable levels of enzyme activity in transfected cells regardless of Nef expression. These data are representative of three independent experiments and have been corrected for the low level of background activity (364 cpm) observed in mock-transfected CEM-SS cells.

T cells expressing Nef are shown to be highly resistant to HIV-1 infection, yet retain the ability to support efficient transcription from the HIV-1 LTR promoter. We therefore hypothesize that the downregulation of cell-surface CD4 has rendered these cells, in effect, resistant to superinfection by SIV or HIV-1.

If downregulation of surface CD4, and the associated inhibition of superinfection, represents the only function of Nef, then why does this enhance virus spread in vivo? It does not appear probable that inhibition of superinfection by exogenous virions is likely to provide a major selective advantage, as the virus load in vivo is low compared with the number of available $CD4^+$ target cells (15). We hypothesize, instead, that downregulation of CD4 is designed to facilitate the efficient release of infectious progeny virions from the infected cell by preventing the sequestration of viral envelope (Env) protein or by preventing continuous CD4-mediated reinfection.

Several groups have demonstrated that CD4 and HIV-1 Env can functionally interact in the endoplasmic reticulum (ER) (31–33). This process can lead to the retention of both proteins within the ER and hence to their functional inactivation (31–33). Extensive superinfection of cultured human T cells by HIV-1 has also been reported (34, 35). Whereas such superinfection results in the accumulation of large amounts of proviral DNA, it does not augment virus production and, therefore, presumably inhibits virus spread (34, 35). Particularly relevant is the recent demonstration by Marshall et al. (36) that high level expression of cell-surface CD4 can effectively block the cell-to-cell transmission of HIV-1 in culture, but does not inhibit infection by exogenously added virus. These authors suggest that this inhibition of virus spread results from the inability of progeny virions to effectively disengage from infected cells that express high levels of cell-surface CD4. Conditions that reduce the ability of released virions to diffuse away from an infected T cell that continues to express surface CD4 would therefore be predicted to maximize the positive effect of Nef on the rate of spread of HIV-1. We hypothesize that such conditions might well include the far greater cell density that exists, for example, in an HIV-1–infected lymph node in vivo when compared with an in vitro T cell culture. Precedent for a viral protein that functions to enhance viral release and spread by removal of receptors from the surface of infected cells exists in the form of influenza virus neuraminidase, which has been shown to destroy such receptors by removal of terminal sialic acid residues (37). These considerations suggest that demonstration of a positive effect of Nef on HIV-1 replication in vitro should be achievable by appropriate modification of cell culture conditions, as has already been demonstrated by some groups (11, 12).

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