HIV-1), HXB2 (X4 HIV-1) or amphotropic murine leukemia virus by transfecting 293T cells with plasmids encoding them, as described previously (23). Single-round viral replication assays were performed as described previously (23).

Multiple-round viral replication assays were performed with HIV-1 89.6 that was prepared by 293T cell transfection with the respective molecular clones, as described previously (21). Viral replication was assessed by reverse transcriptase assays for cell-free supernatants, as described previously (21).

Plasmids and transfection. Plasmid pGL-HIV-1-LTR contains the HIV-1 long terminal repeat (LTR) from strain HXB2 in pGL2-basic (Promega, Madison, Wis.) backbone. LTR sequence was truncated at −116 relative to transcription start site in pGL-HIV-1-LTR(−116), while NF-κB and SP1 binding sites on HIV-1 LTR were deleted in plasmids pGL-HIV-1-LTRΔNF-κB and pGL-HIV-1-LTRΔSP1, respectively (16). Plasmids pCMV-HA/Smad2 and pCMV-Flag/Smad3 were kindly provided by J. L. Wrana (Mount Sinai Hospital, Toronto, Canada) (11), and pCMV-hFAST1 was a gift of B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, Md.) (32). Plasmid pHTLV-luc contains the human T-cell leukemia virus type I (HTLV-I) LTR promoter followed by the luciferase gene (10). One-day-old MCF-7 cell monolayer or 5-day-old monocyte-derived macrophages monolayer were transfected with modified calcium phosphate method, and luciferase assays were performed, as described previously (20, 22).

Reagents. Human lactoferrin were purchased from Sigma Chemical Co., and recombinant human TGF-β2 and polyclonal neutralizing anti-TGF-β antibody were purchased from R&D Systems (Minneapolis, Minn.). Early (<2weeks after delivery) and mature (>4 weeks after delivery) breast milk samples were donated from three healthy breastfeeding mothers. After centrifugation of the samples at 14,000 × *g* for 15 min, milk whey was separated from solid precipitate and stored at −80°C before use.

Nuclear extracts and gel mobility shift assays. Nuclear extracts were prepared from MCF-7 cells or monocyte-derived macrophages that had been untreated or treated with TGF-β (200 pg/ml), as described previously (19). Gel mobility shift assays were performed as described previously (19), with oligonucleotides corresponding to sequences spanning HIV-1 LTR NF-κB binding sites or SP1 binding sites as primers. Antibodies to NF-κB p50 or p65 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

Real-time RT-PCR. MCF-7 cells untreated or treated with TGF-β or milk whey were harvested, total RNA was extracted with a QIAamp RNA kit (Qiagen, Tokyo, Japan) and measurement of induced IκB kinase mRNA levels was performed by real-time RT-PCR with the iCycler iQ real-time detection system (Bio-Rad Laboratories, Inc., Hercules, Calif.) and QuantiTect SYBR Green RT-PCR kit (Qiagen). Primers were 5′-CCCAGCCCTACACGAAAGGACCTGC TTCTC-3′ and 5′-TCAGACATCAGGAGGTGCTGGGACTCTAT-3′. Reactions were incubated at 50°C for 30 min and then 95°C for 15 min, followed by 50 cycles of 95°C for 15 s and 62°C for 60 s. IκB kinase mRNA levels were normalized to tubulin mRNA by calculating the IκB kinase and tubulin ratio for each sample.

RESULTS

MCF-7 cells are susceptible to HIV-1 infection. It has been shown previously that primary mammary epithelial cells as well as certain breast cancer cell lines are susceptible to HIV-1. Therefore, we first tested whether MCF-7 cells are capable of supporting HIV-1 infection. To avoid any soluble factor derived from lymphocytes or macrophages and to precisely delineate susceptibility to the HIV-1 phenotypes, we performed single-round viral replication assays with HIV-1 molecular clone stocks prepared from 293T cell culture supernatants. As shown in Fig. 1A, MCF-7 cells supported infection with certain HIV-1 strains, although infection efficiency was far less than that of amphotropic murine leukemia virus. Compared to monocyte-derived macrophages that were more susceptible to R5 HIV-1 than to X4 HIV-1 (Fig. 1B) or peripheral blood lymphocytes that were susceptible comparably to R5 and X4 HIV-1 (Fig. 1C), MCF-7 cells apparently were more susceptible to dual (R5X4) or X4 HIV-1 than to R5 HIV-1. Since HIV-1 89.6 replicated comparably in all cell types tested, the following experiments were performed exclusively with this virus.

TGF-β inhibits HIV-1 infection of MCF-7 cells but enhances that of monocyte-derived macrophages. Next, we investigated

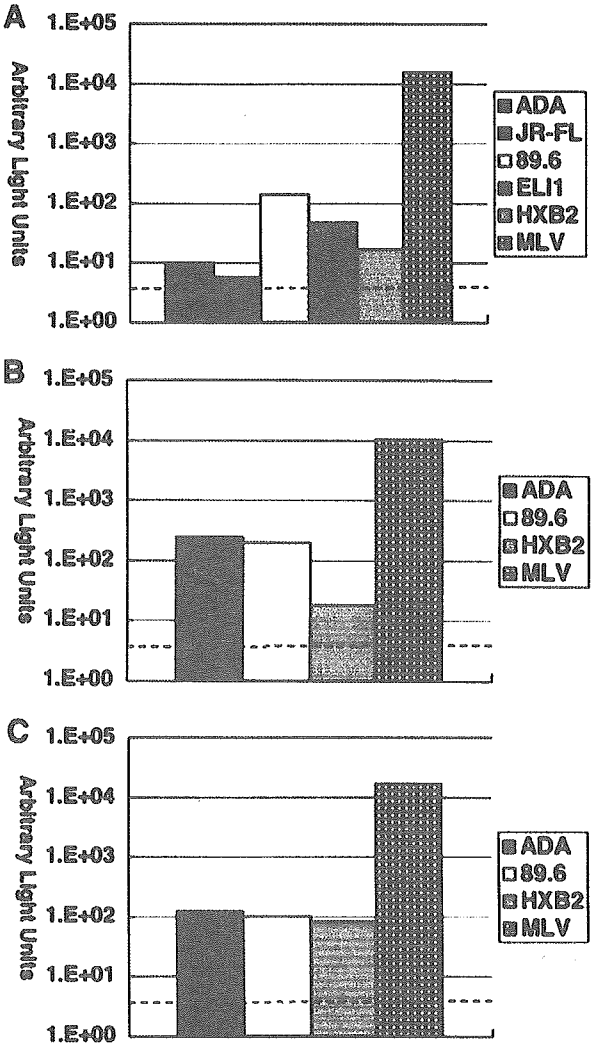

FIG. 1. Susceptibility of breast milk cell analogues to HIV-1 infection. In single-round viral replication assays, MCF-7 cells (A), monocyte-derived macrophages (B), and peripheral blood lymphocytes (C) were infected with NL4-3-Luc-R^-E^- (3×10^5 cpm of reverse transcriptase activity each) supplemented with Env glycoprotein derived from the indicated strains, and the infected cell lysates were subjected to luciferase assays. A broken line indicates background luciferase activity. Representative results from three independent experiments are shown.

whether exposure to milk whey or its components can influence the susceptibility of mammary epithelial MCF-7 cells to HIV-1. While treatment of cells with crude milk whey or lactoferrin, a known anti-HIV-1 factor, suppressed HIV-1 infection, TGF-β had a minimal effect when added to MCF-7 cells prior to infection. In contrast, posttreatment with TGF-β as well as crude milk whey suppressed HIV-1 infection (Fig. 2A), suggesting that TGF-β has inhibitory effects on a postentry step(s) of the HIV-1 replicative cycle in MCF-7 cells. The inhibitory effect of crude milk whey posttreatment was partially restored by neutralizing anti-TGF-β antibody, suggesting that TGF-β played a role in that effect.

