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The Human Immunodeficiency Virus-1 *nef* Gene Product: A Positive Factor for Viral Infection and Replication in Primary Lymphocytes and Macrophages

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Summary

Considerable controversy and uncertainty have surrounded the biological function of the Human Immunodeficiency Virus (HIV)-1 *nef* gene product. Initial studies suggested that this early, nonstructural viral protein functioned as a negative regulatory factor; thus, it was proposed to play a role in establishing or maintaining viral latency. In contrast, studies in Simian Immunodeficiency Virus $(SIV)_{mac}$-infected rhesus monkeys have suggested that Nef is not a negative factor but rather plays a central role in promoting high-level viral replication and is required for viral pathogenesis in vivo. We sought to define a tissue culture system that would approximate the in vivo setting for virus infection in order to assess the role of HIV-1 Nef in viral replication. We show that infection of mitogen-activated peripheral blood mononuclear cells (PBMC) with Nef^+ HIV results in enhanced replication as evidenced by earlier *gag* p24 expression when compared with infections performed with *nef* mutant viruses. Moreover, when unstimulated freshly isolated PBMC are infected with Nef^+ and Nef^- viruses and then subsequently activated with mitogen, the Nef-induced difference in viral replication kinetics is even more pronounced, with the Nef^- viruses requiring much more time in culture for appreciable growth. A positive effect of Nef on viral replication was also observed in primary macrophages infected with a recombinant of YU-2, a patient-derived molecular clone with macrophage tropism. These positive effects of Nef on viral replication are dependent on the initial multiplicity of infection (MOI), in that infections of unstimulated PBMC at low MOI are most dependent upon intact *nef* for subsequent viral growth. We now provide evidence that the Nef^+ HIV is more infectious than Nef^- HIV from both a tissue culture infectious dose analysis, and a single-cell HIV infection assay. In the latter case, we demonstrate that infection with equivalent doses of HIV based on virion-associated *gag* p24 yields five- to sixfold more infected cells if Nef^+ viral stocks were used. Furthermore, we find that the differential infectivity is not dependent on CD4 down-regulation as Nef^+ virus produced from transfected COS cells lacking CD4 is also more infectious. However, normalization of PBMC infections to equivalent infectivity between that of the Nef^+ and Nef^- viruses continues to reveal delayed viral replication in the absence of Nef, suggesting that secondary viral spread in PBMC is also enhanced in Nef^+ infections. We demonstrate this directly by showing a 13–15-fold increase in infectivity of PBMC-derived Nef^+ HIV. In summary, these findings demonstrate a consistent positive role for the HIV-1 *nef* gene in promoting viral infection and replication, and suggest that the basis for this phenotype is the increased infectivity of HIV produced from cells expressing *nef*. These data suggest that HIV-1 Nef, as previously shown for SIV Nef, may play an important role in establishing a fulminant form of viral infection in vivo.

The HIV-1 *nef* gene is located at the 3′ end of the viral genome, partially overlapping the U3 region of the 3′ LTR. Nef is expressed early in the HIV replication cycle from multiply spliced mRNA transcripts that encode a 27-kD myristylated protein that is largely membrane associated (1–5). Interestingly, up to 80% of the early viral transcripts can encode Nef, suggesting an early function for the *nef* gene product (6). This gene was named *nef* because the earliest studies suggested that it behaved as a negative regulatory factor, suppressing both viral replication and transcriptional activity

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of the HIV-1 LTR (7–10). Correspondingly, it was postulated that Nef might function to establish viral latency after infection. However, other investigations failed to confirm these negative effects of Nef (5, 11). In fact, Kim et al. (11) observed that infection of activated $CD4^+$ T lymphocytes with a Nef^+ viral strain resulted in moderately enhanced viral replication as compared with a *nef* mutant. Additionally, the mechanistically intriguing observation that Nef both binds GTP and exhibits GTPase activity (12) was subsequently refuted by three different laboratories employing purified Nef protein isolated from both bacterial and insect sources (13–15). Perhaps the only property of Nef that is not in dispute is its inhibitory effect on cell surface CD4 expression. This has been demonstrated in multiple cell lines stably or transiently expressing HIV *nef* (16–18), and has recently been shown for SIV *nef* as well (19). Although these authors have shown that CD4 down-regulation provides resistance to superinfection with SIV in vitro (19), the in vivo significance of this biological effect of Nef remains to be established.

Thus, the in vitro analyses of HIV-1 Nef to date have produced no uniform insights into its biological function or significance in the viral life cycle. In fact, tissue culture HIV-1 isolates often develop mutations within *nef* with no apparent adverse effect on their replication in various tissue culture cell lines (20). In 1991, Kestler et al. (21) described intriguing results in the SIV_{mac}/rhesus monkey model system of AIDS that emphasized an in vivo significance for *nef*. Three major findings emerged from this work: (*a*) animals infected with SIV_{mac} containing a point mutation within *nef* rapidly reverted back to wild type *nef* in vivo; (*b*) animals infected with a *nef*-deleted virus demonstrated a dramatically reduced virus load in their PBMC; and (*c*) the same *nef*-deleted virus-infected animals did not develop any AIDS pathology. More recently, the animals infected with the *nef*-deleted SIV_{mac} have been shown to be protected from challenge with a high dose of live wild type SIV_{mac} (22). Together, these findings highlight the in vivo importance of the *nef* gene product in viral pathogenesis and suggest that the *nef* gene functions as a positive regulatory factor that is essential for high-level viral replication in vivo.

In this report we demonstrate that HIV-1 Nef acts to dramatically accelerate the replication kinetics of HIV-1 in primary human PBMC that are infected directly after isolation from normal donors and subsequently lectin-activated in vitro. We also show that HIV-1 *nef* contributes to increased replication in primary macrophages. Finally, we show that the infectivity of HIV containing intact *nef* is enhanced in both a tissue culture infectious dose ($TCID_{50}$)[1] analysis and a single-cell infection assay. These observations establish a positive role for HIV-1 *nef* in the viral life cycle and suggest that the basis for the increased replication kinetics of Nef^+ HIV is the increased infectivity of viral particles. The tissue culture–based assay systems described provide for future investigations into the precise biological mechanism of Nef-mediated enhancement of HIV infection and replication in primary cells.

Materials and Methods

Cell Lysates and Western Blots. 8×10^6 COS-7 cells were transfected with 10 μg of HIV proviral DNA (see Fig. 1 *a*) by electroporation at 240 V and 960 μF using the Bio-Rad Gene Pulser (Richmond, CA). A mock transfection was done by electroporating cells in media alone. The cells were cultured for 2 d in Iscove's Complete Medium (Mediatech, Washington, DC) containing 10% FBS (GIBCO BRL, Gaithersburg, MD), 100 U/ml penicillin/streptomycin (pen/strep), and 2 mM L-glutamine (both from Mediatech). The cell layer was washed twice with Mg^{2+}, Ca^{2+} free PBS. The cells were removed from the flask by a 10-min incubation in TEN (40 mM Tris-Cl, pH 7.5, 1 mM EDTA, pH 8.0, 150 mM NaCl) with agitation. The cells were pelleted and washed once with additional TEN. The cell pellet was resuspended in 0.6 ml TEN lysis buffer (TEN plus 1% Triton X-100 and 1 mM PMSF). The cells were centrifuged again, and the soluble portion retrieved and stored at −70°C. The cell pellets were discarded.