We also investigated the effects of milk whey or TGF-β on HIV-1 infection of macrophages, another major source of HIV-1

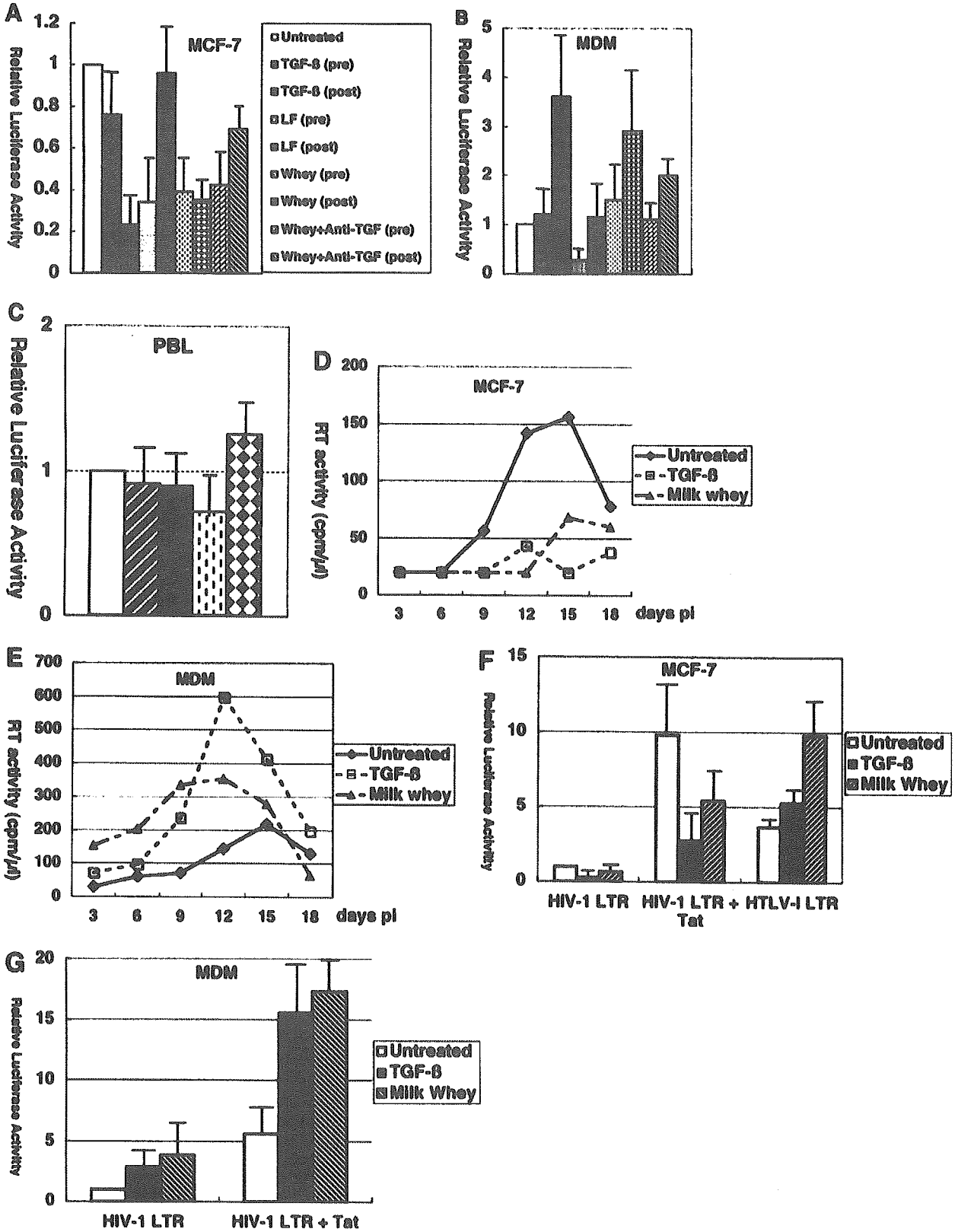

FIG. 2. Cell type-dependent effects of TGF-β on HIV-1 infection. (A, B, and C) In single-round viral replication assays, TGF-β inhibited HIV-1 infection of MCF-7 cells, enhanced HIV-1 infection of monocyte-derived macrophages at a postentry step(s), and had a minimal effect on HIV-1 infection of peripheral blood lymphocytes. MCF-7 cells (A), monocyte-derived macrophages (B), and peripheral blood lymphocytes (C) were either untreated or treated for 16 h before infection (pre) or treated after infection (post) with NL4-3-Luc-R^-E^- supplemented with Env

in breast milk. In striking contrast to HIV-1 infection of MCF-7 cells, posttreatment of monocyte-derived macrophages with crude milk whey or TGF-β enhanced HIV-1 infection (Fig. 2B). The effect mediated by posttreatment with crude milk whey was reduced to some extent by neutralizing anti-TGF-β antibody, suggesting that TGF-β contributed to that effect. Pretreatment of monocyte-derived macrophages with either reagent had a minimal effect.

TGF-β as well as crude milk whey had a minimal effect on HIV-1 infection of peripheral blood lymphocytes (Fig. 2C) at the concentration used (200 pg of TGF-β per ml and 10% crude milk whey).

Since crude milk whey or TGF-β appears to have dichotomous effects on HIV-1 infection, depending on cell types and timing of treatment, we tested their effects on multiple rounds of HIV-1 replication. Cells were treated with the reagents for 16 h before infection and throughout the experiments. As shown in Fig. 2D and 2E, TGF-β suppressed HIV-1 replication in MCF-7 cells but enhanced that in monocyte-derived macrophages.

Crude milk whey samples derived from three different donors had similar effects, and results obtained with early and mature milk were comparable (data not shown). Our early and mature milk whey samples contained 1.5 to 2.5 ng and 1.0 to 2.0 ng of TGF-β2 per ml, respectively (data not shown). Since 10% milk whey was used in our experiments, the final concentration of TGF-β would be approximately 200 pg/ml. Therefore, although breast milk must be diluted in baby's gastrointestinal tract, we consider that concentrations of milk whey (10%) and TGF-β (200 pg/ml) were physiologically relevant.

Thus, the net effects of crude milk whey and TGF-β on HIV-1 infection may be negative in MCF-7 cells and positive in monocyte-derived macrophages.

TGF-β downregulates HIV-1 LTR activity in MCF-7 cells but upregulates it in monocyte-derived macrophages. Previous studies have demonstrated that TGF-β upregulates HIV-1 LTR activity in mesangial cells and murine B cell lines. However, since TGF-β plays pleiotropic roles, depending on the cell types involved and combinations with other factors, we tested its effect on HIV-1 LTR activity in MCF-7 cells. As shown in Fig. 2F, HIV-1 LTR activity, either basal and Tat induced, was downregulated by TGF-β in MCF-7 cells. That effect was specific, because TGF-β did not have inhibitory effect on an irrelevant promoter like the HTLV-I LTR at this concentration.

Macrophages are another major cellular components in breast milk and probably serve as an important source of HIV-1 in this body fluid. In similar experiments, TGF-β upregulated basal and Tat-induced HIV-1 LTR activity in monocyte-derived macrophages (Fig. 2G).

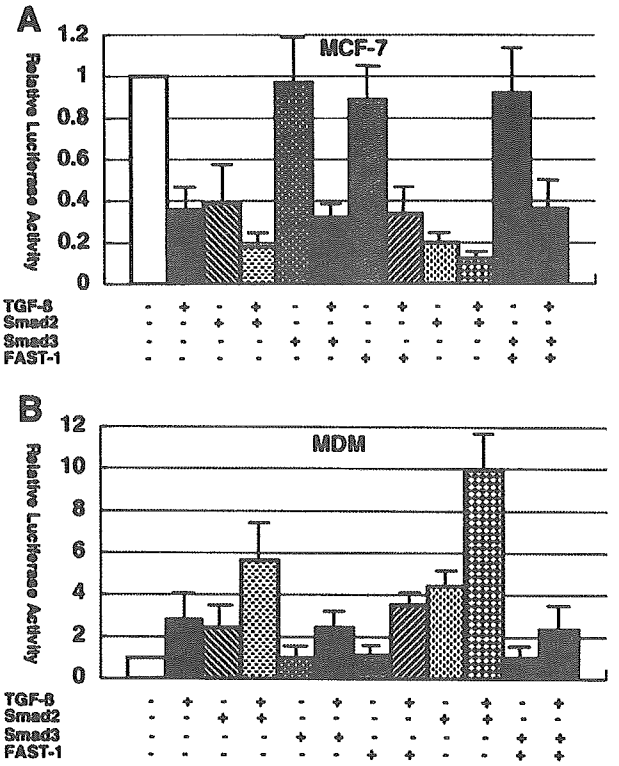

FIG. 3. Augmentation of TGF-β-mediated effects by FAST-1 and Smad2. MCF-7 cells (A) and monocyte-derived macrophages (B) were transfected with pGL-HIV-1-LTR along with pcDNA3.1 or expression vector of the indicated factor and either untreated or treated with TGF-β (200 pg/ml). Results are shown as luciferase activity relative to that of pGL-HIV-1-LTR and pcDNA3.1 when untreated. Error bars represent standard deviations from triplicate experiments.

Overexpression of FAST-1 and Smad2 but not Smad3 augmented TGF-β-mediated effects. TGF-β conveys its signal from membrane receptors into the nucleus by specific mediators called SMADs (reviewed in 11). SMADs activation can lead to transcriptional activation through direct binding to specific DNA sequences and association with another transcription factor such as FAST-1 (32). To further investigate mechanisms whereby TGF-β exerts its effects on HIV-1 LTR, those mediators were overexpressed in transient expression assays.

Cotransfection of Smad2, FAST-1, or both with pGL-HIV-1-LTR augmented TGF-β-mediated effects, downregulation in MCF-7 cells, and upregulation in monocyte-derived macrophages (Fig. 3A and 3B). However, Smad3 alone and in combination with FAST-1 barely influenced HIV-1 LTR activity.

glycoprotein from 89.6 with TGF-β (200 pg/ml), human lactoferrin (LF) (100 ng/ml), or crude milk whey (10%) with and without anti-TGF-β antibody (10 μg/ml). Cells were harvested 48 h after infection, and cell lysates were subjected to luciferase assays. Arbitrary light units are shown. Error bars represent standard deviations from duplicate experiments. (D, E) In multiple-round viral replication assays, TGF-β inhibited HIV-1 infection of MCF-7 cells and enhanced HIV-1 infection of monocyte-derived macrophages. Approximately 2×10^5 MCF-7 cells (D) or 4×10^5 monocyte-derived macrophages (E) were untreated or treated with TGF-β (200 pg/ml) or crude milk whey (10%) for 16 h before and continuously after infection with HIV-1 89.6. The experiments were repeated twice with similar results. Another experiment in which monocyte-derived macrophages were also infected with ELI1 also gave similar results (data not shown). (F, G) Cell type-dependent effects of TGF-β on HIV-1 LTR activity. MCF-7 cells (F) and monocyte-derived macrophages (G) were transfected with pGL-HIV-1-LTR and pSV2-CAT, pGL-HIV-1-LTR and pSV2-Tat, or pHTLV-I-luc and either untreated or treated with TGF-β (200 pg/ml) or crude milk whey (10%). Results are shown as luciferase activity relative to that of pGL-HIV-1-LTR and pSV2-CAT when untreated. Error bars represent standard deviations from triplicate experiments.