Approximately 25 μg of the cell extracts was run on 10% polyacrylamide gels containing 0.1% SDS along with Rainbow molecular weight standards (Amersham Corp., Arlington Heights, IL) and then transferred onto nitrocellulose (Schleicher and Schuell, Inc., Keene, NH). The blots were blocked overnight in 5% nonfat dry milk and 0.1% Tween-20 in PBS. The blocking solution also served as the primary antibody diluent. One blot was incubated in 17.6 μg/ml of an HIV-1 BH10 anti-Nef monoclonal antibody obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Cat. #456) (13) for 1 h. A second blot was incubated for 1 h in a pool of four human HIV patient antisera each diluted to 1:400. Both blots were incubated in horseradish peroxidase-conjugated sheep anti-human or anti-mouse Ig (Amersham Corp.) diluted at 1:2500 in PBS containing 0.1% Tween-20. All incubations were carried out at room temperature, and the blots were extensively washed between incubations with PBS containing 0.1% Tween-20. Immunoreactive bands were visualized using the ECL Detection System (Amersham Corp.).

Virus Stocks. 10 μg of plasmid containing the HIV proviral DNA was transfected into 15×10^6 COS-7 cells by electroporation as described above. After 2 d of culture in complete Iscove's medium, virus-containing supernatants were harvested, filtered through a 0.45-μm filter, and stored at −70°C. Virus stocks were assessed for *gag* p24 concentration using a commercial antigen capture ELISA (Coulter Immunology, Hialeah, FL) according to the manufacturer's instructions. The viral stocks were each normalized to 10 ng/ml of p24. Higher concentrations of virus were prepared by infecting H9 cells overnight with the 10 ng/ml virus stocks from COS cells. The infected H9 cells were passaged in RPMI containing 10% FBS, 100 U/ml pen/strep, and 1 mM Hepes buffer (complete RPMI) for 15 d with changes of the culture medium every other day. Peak days of viral replication were determined by p24 ELISA and those culture supernatants were filtered and stored. The H9-generated HIV stocks were normalized to 250 ng/ml of pelletable virus. Pelletable virus was determined by microcentrifugation for 90 min at 15,000 *g*. Nonpelletable p24 constituted ~50% of the total p24 value in all the virus preparations.

PBMC and Macrophage/Monocyte Isolation. Human PBMC were obtained by Ficoll-Paque (Pharmacia-LKB, Piscataway, NJ) density gradient centrifugation of buffy coats from normal commercial blood donors. To isolate monocytes and macrophages, 50–70 ×

[1] *Abbreviations used in this paper:* fs, frameshift; MOI, multiplicity of infection; $TCID_{50}$, tissue culture infectious dose of 50% infectivity; TEN, Tris-Cl/EDTA/NaCl.

10^6 PBMC were cultured for 3 d in 8 ml of supplemented RPMI containing 20% FBS and 10% pooled human AB serum (Gemini Bioproducts, Calabasas, CA) in T25 flasks (Costar Corp., Cambridge, MA). Nonadherent cells were removed by rigorous washing with warm PBS. The adherent cells were further cultured for 3 d in RPMI/20% FBS before infection. These adherent cell cultures contained more than 95% macrophages as identified by light microscopic observation of nonspecific esterase–stained cultures (23). Unstimulated resting PBMC were cultured in complete RPMI containing 10% pooled human AB serum to avoid FBS-associated T cell activation.

Infections and Cell Culture. All infections were performed by incubating the cells overnight in 1.5–3 ml of a virus supernatant normalized to virion-associated p24 concentration ranging from 1 to 250 ng/ml. 2–20 × 10^6 PBMC were infected freshly after isolation or after 2 d of stimulation with 4 μg/ml of PHA-P (Sigma Chemical Co., St. Louis, MO). After overnight infections, these cultures were washed four times in PBS and then recultured in 4 ml of RPMI/10% human AB serum with (PHA-activated cultures) or without (unstimulated cultures) recombinant human IL-2 added at 10 U/ml (Cetus Corp., Berkeley, CA) in 12-well plates. The unstimulated cultures were activated at various times from 1 to 11 d later by adding a 2× solution of PHA-P and rIL-2. These cultures were incubated for 2 d, washed once, and returned to rIL-2 supplemented RPMI/10% AB. Aliquots of culture supernatants were taken from all cultures every 2–4 d and 50% of the media exchanged every fourth day. Infections of the immortalized T cell lines CEM, C8166, and H9 were performed analogously using 10^7 cells and then culturing in complete RPMI/10% FBS. Every other day ~30% of the cells were removed and fresh culture medium was added to maintain a viable culture. Macrophage infections were performed overnight using 3 ml of 8 ng/ml p24 normalized viral supernatant in the T25 flask. The macrophages were then washed four times in PBS and cultured in RPMI/20% FBS with a 75% media exchange every third day.

T Cell Clone Stimulation and Infection. The influenza hemagglutinin (HA)-specific, $CD4^+$ human T cell clone 103.13 was a generous gift of Wim VanSchutten (Immulogic, Palo Alto, CA) and was maintained by antigen stimulation every 2–3 wk with peptide antigen presented by mitomycin C–treated (Sigma Chemical Co.), autologous EBV-transformed B cells. The culture medium contained 6% pooled human AB serum, and rIL-2 was added at 5 U/ml 4 d after antigen stimulation. 2 wk after stimulation, the IL-2 was removed and the cells allowed to quiesce overnight. 500,000 cells were then infected using 10 or 250 ng/ml of p24 overnight and then washed four times. The infected cells were then stimulated with peptide antigen and expanded in vitro with periodic culture supernatants removed for p24 determination.

$TCID_{50}$ Studies. Sextuplicate infections of 10^4 CEM cells, 10^4 H9 cells, or 10^5 PBMC from two different donors were performed using serial fivefold dilutions of viral supernatants starting at 100 ng/ml of p24. The infected cultures were maintained in 200 μl of RPMI/10% FBS in 96-well plates and visually inspected for cytopathic effect. Infected cultures were confirmed as positive by assaying 100 μl of the supernatant for a p24 concentration activity two standard deviations beyond the residual input level on day 14 for H9 and CEM, and day 21 for PBMC infections. The $TCID_{50}$ is the reciprocal of the dilution of a 100 ng/ml p24 viral supernatant that results in infection of 50% of the wells. $TCID_{50}$ values were calculated according to the method of Reed and Muench (24) by interpolation between the dilutions that bracketed the $TCID_{50}$ endpoint.

Single-Cell Infectivity Assay. This assay was described as the multinuclear activation of a galactosidase indicator (MAGI) assay by Kimpton and Emerman (25). The HeLa-CD4- LTR-β-gal cell line was obtained through the AIDS Research and Reference Program (Cat. #1470) and was maintained in complete Iscove's medium with 10% FBS. 1 d before infection, 7 × 10^3 cells were plated per well in a 96-well plate in 100 μl media. The medium was then removed and replaced with 100 μl of the virus stock at the indicated concentration of pelletable *gag* p24 for an overnight infection. Soluble CD4 was then added at a concentration of 1 μg/ml to prevent potential viral spread and syncytium formation. After a total of 40 h following infection the cells were nearly confluent. The supernatant was aspirated and the cells were fixed for 5 min with 0.2% glutaraldehyde and 1% formaldehyde in PBS. The wells were washed twice with PBS and 50 μl of the β-gal substrate was added (0.4 mg/ml X-gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, and 2 mM $MgCl_2$). The plates were incubated for 50 min at 37°C and then washed twice. Foci of infected blue cells were counted using the light microscope. Many foci consisting of two–four blue cells were observed, likely reflecting cell division events. Wells of uninfected cells did not contain any blue cells, and all infections were performed in triplicate using multiple dilutions of virus.

Results

Construction and Characterization of Proviral Vectors. As summarized diagrammatically in Fig. 1 *a*, the Nef-expressing HIV-1 used for these studies was a modification of the HXB2 molecular clone pHXB2gpt in which the premature stop codon in the *nef* gene was replaced with a portion of a cloned cDNA from an HIV-1_{IIIB}-infected H9 cell line (26, 27). This repaired HXB2 clone, HXB Nef^+, has been completely sequenced within the *nef* reading frame and contains four amino acid substitutions with respect to the published HIV LAI sequence (28) at positions 11 (V→I), 43 (A→V), 64 (A→D), and 168 (K→E) and one additional substitution with respect to the published HXB2 sequence (29) at position 14 (L→P). None of the amino acid substitutions within Nef are unique to this molecular clone when the sequence is compared to other published Nef amino acid sequences (30). HXB Nef^+ was mutagenized at the unique XhoI site in *nef* to create a frameshift (fs) mutant generating a stop codon 33 bases downstream (HXB fs). The second *nef* mutant, HXB Δstart, (obtained from M. Reitz, National Cancer Institute, Bethesda, MD), contains a ClaI site in place of the primary *nef* ATG as well as the unrepaired premature stop codon. The *nef* deletion mutant, ΔN-term, was prepared by cleaving at the introduced ClaI site and at the BglII site in *nef*. As HIV in the HXB2 background does not infect primary macrophages efficiently, the brain tissue–derived molecular HIV-1 clone YU-2 (31, 32) was utilized for the macrophage infection studies. The cloned YU-2 virus was modified by subcloning the entire BssHII-XhoI fragment from the pTZ vector into the HXB Nef^+ vector described above, thus generating YU-2b. A *nef* frameshift mutant of this virus was then made at the XhoI site (YU-2b fs). All of these HIV proviral DNA plasmids, except HXB2, were transfected by electroporation into COS-7 cells, and detergent lysates were assessed for Nef expression by immunoblotting with a mouse anti-Nef monoclonal antibody raised against recombinant BH10 Nef (Fig.

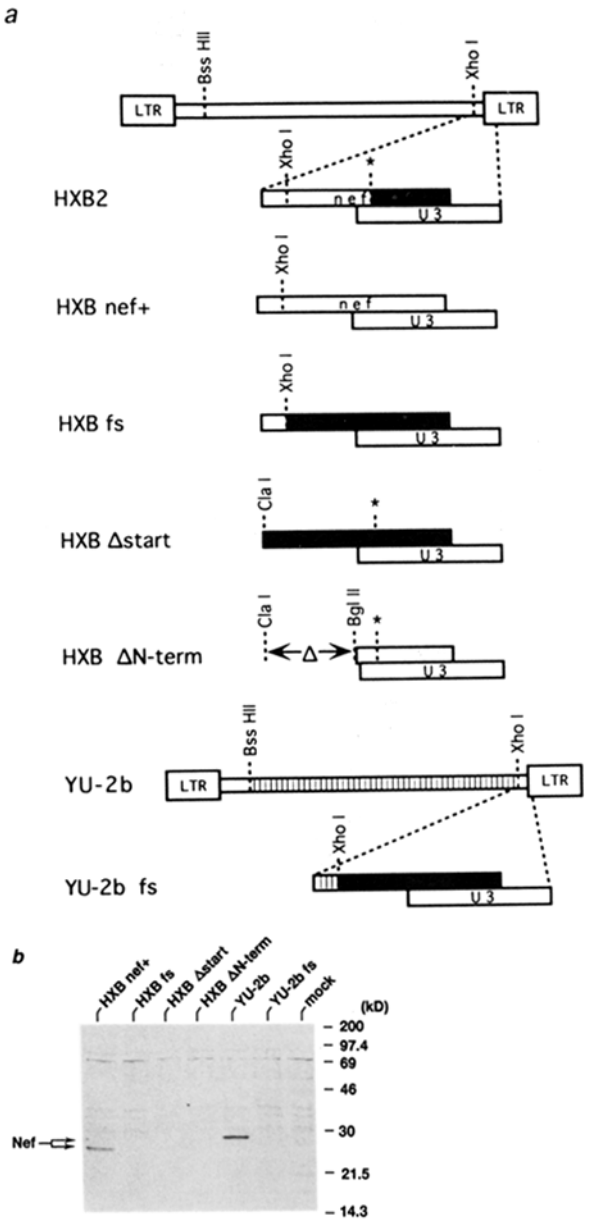

Figure 1. (*a*) Construction of HIV recombinants. The premature stop codon in HXB2 was repaired as previously published (26, 27), creating HXB Nef$^+$. The frameshift mutants were created by cutting at the unique XhoI site, blunting with Klenow, and religating. The HXB Δstart contains a ClaI site in place of the start codon (M. Reitz, NCI), and HXB ΔN-term contains a deletion delimited by the introduced ClaI site and the BglII site. YU-2b is a recombinant containing the entire BssHII-XhoI fragment from YU-2 in the HXB Nef$^+$ plasmid. (*b*) Nef is expressed from the HXB Nef$^+$ and YU-2b proviral constructs. The proviral HIV constructs described in *A* were transfected into COS-7 cells and cell lysates were electrophoresed in a 10% acrylamide gel. The gel was blotted and then incubated with a mouse anti-Nef monoclonal antibody generated against recombinant BH10 Nef. Bands corresponding to Nef appear at ~27 and 28 kD only in the lanes containing lysates from the transfections of proviral HIV with intact *nef* reading frames.

1 *b*). As expected, both the HXB Nef$^+$ and the YU-2b transfections produced detectable levels of the Nef protein. The difference between the migrations of the two Nef species is likely due to a 10-amino acid insertion in the 5′ end of the YU-2 *nef* that is included in the YU-2b recombinant. In contrast, the lysates from the *nef* mutant transfections did not demonstrate any detectable Nef-specific immunoreactive product. The *nef* frameshift mutants would be expected to have generated a 6-kD Nef polypeptide too small to identify in these polyacrylamide gels. However, both the Nef$^+$ and Nef$^-$ HIV proviral DNA transfections generated normal titers of viral particles as measured by *gag* p24 ELISA. Furthermore, immunoblotting the cell lysates with an anti-HIV human antisera demonstrated detectable HIV *gag* p24 and HIV envelope glycoproteins in all cases (not shown).

Infection of Transformed T Cell Lines. To confirm the infectious properties of these viral stocks, human T lymphoblastoid cell lines were infected with the HXB Nef$^+$ as well as the three *nef* mutant viruses using 10 ng/ml of *gag* p24 from the COS-7 cell transfections. The culture supernatants were subsequently monitored for viral production by p24 ELISA. As shown in Fig. 2, all of the viruses were competent to replicate in CEM, C8166, and H9 cells. There appeared to be no consistent difference among the infections in terms of kinetics or magnitude of viral replication. These findings are in agreement with earlier observations, which indicated that Nef is a dispensable gene product for viral replication in transformed human T cell lines (11, 20).

Infection of Primary PBMC. Primary PBMC were isolated from a healthy donor and then activated with PHA-P for 2 d. These cells were then infected with the Nef$^+$ and Nef$^-$ HXB-based viruses generated from COS-7 cell transfections and placed into IL-2 supplemented medium. As shown in Fig. 3 *a*, the HXB Nef$^+$ virus demonstrated increased viral replication as compared with the frameshift and Δstart *nef* mutants in these mitogen-activated PBMC cultures. To more closely approximate possible in vivo conditions of HIV-1 infection, unstimulated freshly isolated PBMC from the same donor were also infected with the Nef$^+$ and Nef$^-$ viruses and then placed into culture without mitogen. 1 or 4 d later, these cultures were activated with PHA-P and IL-2 and the culture supernatant was monitored for virus production by the p24 ELISA. Under these conditions a dramatic difference in replication between the Nef$^+$ and Nef$^-$ isogenic viruses was observed. Specifically, the three *nef* mutant viruses displayed little or no detectable growth at the time of peak viral replication in the HXB Nef$^+$ infection and did not begin to detectably replicate until 3 wk after infection (Fig. 3, *b* and *c*). In other experiments, the PBMC were activated 7 or 11 d after infection with similar results (not shown). Given the propensity of HIV to mutate in vitro, it was possible that the HXB fs and Δstart *nef* mutants may have reverted to wild type *nef* to generate the late viral growth. To address this possibility, we infected unstimulated PBMC from another donor with the *nef* deletion mutant virus (ΔN-term). Fig. 3 *d* shows delayed but eventual growth of this mutant, suggesting that reversion is not the proximal cause for the