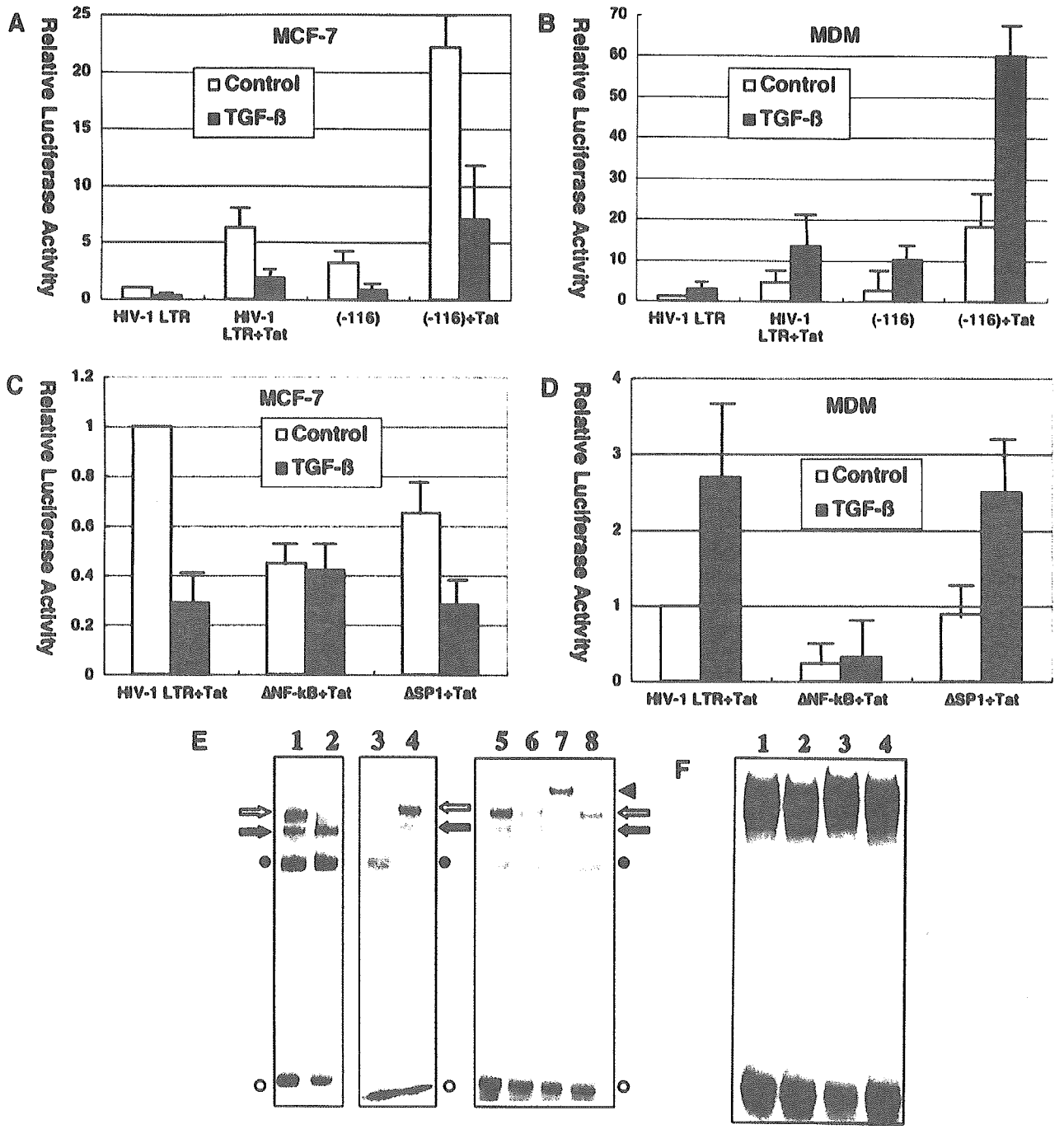

FIG. 4. TGF-β effects on HIV-1 infection are mediated by NF-κB activity. (A to D) Critical role of NF-κB binding sites in TGF-β-mediated effects on HIV-1 LTR. MCF-7 cells (A) or monocyte-derived macrophages (B) were transfected with pGL-HIV-1-LTR or pGL-HIV-1-LTR(−116) along with pSV2-Tat and either untreated or treated with TGF-β (200 pg/ml). Also, MCF-7 cells (C) or monocyte-derived macrophages (D) were transfected with pGL-HIV-1-LTR, pGL-HIV-1-LTRΔNF-κB, or pGL-HIV-1-LTRΔSP1 along with pSV2-Tat and either untreated or treated with TGF-β (200 pg/ml). Error bars represent standard deviations from triplicate experiments. (E, F) TGF-β effects on NF-κB binding to HIV-1 LTR. (E) ^{32}P-labeled oligonucleotides corresponding to sequences spanning the HIV-1 LTR NF-κB binding sites were incubated with nuclear extracts (15 μg of protein) from MCF-7 cells (lanes 1, 2, and 5 to 8) or monocyte-derived macrophages (lanes 3 and 4) and analyzed by gel mobility shift assays. Those cells were either untreated (lanes 1 and 3) or treated (lanes 2 and 4 to 8) with TGF-β (200 pg/ml) for 10 min before harvest. In addition, anti-p65 antibody (lane 6), anti-p50 antibody (lane 7), or control serum (lane 8) was added to the reaction. An open arrow and a solid arrow indicate the p50/p65 heterodimer (NF-κB) and p50 homodimer, respectively. An open circle and a solid circle indicate free probe and nonspecific complex, respectively. A solid triangle indicates a complex supershifted by anti-p50 antibody. (F) ^{32}P-labeled oligonucleotides corresponding to sequences spanning the HIV-1 LTR SP1 binding sites were incubated with nuclear extracts from MCF-7 cells (lanes 1 and 2) or monocyte-derived macrophages (lanes 3 and 4) and analyzed by gel mobility shift assays. Those cells were either untreated (lanes 1 and 3) or treated with TGF-β (200 pg/ml) for 10 min before harvest (lanes 2 and 4).

Thus, Smad2-FAST-1 association apparently plays a role in TGF-β-mediated effects on HIV-1 LTR. It has been shown that hFAST-1 is expressed in all normal human tissues including breast tissue and mediates responses to TGF-β by interacting with Smad2 and Smad4 (32).

Next, we investigated whether Smad2-FAST-1 complex can bind to HIV-1 LTR; however, extensive search with gel mobility shift assay using a panel of oligonucleotides spanning HIV-1 LTR sequence failed to identify a Smad2-FAST-1 binding site(s) (data not shown). Therefore, TGF-β/Smad2/FAST-1 may mediate its effects on HIV-1 LTR indirectly, possibly through transcriptional regulation of other gene(s). Alternatively, TGF-β may mediate SMAD-independent signaling pathway (reviewed in 4).

NF-κB binding sites are critical for TGF-β-mediated effect on HIV-1 LTR. As mentioned above, TGF-β-mediated effects on HIV-1 LTR may be independent of direct binding of SMADs/FAST-1 to HIV-1 LTR and TGF-β signaling may exert its effects on HIV-1 LTR through other *cis*-acting element(s). To demonstrate which *cis*-acting element(s) are involved in TGF-β-mediated effects, a panel of HIV-1 LTR-reporter constructs were tested in transient expression assays. Since a 5′-truncation to −116 relative to transcription start site had little effect on TGF-β-mediated activity (Fig. 4A and 4B), we focused on NF-κB binding sites and SP1 binding sites, major elements located downstream of −116. Deletion of SP1 binding sites had little effect, while deletion of NF-κB binding sites almost abrogated TGF-β-mediated activity in both MCF-7 cells and monocyte-derived macrophages (Fig. 4C and 4D).

To confirm the aforementioned results obtained from transient expression assays, we performed gel mobility shift assays using nuclear extracts from MCF-7 cells that had been untreated and treated with TGF-β. Untreated MCF-7 cells had weak but detectable NF-κB activity. TGF-β treatment of MCF-7 cells abrogated NF-κB activity on HIV-1 LTR, but had little effect on SP1 activity (Fig. 4E and 4F). On the contrary, NF-κB activity in nuclear extracts from untreated monocyte-derived macrophages was enhanced in those from TGF-β-treated monocyte-derived macrophages (Fig. 4E and 4F). It is noteworthy that TGF-β stimulation of HIV-1 LTR through NF-κB activity has been shown in HaCat cells (human keratinocytes) and 300.19 (mouse pre-B) cells (14). These results indicate that TGF-β mediates both inhibitory and stimulatory effects on HIV-1 LTR, at least in part, through the NF-κB pathway.

TGF-β downregulates expression of IκB kinase. In a recent study, TGF-β has been shown to downregulate expression of IκB kinase in mouse mammary epithelial cells. IκB kinase is induced upon various stimuli, degrades IκB, and induces NF-κB activity. To demonstrate whether TGF-β is capable of downregulating IκB kinase expression in MCF-7 cells, real-time RT-PCR was performed. As shown in Fig. 5, TGF-β treatment reduced IκB kinase mRNA levels in MCF-7 cells in a dose-dependent manner but had minimal effect in monocyte-derived macrophages. Thus, IκB kinase appears to be one of the targets of TGF-β action on mammary epithelial cells.

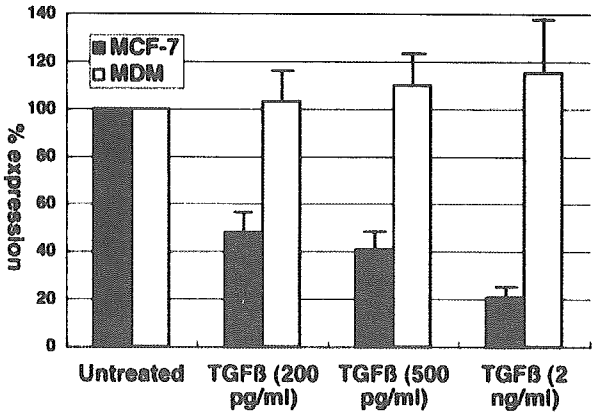

FIG. 5. Regulation of IκB kinase gene expression by TGF-β. MCF-7 cells (solid bars) or monocyte-derived macrophages (open bars) were either untreated or treated with the indicated amount of TGF-β for 60 min, and IκB kinase mRNA levels were determined by real-time RT-PCR. In untreated MCF-7 cells and monocyte-derived macrophages, IκB kinase mRNA levels were approximately 0.1% and 0.3%, respectively, of that of the housekeeping gene tubulin mRNA. The change in expression is shown as expression relative to that in untreated cells. Error bars represent standard deviations from triplicate experiments.

DISCUSSION

Although milk-borne infection accounts for a considerable number of mother-to-child transmissions of HIV-1, the precise molecular and cellular mechanisms by which maternal cell-derived virus is transmitted through the gastrointestinal mucosa to infants remain obscure. In addition to macrophages and lymphocytes, mammary epithelial cells are thought to be the sources of HIV-1 in breast milk. Interestingly, the susceptibility of those cells to HIV-1 strains differs: macrophages support replication of R5-tropic HIV-1 much better than that of X4-tropic HIV-1, lymphocytes support replication of R5- and X4-tropic HIV-1 equally well, and mammary epithelial cells support replication of X4-tropic rather than R5-tropic HIV-1 (18, 28, 30). It is, however, unclear which cell type plays a major role in mother-to-child transmission.

A recent study has shown a differential distribution of HIV-1 variants between breast milk and peripheral blood: the major variant in one compartment corresponded to a minor variant in the other compartment (2). Such observations suggest that a host factor(s) in breast milk can modulate HIV-1 infection. Interestingly, while peripheral blood often harbored both R5- and X4-tropic viruses, HIV-1 variants in breast milk were always R5 tropic (2). Therefore, a breast milk-derived host factor(s) may favor replication of R5-tropic HIV-1 but not X4-tropic HIV-1.

In this study, we identified TGF-β, a major cytokine in breast milk, as a bifunctional modulator of HIV-1 infection. TGF-β favored HIV-1 infection of macrophages but suppressed that of mammary epithelial MCF-7 cells. The pro-HIV-1 effect of TGF-β on macrophages was already reported (13), but it has not been demonstrated how TGF-β influences HIV-1 infection of mammary epithelial cells. TGF-β appears to have dichotomous effects on HIV-1 infection of lymphocytes: it enhanced HIV-1 infection at 10 ng/ml or lower, but suppressed it at 1 ng/ml or higher (26). Peripheral blood lymphocytes were relatively refractory to TGF-β at the concentration (200 pg/ml) used in this study. Since the TGF-β concentration in breast milk is as high as 1 to 2 ng/ml, HIV-1 infection of lymphocytes may be inhibited in this setting.