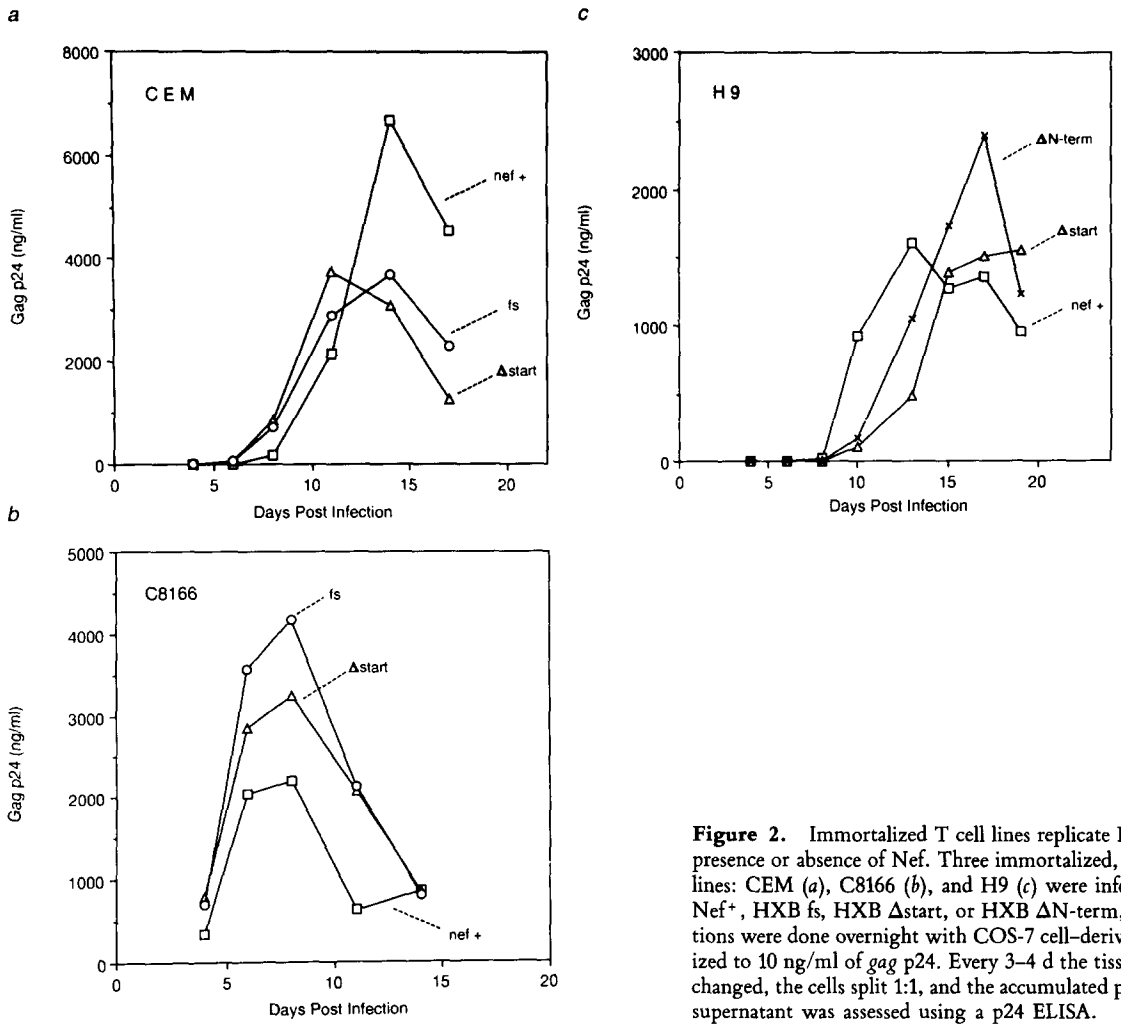

Figure 2. Immortalized T cell lines replicate HIV equally well in the presence or absence of Nef. Three immortalized, long-term cultured cell lines: CEM (*a*), C8166 (*b*), and H9 (*c*) were infected with either HXB Nef$^+$, HXB fs, HXB Δstart, or HXB ΔN-term, as indicated. All infections were done overnight with COS-7 cell-derived virus stocks normalized to 10 ng/ml of *gag* p24. Every 3–4 d the tissue culture medium was changed, the cells split 1:1, and the accumulated p24 concentration in the supernatant was assessed using a p24 ELISA.

delayed growth exhibited by the Nef$^-$ viruses. In fact, in extended PBMC cultures, the growth of the Nef$^-$ virus, although very delayed, can approach that found with wild type virus (not shown). To date, this striking difference in viral replication kinetics between Nef$^+$ and Nef$^-$ viruses has been repeated using primary PBMC from 10 random donors. Thus, infection of unstimulated PBMC with HIV and subsequent activation provide for a robust biological phenotype that reveals a positive role for Nef in accelerating virus replication in primary PBMC.

Infection of Primary Macrophages. To determine whether HIV-1 Nef might also positively contribute to viral replication in a different host cell, primary peripheral blood monocyte-derived macrophages were purified by adherence to plastic and then infected 6 d later with macrophage-tropic YU-2-derived Nef$^+$ and *nef* frameshift viruses, as well as the HXB Nef$^+$ virus. As expected, the T cell–adapted HXB Nef$^+$ virus replicated poorly in these adherent macrophage cultures (Fig. 4 *a*). In contrast, the YU-2b Nef$^+$ virus grew quite well, establishing a persistent infection that produced virus over the entire 1-mo culture period. In the case of the YU-2b *nef* frameshift mutant, viral infection was established; however, the magnitude of *gag* p24 production was reduced, suggesting that the spread of HIV infection in vitro was less efficient. This finding of reduced but not delayed viral production has been reproducibly demonstrated using monocyte-derived macrophages from five different donors. However, infection of unstimulated PBMC with the Nef$^+$ and Nef$^-$ YU-2b viruses demonstrated the delayed kinetics of replication for the *nef* mutant as previously observed with the HXB-derived virus infection of PBMC (Fig. 4 *b*). These findings may highlight the differences in the viral infection parameters active in tissue culture models of PBMC and macrophage infection, and are consistent with the observations that the capacity to infect macrophages is dependent on the differentiation state of the cells, becoming less efficient during prolonged culture (33–35). Thus, the capacity of the Nef$^-$ virus infection of macrophages to eventually achieve the high level of viral replication observed in PBMC infections is reduced due to the extended culture.

Infection of an Antigen-specific CD4$^+$ T Cell Clone. The capacity of Nef to enhance viral growth in an infected human

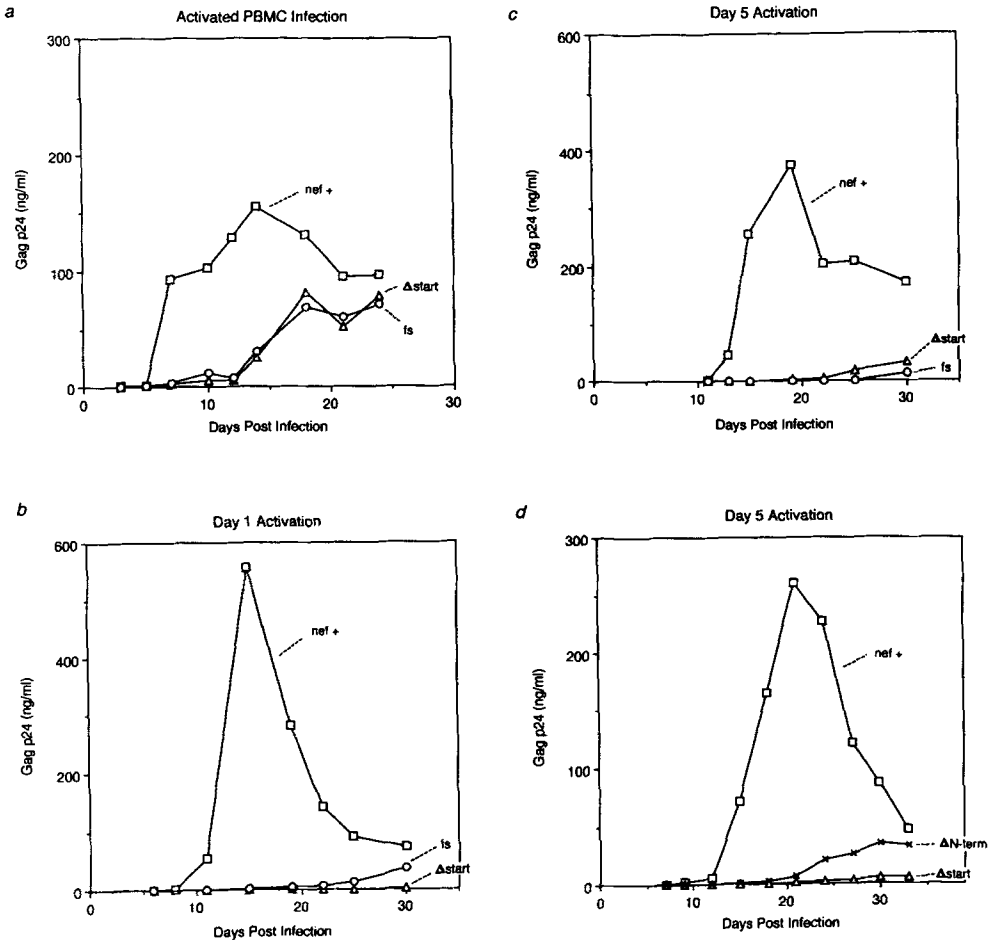

Figure 3. HIV replication in unstimulated primary PBMC is accelerated in the presence of Nef after lectin activation. (*a*) PBMC were isolated from healthy donors and activated with PHA-P before infection with 10 ng/ml of the indicated HIV stocks derived from COS-7 cell transfections. The virus was washed off and then the PBMC placed into IL-2–supplemented media. Every 3–4 d one half of the culture medium was exchanged with fresh IL-2 media and the supernatant was assayed for p24 content. (*b*, and *c*) Unstimulated PBMC were infected directly after isolation with the indicated viruses. The following day, the cells were extensively washed and then activated immediately (*b*) or 4 d later (*c*) with PHA-P and IL-2 and cultured as described. (*d*) PBMC from another donor were infected with the indicated viruses, including the HXB ΔN-term deletion mutant, and cultured as above. PBMC infections from over 10 different donors have generated analogous results, using HIV prepared both from COS-7 cell transfections and HIV prepared from H9 cells infected with COS-7 virus stocks.

T cell following more physiological activation with antigen was addressed using a $CD4^+$, influenza hemagglutinin-specific T cell clone restricted by HLA-DR4. The clone is routinely passaged in vitro by periodic antigen stimulation using peptide antigen and autologous antigen presenting B cells followed by IL-2-mediated cell proliferation. Three weeks after antigen stimulation, the clone was allowed to enter a state of growth arrest by removing IL-2, and the cells were then infected with either HXB Nef^+ or *nef* frameshift HIV generated from H9 cell infections at two virus concentrations, 10 ng/ml and 250 ng/ml of *gag* p24. The clones were then restimulated with antigen and expanded in vitro, and the culture supernatant was monitored for virus production. The low-dose HXB Nef^+ infection resulted in an 8- to 28-fold increase in virus production at all time points assayed, while the high-dose infection resulted in very low levels of replication enhancement (Table 1). These data demonstrate that an antigen-specific T cell clone infected at a low multiplicity of infection (MOI) and subsequently stimulated with antigen produces greater quantities of virus in the presence of an intact *nef* reading frame during one cycle of antigen restimulation. However, this viral growth advantage is significantly reduced at high multiplicities of infection.

Multidose Infection of Primary PBMC. The observation of an MOI dependence on Nef function in the T cell clone infections prompted us to expand upon our initial observations in the unstimulated PBMC infections. In these studies, we infected 20×10^6 PBMC with two dilutions of HXB Nef^+ and HXB fs. The high dose of virus, 250 ng/ml of p24, exhibited only up to a 15-fold increase in *gag* p24 over the culture period, while the lower dose exhibited the more dramatic accelerated replication kinetics typical of PBMC infections with Nef^+ virus (Table 2). In experiments not shown, PBMC were infected with as little as 1 ng/ml of *gag* p24, in which case the *nef* mutant HIV would occasionally fail to establish an infection whereas the Nef^+ virus would eventually establish a fulminant in vitro infection. Thus, as with the antigen-specific T cell clones, these results demonstrate an MOI dependence on the capacity of Nef to enhance viral replication in primary PBMC.

Tissue Culture Infectious Dose ($TCID_{50}$) Studies. The possibility that the differential replication kinetics of Nef^+ and Nef^- HIV might directly result from intrinsic differences in the infectivity of the Nef^+ and Nef^- virus preparations was addressed by determining $TCID_{50}$ values. Two immortalized T cell lines and PHA-activated PBMC from two donors were

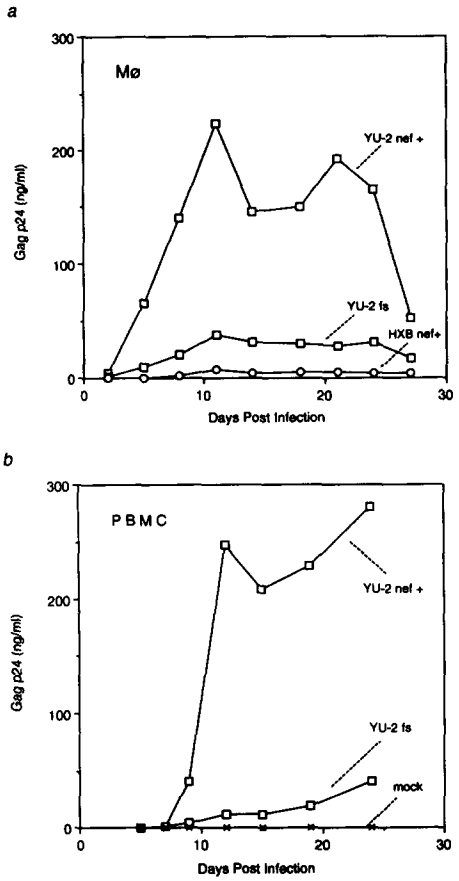

Figure 4. HIV replication in macrophages is increased in the presence of Nef. (*a*) Monocytes and macrophages were isolated from the PBMC of healthy donors by adherence to plastic and multiple washings. The cells were infected after 6 d of adherence culture and were >95% nonspecific serine esterase positive at the time of infection. The cultures were infected overnight with the indicated macrophage-tropic YU-2–derived virus stocks using 8 ng/ml of *gag* p24. 75% of the media was exchanged every third day and the accumulated concentration of p24 was assessed at those times. These are representative data from macrophage infections of five independent donors. (*b*) PBMC were infected with the indicated YU-2 viruses as described in the legend to Fig. 3 and activated just after infection. The virus stocks utilized were prepared from COS-7 cell transfections. Similar results have been obtained using YU-2b and YU-2b fs HIV prepared from PBMC infections.

infected with both COS-7 cell- and H9-derived HIV in serial fivefold dilutions. As shown in Table 3, $TCID_{50}$ values were reduced from 1.5- to 5.6-fold for infections with the two Nef^- viruses as compared with HXB Nef^+. The reduction in $TCID_{50}$ was more notable in the PBMC infections, but was also apparent in both H9 and CEM cell infections. In another experiment, H9 and C8166 cells were infected, but only the infections of H9 cells showed a reduced $TCID_{50}$ (not shown). Thus, a positive effect on viral replication can be demonstrated for Nef in some but not all immortalized T cell lines in the $TCID_{50}$ assay. However, as described above (Fig. 2), there appears to be no consistent difference in the viral replication kinetics in the same immortalized cell lines. These data suggest that properties inherent to virus infection and propagation in transformed cell lines may mask a positive biological function for the *nef* gene under all but the most limiting conditions of infection. This is in contrast to the more broadly defined positive effect that Nef exerts during infection of primary lymphocytes and monocytes over a wide range of viral MOI.