Thus, the milk-borne cytokine TGF-β may be most beneficial to HIV-1 infection of macrophages, and therefore R5-tropic HIV-1 predominance in breast milk and preferential mother-to-child transmission of R5-tropic HIV-1. However, breast milk contains a number of host factors that variably modulate HIV-1 infection. Lactoferrin inhibits replication of both R5- and X4-tropic HIV-1 in various cell types (24). Prostaglandin E2 inhibits or enhances HIV-1 infection, depending on the virus strains, cell types infected, and the timing of treatment (5, 29). Interestingly, an identified factor in breast milk that is sensitive to cathepsin D has been shown to enhance X4 HIV-1 infection of MCF-7 cells (18). Thus, it appears that the net effect of breast milk on HIV-1 infection depends on the balance of those pro- and anti-HIV-1 factors. Further extensive studies are required for a better understanding of the interaction between HIV-1 and milk-derived host factors.

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Norepinephrine inhibits human immunodeficiency virus type-1 infection through the NF-κB inactivation

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Abstract

Exercise or acute stress can exert significant effects on immune system as well as cardiovascular and respiratory systems through catecholamines. In this study, we investigated effects of norepinephrine (NE), a catecholamine neurotransmitter on human immunodeficiency virus type-1 (HIV-1) infection. NE inhibited in vitro HIV-1 infection of peripheral blood mononuclear cells (PBMC) from healthy donors and ex vivo HIV-1 replication in patients' PBMC. In transient expression assays, NE downregulated HIV-1 long terminal repeat, but site-directed mutagenesis on NF-κB-binding sites or cotreatment with H89 (a protein kinase A inhibitor) abrogated the NE-mediated effect. Gel-shift assays showed suppression of NF-κB activity in NE-treated cells. NE increased cytoplasmic levels of IκB-α, a natural inhibitor of NF-κB. Thus, NE apparently inhibits HIV-1 infection, at least in part through NF-κB inactivation.

Keywords: HIV-1; Norepinephrine; Catecholamines; Stress; NF-κB; Long terminal repeat

Introduction

A number of host, viral and environmental factors have been shown to influence the pathogenesis of human immunodeficiency virus type-1 (HIV-1) disease. Events in daily lives may thus have some impacts on HIV-1 infection through interactions between those factors. Exercise as well as acute psychological stress leads to activation of the sympathetic nervous system (SNS), which is mediated by neurotransmitters such as norepinephrine (NE). NE exerts not only cardiotonic and bronchodilating actions but also anti-inflammatory effects.

A number of previous studies have demonstrated that β-adrenergic agonists suppress production of proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β and IL-6 (Cole et al., 1998; Farmer and Pugin, 2000; Heneka et al., 2003; Koff et al., 1986; Severn et al., 1992; Talmadge et al., 1993; Van der Poll et al., 1994). Since the pathogenesis of HIV-1 infection is deeply involved in immune activation, NE may influence HIV-1 infection as well as cytokine networks. In this study, we show that NE inhibited HIV-1 infection, at least in part, by downregulation of NF-κB activity.

Results and discussion

NE suppresses HIV-1 replication

NE effects on HIV-1 infection were investigated in two systems. In in vitro or acute infection system, peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) were isolated from healthy individuals, and were infected in vitro with R5 (AD8)- or X4 (NL4-3)-HIV-1. PBMC that had been prestimulated with anti-CD3 and anti-CD28 antibodies were treated with NE prior to infection and throughout the incubation period. The concentrations of NE used in these experiments were between 10 nM and 1 μM, since serum NE levels reach 10 nM upon activation of the SNS (Bierhaus et al., 2003) and NE is released neuronally into the

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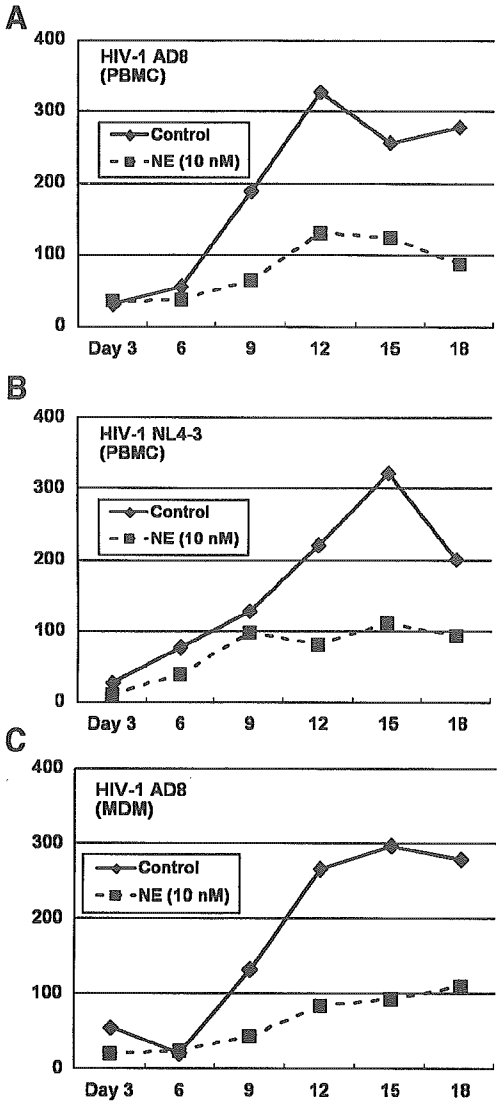


Fig. 1. NE inhibited HIV-1 infection of PBMC or MDM. (A, B) PBMC from a healthy donor were prestimulated with anti-CD3 and anti-CD28 antibodies for 2 days and infected with HIV-1 AD8 (A) or HIV-1 NL4-3 (B). One set of culture was also treated with NE (10 nM) 24 h prior to and after infection and RT assays were performed for cell-free culture supernatants. Similar results were obtained from 3 different donors (data not shown). (C) MDM from a healthy donor were either untreated or treated with NE (10 nM) 24 h prior to and after infection with HIV-1 AD8, and RT assays were performed for cell-free culture supernatants. Similar results were obtained from 3 different donors (data now shown).

lymphoid tissue at micromolar levels (Felten et al., 1987). As shown in Fig. 1, HIV-1 replication was reduced in the presence of 10 nM of NE, irrelevantly to HIV-1 phenotypes (R5 versus X4) or target cells (PBMC versus MDM). Higher concentrations of NE (100 nM or 1 μM) had slightly more potent anti-HIV-1 effects, but prolonged (>1 week) exposure to NE higher than 10 nM was variably toxic to PBMC in trypan blue exclusion assays and 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assays (data not shown); therefore, higher concentrations were not applied to infection experiments thereafter. In contrast, percentages (mean ± SD) of viable cells in the presence of NE (10 nM) were comparable to those in the absence of NE (86% ± 8% versus 89% ± 9%).

NE effects were also investigated in PBMC derived from HIV-1-infected individuals, some of which had been on highly active antiretroviral therapy (HAART) (Table 1). Ex vivo stimulation of $CD8^+$ T cell-depleted PBMC with anti-CD3 and anti-CD28 induced endogenous HIV-1 replication; however, cotreatment with NE precluded such HIV-1 outgrowth (Table 1). Thus, NE appears to suppress HIV-1 infection in both in vitro (acute) and ex vivo (endogenous) experimental conditions.

To demonstrate whether NE-mediated effect is specific to HIV-1, we also tested NE effects on infection with human T-cell leukemia virus type I (HTLV-I), another human retrovirus. PBMC derived from asymptomatic HTLV-I carriers were incubated in the presence or absence of NE (10 nM), and release of HTLV-I virions into culture medium as well as proviral load in cells was determined. NE did not suppress ex vivo HTLV-I infection, and contrarily, did enhance it slightly (Table 2). Therefore, NE-mediated anti-HIV-1 activity does not appear to be a non-specific effect.