Single-Cell Infection Analysis. The definition of reduced $TCID_{50}$ values for Nef^- HIV could either indicate that Nef^- virus is inefficient at initially infecting target cells or that the subsequent in vitro spread of virus, necessary for a positive TCID culture, is impaired in the Nef^- virus infections. To further characterize the mechanism of Nef activity we utilized a single-cell infection assay developed by Kimpton and Emerman (25). The HeLa-CD4 indicator cell line is stably transfected with β-gal under the control of the HIV-1 LTR and is strictly dependent upon *tat* expression, provided by successful infection with HIV, for β-gal expression and activity. After a 40-h infection of a monolayer in a microtiter well, the X-gal substrate solution was added and the number of blue cells scored by light microscopy. The assay is quite reproducible and behaves linearly over a reasonable counting range of 10–400 infected cells. During the period of infection and *tat*-expression cell division is occurring; thus, equally infected daughter cells are apparent but are only scored once. As shown in Fig. 5 *a*, the H9-derived Nef^+ HIV infected five to six times as many cells as either *nef* mutant HIV at a concentration of 50 ng/ml of pelletable *gag* p24. Similarly, a four- to eightfold enhancement in infectivity has also been observed using Nef^+ and Nef^- HIV generated through transfection of COS-7 cells (not shown). Thus, the infection events leading to Tat production in the first round of infection are facilitated in the presence of intact *nef*.

PBMC Infection Normalized to Infectivity. Given the observations from both the $TCID_{50}$ studies and the single-cell infectivity assay that Nef^+ HIV is as much as six times more infectious than Nef^- HIV, it was possible that the initial infectivity difference alone accounted for the differential replication kinetics presented in Fig. 3. To address this issue, unstimulated PBMC were exposed to 4-fold and 16-fold more Nef^- HIV, and then activated and monitored for virus replication over time. The 16-fold excess of Nef^- HIV would ensure that these infections contained even greater numbers of initially infected cells than the Nef^+ infection, based on both the single-cell infectivity assay and the $TCID_{50}$ assay. As shown in Fig. 5 *b*, under both of these conditions the production of virus in the Nef^- virus infection appreciably lagged behind the infection with Nef^+ HIV. Interestingly, the earliest time points after infection, although only demonstrating p24 in the pg/ml range, showed comparable initial virus production from both the Nef^+ and Nef^- infections. Together, these observations suggested that the virus produced from the infected PBMC was also differentially infectious depending on the presence or absence of Nef. To assess this, the HIV produced from the PBMC infections was also analyzed for infectivity in the HeLa β-gal single-cell infectivity assay. Fig. 6 demonstrates that HIV produced from PBMC infection with Nef^+ HIV is 13–17 times more infectious than that produced from Nef^- HIV PBMC infec-

Table 1. *Infection of an Antigen-specific T Cell Clone with Nef⁺ vs. Nef⁻ HIV Results in More Efficient Viral Replication after Activation with Antigen*

Virus dose*	Days after infection	*gag* p24		Fold Nef induction‡
		HXB Nef⁺	HXB fs	
		ng/ml		
10 ng/ml	6	6.6	0.5	13.9
	8	69.4	3.1	22.5
	10	289	10.3	28.0
	13	934	54.9	17.0
	16	1258	78.2	16.2
	19	856	70.5	12.1
	22	495	61.3	8.1
250 ng/ml	6	10.3	9.9	1.0
	8	191	72.3	2.6
	10	1005	403	2.5
	13	4190	1201	3.5
	16	1625	1082	1.5
	19	1462	926	1.6
	22	976	776	1.3

* An influenza hemagglutinin-specific CD4⁺ T cell clone (0.5×10^6 cells) was infected with HIV on day 0 with either 10 or 250 ng/ml of *gag* p24, employing either HXB Nef⁺ or the HXB *nef* frameshift mutant, HXB fs, generated from H9 cells. Virus was washed off the cells with PBS on the following day and the cells were activated with peptide antigen presented by autologous B cells. Il-2 was added to the media 4 d later. Viral supernatant samples were taken on the indicated days after infection and the quantity of *gag* p24 determined by ELISA.
‡ Fold Nef induction is the ratio of *gag* p24 from the HXB Nef⁺ infection vs. the HXB fs infection.

tion. This differential infectivity of PBMC-derived HIV persists over the entire culture period of virus production. Thus, the capacity of Nef to enhance viral replication in primary cells is due to its capacity to facilitate initial HIV infection as well as subsequent virus spread in culture.

Discussion

The results presented in this report demonstrate that HIV-1 *nef* significantly augments the in vitro replication of two different molecular clones of HIV upon infection of two natural target cells of HIV-1: primary T lymphocytes and macrophages. This positive Nef function is most pronounced when unstimulated freshly isolated PBMC are infected and then subsequently activated with mitogen. Furthermore, this function is related to the multiplicity of viral infection, with the lowest MOI demonstrating the greatest enhancement of viral replication in the Nef⁺ virus. The MOI-dependence indicates a potential infectivity difference between Nef⁺ and Nef⁻ HIV that is supported by reduced $TCID_{50}$ values and reduced infectivity titers in a single-cell infectivity assay with Nef⁻ virus. Together, these data suggest that *nef* acts in the cells producing virus to increase the infectivity of progeny viral particles.

Interestingly, the described positive function of Nef was not definable through conventional infection at moderate MOI of numerous immortalized T cell lines (Fig. 2). In sharp contrast to these results, several groups have shown negative effects of Nef on virus replication employing various immortalized T cell lines (7, 9, 10). However, other investigations have shown, in agreement with our studies, that the presence of intact *nef* resulted in little or no difference in viral replication in immortalized T cell lines (5, 11). In one case, Kim et al. (11) utilized the same cells and the same molecular clones of HIV as in the earlier studies, and were still unable to demonstrate a negative effect on replication. Interestingly, this group observed a moderate positive effect upon infection of activated PBMC. In this regard, de Ronde et al. (36) have transfected Nef⁺ and Nef⁻ proviral HIV constructs into activated PBMC and also demonstrated a moderate positive effect on virus replication. Similarly, Terwilliger et al. (37) have shown differences in the growth of HIV containing a recombinant of the Eli allele of Nef in the HXB2 background in activated PBMC. In that report, however, there was no detectable growth of the Nef⁻ variants in activated PBMC at any time after infection. This is in contrast with our data, which show only a substantial delay in viral replication in the absence of Nef at the lowest MOI and near wild type replication of a Nef⁻ variant at the highest MOI. In this report we expand upon these earlier observations of Nef func-

Table 2. *Nef-mediated Enhancement of HIV Replication Is MOI Dependent in Unstimulated PBMC Infections*

Virus dose*	Days after infection	*gag* p24		Fold Nef induction‡
		HXB Nef+	HXB fs	
		ng/ml		
10 ng/ml	11	0.034	ND§	-
	13	0.70	0.012	58
	15	18.6	0.021	885
	17	59.1	0.12	492
	19	258	2.8	92
	24	632	30.0	21
	28	217	69.7	3.1
250 ng/ml	11	1.2	0.28	4.1
	13	82.1	7.4	11
	15	313	20.5	15
	17	423	45.0	9.4
	19	288	58.2	4.9
	24	359	264	1.3
	28	390	503	0.8

* Unstimulated PBMC (20×10^6 cells) were infected with the indicated concentration of HIV, employing either HXB Nef+ or the HXB *nef* frameshift mutant, HXB fs, generated from H9 cells. Virus was washed off the cells with PBS on the following day and the cells were activated with PHA/IL-2 on day 5. Viral supernatants samples were taken on the indicated days after infection and the quantity of *gag* p24 determined by ELISA.
‡ Fold Nef induction is the ratio of *gag* p24 from the HXB Nef+ infection vs. the HXB fs infection.
§ ND, not detectable.