To investigate at which step(s) in the HIV replication cycle NE exerts its influence, we performed single-round viral replication assays using replication-incompetent luciferase-reporter molecular clone NL43-Luc-R^-E^- that had been complemented with R5-tropic (ADA), X4-tropic (HXB2) or amphotropic (murine leukemia virus [MLV]) Env glycoprotein. Either pretreatment or post-treatment with NE inhibited HIV-1 infection of anti-CD3/anti-CD28-stimulated PBMC or MDM in physiologically relevant concentrations in serum (10 nM) or the lymphoid tissue (1 μM), and NE had similar influence on R5-tropic, X4-tropic or amphotropic virus (Figs. 2A–B).

Table 1
NE suppressed HIV-1 outgrowth by cellular activation

Patient no. (age/gender)	Antiviral therapy	Viral RNA (copies/ml)	CD4 T cell counts (cells/mm^3)	RT activity (cpm/μl)		
				No stimulation	Anti-CD3/CD28	Anti-CD3/CD28 + NE
1 (57 M)	ZDV/3TC/NFV	<50	802	<40	224	<40
2 (54 F)	ZDV/ddC/NFV	<50	432	<40	333	<40
3 (34 M)	No therapy	6900	592	<40	1264	168
4 (25 M)	No therapy	4400	451	<40	658	<40

ZDV, zidovudine; 3TC, lamivudine; ddC, zalcitabine; NFV, nelfinavir.
Approximately one million CD8-depleted PBMC derived from the above patients were incubated in the presence or absence of anti-CD3 and anti-CD28 antibody ± NE (10 nM). Maximal RT activities obtained from cell-free supernatants during 21-day culture period are shown.

Table 2
NE slightly enhanced HTLV-I replication

Patient no.	Proviral load (copies/100 PBMC)		p19 (pg/ml)	
	No stimulation	NE (10 nM)	No stimulation	NE (10 nM)
1	0.24	0.30	65	80
2	0.12	0.29	<25	62
3	0.082	0.19	<25	<25
4	0.82	0.91	286	342

Approximately one million PBMC were incubated in the presence or absence of NE (10 nM). Cell-free culture supernatants were collected for p19 assays, and DNA was extracted from cell pellets for real-time PCR of HTLV-I provirus.

To further investigate at which step(s) in the HIV replication cycle NE exerts its influence, we quantified HIV-1 proviral DNA loads 24 h after infection by real-time PCR. Pretreatment of PBMC or MDM with NE had little effect on HIV-1 proviral DNA loads in those experiments (Fig. 2C), indicating that NE could not inhibit HIV-1 entry into cells. Furthermore, NE treatment had little effects on cell surface expression of CD4, CCR5 or CXCR4 (data not shown). Those results suggested that post-entry step(s) are involved mostly in NE-mediated suppression of HIV-1 infection.

NE downregulates HIV-1 long terminal repeat (LTR) promoter

To investigate mechanisms whereby NE inhibits HIV-1 infection, we tested NE effects on HIV-1 transcription, one of the most critical post-entry steps of viral replicative cycle.

NE downregulated HIV-1 LTR activity in either cell type (Figs. 3A–B; data not shown), and deletion of NF-κB-binding sites, but not of SP1-binding sites abrogated the NE-mediated effect (Figs. 3A–B).

NE-mediated downregulation of HIV-1 LTR involves inhibition of NF-κB activity

Since the aforementioned results suggested that NF-κB-binding sites were required for NE-mediated downregulation of HIV-1 LTR activity, NF-κB activity was determined by gel-mobility shift assay in nuclear extracts from NE-treated or untreated THP-1 cells. THP-1 cells had constitutional NF-κB activity (formation of p50/p65 heterodimer) (Fig. 3C), which was enhanced by stimulation with phorbol 12-myristate 13-acetate (PMA) (Fig. 3D); however, NE treatment markedly suppressed p50/p65 heterodimer formation in a dose-dependent manner (Figs. 3D–E). In contrast, NE had no or little effect on SP1 activity (Fig. 3D).

Inhibition of NF-κB activity could result either from competition of NF-κB-binding sites on HIV-1 LTR with other transcription factor(s) or from the regulation by its natural inhibitor, IκB-α. If the former postulation was the case, gel-mobility shift assays should have shown formation of a novel complex on the NF-κB element. However, since we only observed diminishment of NF-κB complex, not formation of a novel complex, downregulation of NF-κB activity by IκB-α is more likely. To verify this postulation, two experiments were done. First, transient expression assays were performed in the presence of NE ± H89, a protein kinase A (PK-A) inhibitor. It has been shown that signaling of NE or other β-agonists via β-adrenergic receptor is mediated by PK-A, and that PK-A inhibitors prevent such activity. As expected, NE-mediated downregulation of HIV-1 LTR was significantly neutralized by H89 (Fig. 3F). Second, Western blot analysis was performed to determine cytoplasmic IκB-α levels inTHP-1 cells that had been untreated or treated with NE ± H89. As shown in Fig. 3G, NE treatment increased cytoplasmic IκB-α levels, but H89 counteracted NE. Those results suggested that NE down-

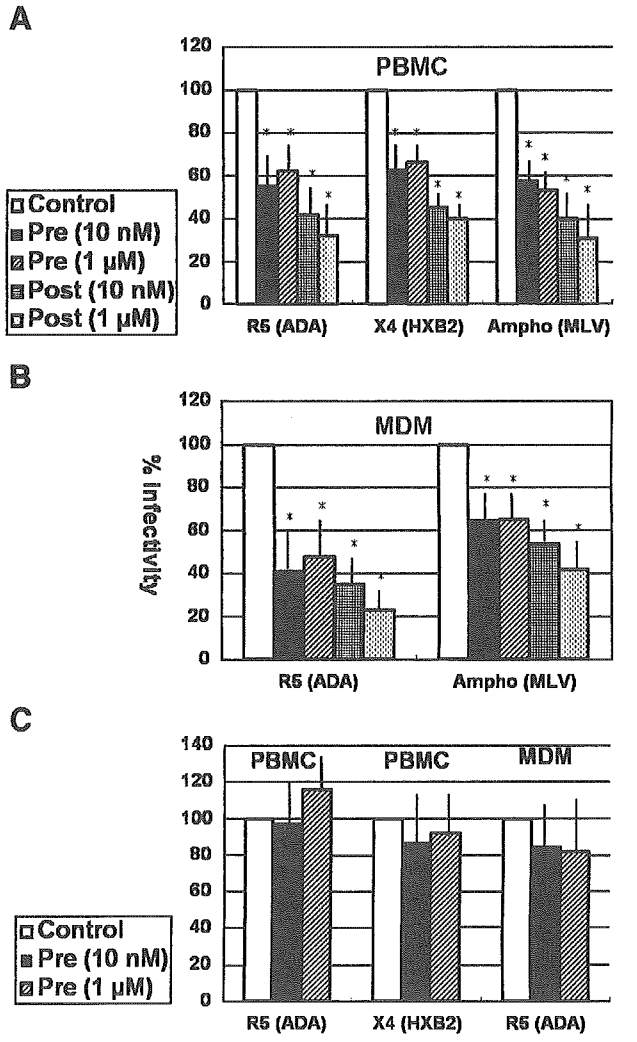


Fig. 2. NE inhibited HIV-1 infection at post-entry step(s). (A, B) Either pretreatment or post-treatment with NE inhibited HIV-1 infection. PBMC prestimulated with anti-CD3 and anti-CD28 (A) or MDM (B) from healthy donors were incubated with HIV-1 NL4-3-Luc-R$^-$E$^-$ complemented with the indicated Env glycoprotein. Where indicated, the cells were treated with 10 nM or 1 μM of NE for 24 h prior to infection (Pre) or immediately after infection (Post). The data shown are representative of three independent experiments from separate donors yielding similar results, as the mean (±SD) of triplicate determinations. Statistically significant differences between control and NE treatment are indicated by $^*P < 0.05$. (C) NE had little effect on HIV-1 entry into cells. PBMC prestimulated with anti-CD3 and anti-CD28 or MDM from healthy donors were infected with the indicated viruses, and HIV-1 proviral loads were quantified by real-time PCR 24 h after infection. The data shown are representative of three independent experiments from separate donors yielding similar results, as the mean (±SD) of triplicate determinations. Statistically significant differences between control and NE were not obtained.

regulated HIV-1 LTR, at least in part, through inhibition of NF-κB activity.

In this study, we have shown that NE inhibited HIV-1 replication in both acute and endogenous infection systems, and in both PBMC and MDM. Our results also suggested that downregulation of NF-κB activity was involved in NE-mediated anti-HIV-1 effect.

NE effects on NF-κB activity have been controversial in the previous reports. While several studies demonstrated that NE increases expression of I-κBα, which inhibits NF-κB activity (Farmer and Pugin, 2000; Gavrilyuk et al., 2002; Heneka et al., 2003), a recent study has shown that NE induces NF-κB activity (Bierhaus et al., 2003). The reason why NE and/or other catecholamines apparently mediated quite different effects on NF-κB activity is unknown. The inhibitory effect was demonstrated in THP-1 cells (Farmer et al., 2000; this study), PBMC (this study), astrocytes (Gavrilyuk et al., 2002) or brain tissue (Heneka et al., 2003); induction of NF-κB activity was shown in PBMC and THP-1 cells (Bierhaus et al., 2003). Therefore, such discrepancy cannot be explained by difference in cell types tested. Although our study suggested that PK-A-mediated NE signaling increased I-κBα levels, more precise molecular mechanisms remain obscure. Further studies are needed to clarify the apparent controversy and to define more detailed cellular and molecular mechanisms. We are currently investigating them.

It may be difficult to precisely determine net effects of exercise or acute stress on HIV-1 infection, since a number of factors are involved in those conditions. Although we have demonstrated that NE, one of the mediators involved in exercise or acute stress, inhibited HIV-1 infection, we do not believe that adequate burden of stress simply leads to modulation of HIV-1 replication in infected individuals. However, it may be worthwhile investigating potential benefit of exercise in HIV-1-infected individuals, since exercise induces the SNS activity but probably not other detrimental effects observed in stress reactions (Cole and Kemeny, 1997). In this regard, it has been reported that NE response to stress is blunted in HIV-1-infected individuals (Kumar et al., 1991), and that a cerebrospinal fluid catecholamine metabolite is lower in AIDS patients (Lasson et al., 1991). In addition, Cole et al. (2001) have recently shown that high autonomic nervous activity is associated with impaired response to HAART in HIV-infected individuals. Thus, it might be possible that individuals with high autonomic nervous system activity may be somehow different from those with low activity in their immunological activity or drug metabolism, and that HIV-1 infection itself may lead to autonomic dysfunction. Further investigations are needed to clarify these issues.

Materials and methods

In vitro or acute HIV-1 infection experiments

PBMC were isolated from healthy individuals, and propagated as described previously (Moriuchi et al., 1998b). Monocytes were isolated from PBMC by sorting $CD14^+$ cells with AutoMACS (Moriuchi and Moriuchi, 2004b), and monocyte-derived macrophages (MDM) were propagated as described previously (Moriuchi et al., 1998b). R5 (AD8)- and X4 (NL4-3)-HIV-1 stocks were propagated by transfecting 293T cells with the corresponding plasmids (Moriuchi et al., 1998b). PBMC that had been prestimulated with anti-CD3 and anti-CD28 antibodies (1 μg/ml each) for 2 days, or 7-day-old MDM, were treated with NE (Sigma Chemical Co., St. Louis, MO) 24 h prior to infection and throughout the incubation period. Cell-free culture supernatants were collected every 3 days for reverse transcriptase (RT) assays (Moriuchi et al., 1998a).

Ex vivo or endogenous HIV-1 infection experiments

PBMC were isolated from HIV-1-infected patients outlined in Table 1, and $CD8^+$ T cells were depleted as described previously (Moriuchi et al., 1998a). Approximately one million CD8-depleted PBMC were incubated in RPMI-1640 with 10% fetal bovine serum in the presence or absence of anti-CD3 and anti-CD28 antibody (1 μg/ml each) ± NE (10 nM). Cell-free supernatants were collected every 3 days until day 21, and tested for RT activity.

Ex vivo or endogenous HTLV-I infection experiments

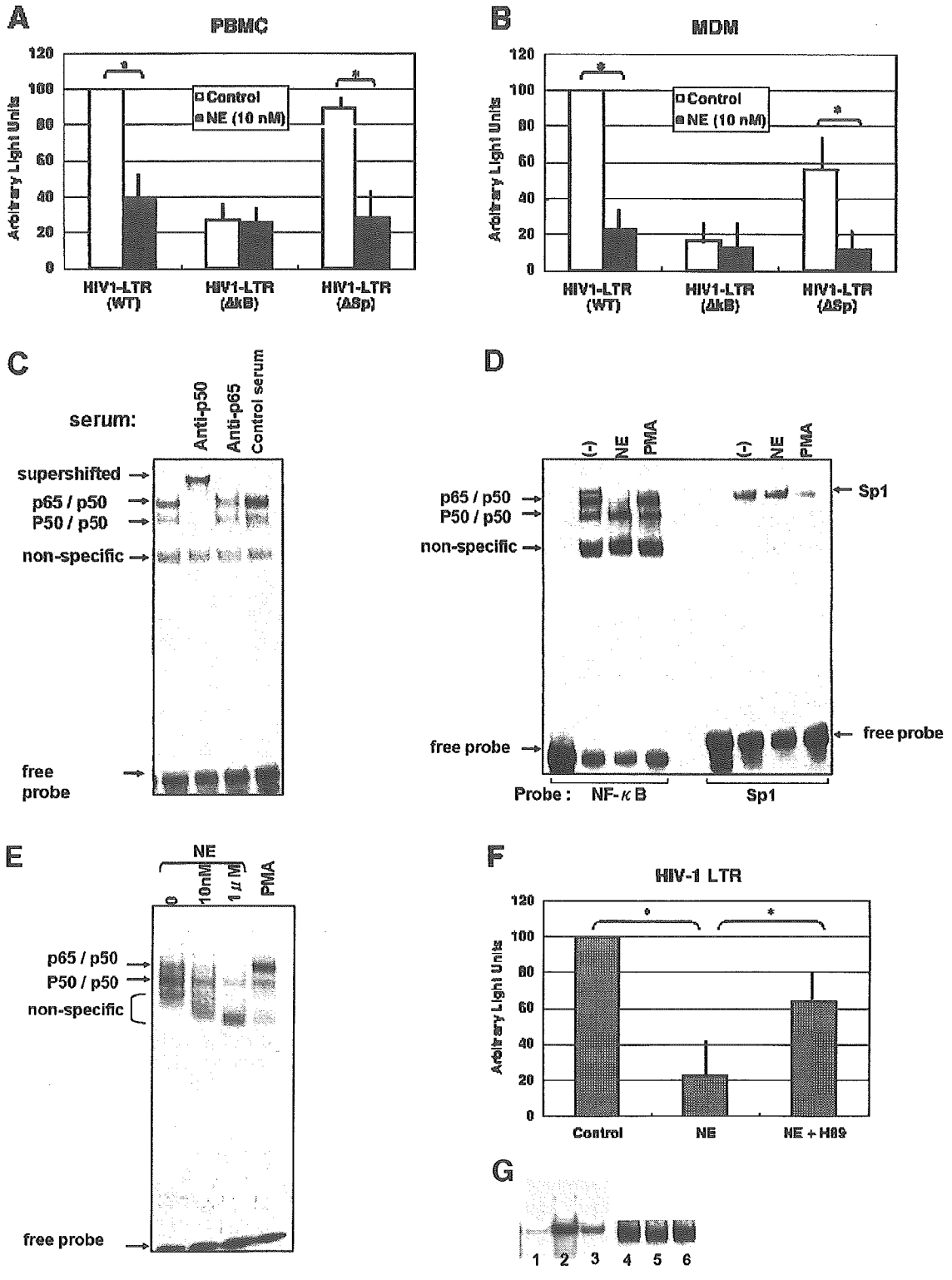
PBMC were isolated from asymptomatic HTLV-I carriers, and approximately one million PBMC were incubated in RPMI-1640 with 10% fetal bovine serum in the presence or absence of