Table 3. *Limiting Dilution Infection of H9, CEM, and PBMC Demonstrate a Reduction in Infectivity for Nef- HIV.*

Target cells	Source of virus§	$TCID_{50}$ value* at 100 ng/ml p24 (Fold reduction‡)		
		HXB Nef+	HXB ΔN-term	HXB fs
H9	COS transfection	12.6×10^3	4.6×10^3 (2.7)	NT‖
	H9 infection	2.5×10^3	1.4×10^3 (1.8)	1.1×10^3 (2.3)
CEM	COS transfection	8.4×10^3	1.7×10^3 (4.9)	NT
	H9 infection	2.5×10^3	0.90×10^3 (2.8)	0.75×10^3 (3.3)
PBMC	COS transfection	2.5×10^3	1.7×10^3 (1.5)	NT
Donor A	H9 infection	7.5×10^2	2.2×10^2 (3.4)	2.0×10^2 (3.8)
PBMC	COS transfection	6.7×10^3	1.3×10^3 (5.1)	NT
Donor B	H9 infection	1.3×10^2	0.29×10^2 (4.5)	0.23×10^2 (5.6)

* The $TCID_{50}$ value is the reciprocal of the dilution of 100 ng/ml *gag* p24 supernatant that results in the infection of 50% of the wells containing target cells. 10^4 (H9 and CEM) or 10^5 (PHA-activated PBMC) target cells were infected in sextuplicate and cultures were scored positive if the p24 concentration 14 d (H9 and CEM) or 21 d (PBMC) after infection was greater than 2 SD away from background. 50% endpoints were interpolated when necessary (24).
‡ Fold reduction in $TCID_{50}$ value of *nef* mutant viral stock as compared with the HXB Nef+ viral stock on the same target cells.
§ HIV stocks analyzed for infectivity were isolated from COS-7 cell transfections of proviral plasmid DNA 2 d after transfection and from H9 cells infected with the COS-7 cell virus stocks 11 d (Nef+), 13 d (fs) or 15 d (ΔN-term) after infection.
‖ NT, not tested.

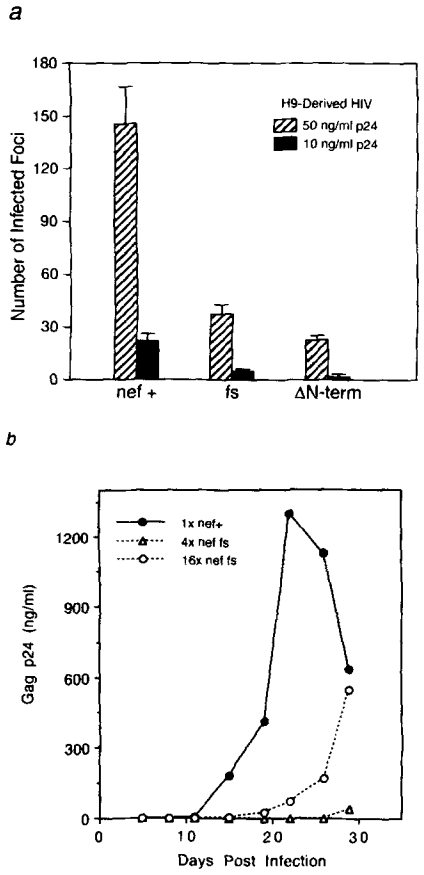

Figure 5. (*a*) Viral infectivity is reduced in Nef⁻ viral stocks of H9 cells. Viral infectivity was quantified in a single-cell infection assay utilizing a HeLa-CD4 cell line stably transfected with β-gal under the control of a Tat-dependent HIV-1 LTR. Cells in microtiter plates were infected with H9-produced HXB Nef⁺ and the two *nef* mutants, *nef* fs and ΔN-term, at 50 and 10 ng/ml of pelletable *gag* p24. 40 h after infection the cells were incubated with the β-gal substrate X-gal, and the number of infected cells expressing Tat was scored by light microscopy. Cell division events over the infection period resulted in daughter cells each expressing Tat and were counted as one infection event. Each infection was performed in triplicate and the standard deviation of the triplicate average is indicated above the bar. The *nef* fs mutant was 4.4-fold less infectious and the ΔN-term mutant 6.3-fold less infectious than the Nef⁺ virus at 50 ng/ml of p24. (*b*) PBMC infection infection normalized to viral infectivity with a 16-fold excess of Nef⁻ HIV demonstrates delayed viral replication kinetics. Unstimulated PBMC (20×10^6 cells) were infected with 4 ng/ml of HXB Nef⁺, 16 ng/ml HXB *nef* fs, or 64 ng/ml of HXB *nef* fs as described in the legend to Fig. 3. 1 d after infection the PBMC were activated with PHA and the culture supernatant monitored for the production of virus over time by *gag* p24 ELISA.

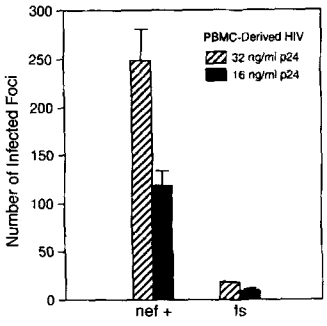

Figure 6. HIV produced during PBMC infection with Nef⁺ HIV is more infectious than that produced from PBMC infected with Nef defective HIV per ng of *gag* p24. Viral supernatants were harvested after infection of PHA-activated PBMC with 8 ng/ml of COS-7 cell produced HXB Nef⁺ and *nef* fs HIV on day 13 after infection. At this late time point there is comparable viral production in both cultures. The HeLa-CD4-LTR-β-gal cell line was then infected with either 32 or 16 ng/ml of each viral supernatant as described in the legend to Fig. 5, and blue foci were counted after 40 h of infection. The virus derived from the Nef⁺ PBMC infection was 13.5 times (at 32 ng/ml) or 17.5 times (at 16 ng/ml) more infectious than virus derived from the *nef* mutant PBMC infection.

tion in primary cells and provide evidence that the basis for the positive effect of Nef on viral replication is due to the increased infectivity of Nef⁺ viral particles. We now postulate that the lack of a Nef-associated replication phenotype in immortalized T cell lines is due to the greater ease with which HIV, even the less infectious Nef⁻ HIV, can spread in these highly permissive culture conditions using tissue-culture adapted HIV strains. Of note, however, are the differential effects of Nef on infection of both H9 and CEM cells observed in the presented $TCID_{50}$ studies. Thus, at extreme limiting dilution of virus some immortalized T cell lines can demonstrate a replication advantage for Nef⁺ HIV infection.

The nucleotide sequence of *nef* varies considerably from isolate to isolate, and some variants might conceivably encode dysfunctional gene products (30, 38). Of particular note is a repetitive sequence of variable length near the 5′ end of the *nef* gene, beginning at nucleotide 8464 (30). The length of this sequence depends on the isolate and can give rise to a 2–14-amino acid insertion in Nef. The HXB Nef⁺ HIV used in these studies does not contain such an insertion; however, the YU-2b recombinant contains a 10-amino acid insertion in this region. The capacity of the YU-2b recombinant *nef* also to enhance viral replication in PBMC and macrophages demonstrates that even a 10-amino acid insertion in this region does not impair the capacity of Nef to mediate enhanced replication. However, in analyses of Nef activity that are based on cell-surface CD4 down-regulation, dysfunctional alleles of *nef* have been identified (18, 39). The infectivity and replication assays described in this report can be harnessed to determine whether the capacity of Nef to down-regulate CD4 expression is related to the increased infectivity of Nef⁺ HIV particles. Moreover, an analysis of naturally occurring Nef isolates for functional capacity in vitro should reveal whether dysfunctional *nef* alleles are present in HIV-infected people. Given the reduced capacity of Nef-defective virus to infect and replicate, the presence of defective *nef* genes may play a role in determining the variable pathogenic courses observed in HIV-infected people.

Now with the consistent demonstration of a positive rather than a negative effect of Nef on the infection and replication of HIV in multiple cell types, including primary PBMC, primary macrophages, and antigen-specific T cell clones, as well as the positive effects of Nef on SIV_{mac} replication in vivo in monkeys, one can begin to determine the precise mechanism of the positive regulatory effects of Nef. It is possible that the increase in infectivity is related to the apparent capacity of Nef to modulate T cell activation and thymocyte ontogeny (18, 40). In the case of the Nef-transgenic mice,

the expression on Nef in thymocytes was associated with decreased cell surface CD4 expression, and elevated intracellular Ca^{2+} flux after anti-CD3 treatment. These two observations may be related as studies in T cell clones have demonstrated an increased sensitivity to TCR-mediated activation in the absence of CD4 (41), presumably due to the dissociation of CD4 from the tyrosine kinase $p56^{lck}$ (42, 43). In this regard, preliminary results were presented demonstrating a dissociation between CD4 and $p56^{lck}$ in Nef-expressing CEM cells (44). Thus, it is possible that a Nef-induced dysregulated intracellular activation environment might be more conducive to replication and the production of infectious HIV. However, the data presented herein demonstrate a broadly defined function of Nef that can be observed in multiple cell types, including macrophages that lack $p56^{lck}$. Most interestingly, the transfection of COS cells, a cell line which expresses neither CD4 or $p56^{lck}$, results in Nef^+ HIV particles which are more infectious than Nef^- HIV in both the $TCID_{50}$ and single-cell infectivity assays. Thus CD4 expression, per se, is not absolutely required for Nef-mediated enhancement of viral infectivity.

We feel that our results argue against a mechanism of Nef activity that is strictly dependent upon a particular cellular environment or upon a specific cellular process, e.g., T cell activation. In fact, although the most pronounced effect of Nef was demonstrated by exposing unstimulated cells to virus and subsequently activating these cells, we have not yet unequivocally established that resting cells are truly infected before activation. Previous reports have defined the capacity of resting T cells to be infected based on DNA PCR for evidence of early products of reverse transcription (45–47). However, two groups have subsequently shown the presence of such partial reverse transcripts within purified viral particles (48, 49), and this may have confounded the interpretation of the earlier PCR-based analyses of resting cell infection. In our DNA PCR analysis, we observed no evidence of reverse transcription beyond the level that can be defined within the viral particles that are simply absorbed to cell surface membranes (our unpublished observations). Rather, we interpret the more pronounced positive effects of Nef in this tissue culture setting to be due to a relatively inefficient infection process, which is more dependent upon intact Nef function for initial infection and viral amplification during the subsequent rounds of infection.

As the HeLa β-gal single-cell infectivity assay models only a portion of the HIV replication cycle, it defines a temporal restriction to Nef function within the replication cycle. Specifically, the results from the assay indicate that either the particles produced from cells infected or transfected with Nef^+ HIV are intrinsically more infectious, or that the post-binding events leading to Tat expression are facilitated in the case of Nef^+ HIV infection. As the Nef protein has not been found within purified virions and as *tat* and *nef* expression are concomitant in the newly infected cell (3), we predict that Nef most likely exerts its function within the virus-producing cells. Most simply, we speculate that Nef acts in the virus-producing cells to create a more highly infectious viral particle, perhaps by increasing the efficiency of gp120 incorporation into the viral membrane or in some other way affecting the assembly of complete and fully infectious viral particles. Thus, a careful analysis of the composition of Nef^+ and Nef^- HIV particles is warranted.

Although we have shown that CD4 expression is not required for Nef-mediated enhancement of viral infectivity, it is possible that the ability of many HIV-1 *nef* genes to down-regulate cell surface CD4 expression (16–18) may also contribute to the production of more highly infectious viral particles. Nef-mediated reduction of CD4 from the cell surface may result in a greater pool of envelope glycoproteins that are not associated with CD4 and that may, therefore, be successfully incorporated into the membranes of budding viral particles. In the absence of Nef-mediated CD4 down-regulation, the viral particles would contain a decreased number of envelope glycoproteins in the viral envelope and, hence, would be less infectious (50). Thus, the capacity of Nef to mediate CD4 down-regulation may be important in the production of infectious HIV. Alternatively, these functions of Nef may be distinct. We can now compare the genetic determinants of CD4 down-regulation and increased viral infectivity by Nef as a means of addressing this issue. Interestingly, HIV-1 *vpu* has also been shown to affect CD4 expression by mediating the degradation of CD4 bound to newly synthesized gp160 in the endoplasmic reticulum (51). Thus, HIV-1 appears to regulate cell surface CD4 expression via a multiple gene strategy that emphasizes the importance of regulating CD4 receptor expression in the viral life cycle.

As previously noted, infection of rhesus monkeys with Nef^+ and Nef-deleted SIV_{mac} demonstrated the establishment of significantly higher levels of viral burden, a greater extent of viral replication, and virus-induced pathogenicity in animals infected with the Nef^+ virus (21). Moreover, the data from that study demonstrated a strong selective pressure for reversion to a functional *nef* gene, which correlated with an increased in vivo viral burden. The authors inferred that the in vivo replication of SIV must be enhanced in the presence of an intact or reverted *nef* gene. Curiously, the authors were not able to demonstrate any clear enhancement in viral replication through infection of activated primary macaque PBMC, even at low MOI. In view of our results, infection of unstimulated macaque PBMC with Nef^+ and Nef^- SIV might have been more revealing. We predict that Nef^- SIV, like Nef^- HIV, would be less infectious than Nef^+ virus, and surmise that this decrease in infectivity could greatly debilitate viral spread in vivo. Thus, a seemingly minor 5- to 10-fold decrease in infectivity of the Nef^- virus might result in dramatically reduced viral spread and pathogenicity in an in vivo environment where it was also opposed by an antiviral immune response. In parallel with the SIV model, we propose that HIV-1 Nef may function in vivo to increase viral infectivity and viral burden in HIV-infected humans, and that this would be linked to viral pathogenesis. In support of this, the SCID-Hu mouse model of HIV infection has yielded preliminary results confirming a greater in vivo viral burden and more accelerated pathogenic

consequences as a result of infection with a Nef$^+$ HIV in the absence of a significant immune response (52). Given these pathologic and viral replication results in model systems, it is possible that specific therapeutic approaches designed to inhibit HIV-1 Nef function in vivo may one day be of value in the treatment of HIV-1 infected people. The definition of reproducible tissue culture assays for Nef augmentation of viral replication provide an important starting point for the development of potential therapeutic agents.

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