Fig. 3. NE downregulated HIV-1 LTR activity through inhibition of NF-κB activity. (A, B) NE downregulated HIV-1 LTR, and NF-κB-binding sites are apparently involved in the NE-mediated effect. PBMC (A) or MDM (B) were transfected with the indicated reporter plasmid along with pSV2-Tat, either untreated or treated with NE (10 nM) for 24 h, and harvested for luciferase assays 2 days after transfection. Arbitrary light units of promoter-less pGL2-basic transfectants were always less than 3 (data not show). The data shown are representative of three independent experiments from separate donors yielding similar results, as the mean (±SD) of triplicate determinations. Statistically significant differences between control and NE treatment are indicated by $^*P < 0.05$. (C) Constitutive NF-κB activity in THP-1 cells. Nuclear extracts were prepared from THP-1 cells, and gel-mobility shift assays were performed using an oligonucleotides corresponding to HIV-1 LTR NF-κB-binding sites as a probe (Moriuchi and Moriuchi, 2004a, 2004b). Where indicated, control serum or antibodies to p50 or p65 were added to the reaction. Identification of protein–DNA complexes was indicated. (D, E) Inhibition of NF-κB, but not SP1 in NE-treated THP-1 cells. THP-1 cells were either untreated or treated with NE (10 nM or 1 μM) or PMA before harvest for nuclear extract preparation. Gel shift assays were performed using probes corresponding to HIV-1 LTR NF-κB-binding sites or SP1-binding sites. Similar results were obtained in nuclear extracts of PBMC derived from 3 different donors (data not shown). (F, G) NE-mediated inhibition of NF-κB activity involves PK-A pathway. (F) Transient expression assays. THP-1 cells were transfected with pGL-HIV-1-LTR and pSV2-Tat, were untreated or treated with NE (1 μM) ± H89 (10 μM), and cell lysates were tested for luciferase activity. The data shown are representative of three independent experiments from separate donors yielding similar results, as the mean (±SD) of triplicate determinations. Statistically significant differences between control and NE treatment are indicated by $^*P < 0.05$. (G) Western blot analysis. Cell lysates from untreated THP-1 cells (lane 1), THP-1 cells treated with NE (1 μM) alone (lane 2) or THP-1 cells treated with both NE and H89 (10 μM) (lane 3) were analyzed by Western blotting for cytoplasmic IκB-α levels. The same set of cell lysates were also analyzed for actin (lanes 4–6).

NE (10 nM) for 7 days. Assays of p19 antigen levels in cell-free supernatants and of proviral DNA in PBMC were performed as described previously (Moriuchi and Moriuchi, 2004a).

Single-round viral replication assays

Replication-incompetent luciferase-reporter molecular clone NL43-Luc-R$^-$E$^-$ was complemented with R5-tropic (ADA), X4-tropic (HXB2) or amphotropic (murine leukemia virus [MLV]) Env glycoprotein by cotransfecting 293T cells with plasmids encoding the corresponding Env glycoproteins (Moriuchi et al., 1998b). Anti-CD3/anti-CD28-prestimulated PBMC or MDM were either untreated or treated with NE (10 nM) for 24 h, infected with those virus stocks for 3 h, and infectivity was estimated by luciferase assays that were performed 48 h after infection (Moriuchi et al., 1998a).



HIV-1 entry assays

PBMC that had been prestimulated with anti-CD3 and anti-CD28 antibodies or MDM were untreated or pretreated with NE (1 μM) for 24 h before infection with the indicated viruses. The infected cells were extensively washed 4 h after, and harvested 24 h after infection, RNA-free DNA was extracted, according to manufacturer's instruction (QIAamp DNA Mini Kits, QIAGEN K.K., Tokyo, Japan) and real-time quantitative PCR for HIV-1 proviral DNA of *gag* region was performed as described previously (Hoshino et al., 2004).

Cytotoxicity tests

Viability of cultured cells was assessed with trypan blue exclusion assays or 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assays after NE treatment, according to the manufacturer's instructions (the CellTiter-Blue Cell Viability Assay, Promega K.K., Tokyo, Japan).

Plasmids and transfection

Plasmid pGL-HIV-1-LTR contains HIV-1 long terminal repeat (LTR) from HXB2 strain in pGL2-basic (Promega, Madison, WI) backbone. NF-κB and SP1 binding sites on HIV-1 LTR were deleted in plasmids pGL-HIV-1-LTRΔNF-κB and pGL-HIV-1-LTRΔSP1, respectively (Margolis et al., 1992). Transfection of PBMC and THP-1 cells was performed by electroporation (Moriuchi et al., 1997), and MDM were transfected by modified calcium phosphate method (Moriuchi et al., 1994).

Nuclear extracts, antibodies and gel-mobility shift assays

Nuclear extracts were prepared from THP-1 cells untreated or treated with NE for 60 min or PMA for 15 min (Sigma Chemical Co., St. Louis, MO), and gel-mobility shift assays were performed, as described previously (Moriuchi et al., 1997). Antibodies to p50 and p65 were kindly provided by U. Siebenlist (National Institutes of Allergy and Infectious Diseases, Bethesda, MD).

Western blotting

THP-1 cells were untreated or treated with NE ± H-89 (10 μM) (Sigma Chemical Co., St. Louis, MO) for 60 min, and lysed in RIPA buffer (10 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxy cholate, 0.1% SDS and 100 μg/ml phenylmethylsulfonyl fluoride). Lysates from 5×10^5 cells were loaded in each lane, separated by SDS-PAGE (8% acrylamide-bis-acrylamide gel) and electrotransferred onto a nitrocellulose membrane (0.45 μm). IκB-α or actin was detected with rabbit anti-human IκB-α antibody (Santa-Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-actin antibody (SIGMA-ALDRICH Japan, Tokyo, Japan), mouse anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) and enhanced chemiluminescence reaction with ECL Plus Western Blotting Detection System (Amersham Biosciences).

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Pathways for the emergence of multi-dideoxynucleoside-resistant HIV-1 variants

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Objective: To investigate the mechanism by which the Q151M mutation in reverse transcriptase (RT) that confers multi-dideoxynucleoside resistance on HIV-1 and that requires a two base change (CAG→ATG) develops, and to understand the reason for the relatively lengthy period of time required for its emergence under therapy with multiple nucleoside RT inhibitors (NRTI).

Design and methods: Propagation assays and competitive HIV-1 replication assays were used to evaluate the fitness of various infectious clones, including two putative intermediates (HIV-$1_{Q151K(AAG)}$ and HIV-$_{1Q151L(CTG)}$) for HIV-$1_{Q151M(ATG)}$, in terms of sensitivity to zidovudine and didanosine. Steady-state kinetic constants of recombinant RT were also determined.

Results: HIV-1_{Q151L} replicated relatively poorly while HIV-1_{Q151K} failed to replicate. When HIV-1_{Q151L} was propagated further, it took three pathways in continuing to replicate: (i) HIV-1_{Q151L} changed to HIV-1_{Q151M} in eight of 16 experiments; (ii) HIV-1_{Q151L} reverted to wild-type HIV-1 (HIV-1_{WT}) in four of 16 experiments; and (iii) HIV-1_{Q151L} acquired an additional mutation M230I in four of 16 experiments improving HIV-1 fitness. The relative order of replicative fitness without drugs was: HIV-1_{Q151M} > HIV-1_{WT} > HIV-$1_{Q151L/M230I}$ > HIV-1_{M230I} >> HIV-1_{Q151L} >>> HIV-1_{Q151K}, HIV-$1_{Q151K/M230I}$. HIV-1_{Q151M} was less susceptible to drugs, while HIV-$1_{Q151L/M230I}$ was as sensitive as HIV-1_{WT}. Enzymatic assays corroborated that HIV-1_{Q151L} is more replication-competent than HIV-1_{WT} and HIV-1_{Q151K} in the presence of drugs.

Conclusion: HIV-1_{Q151M} probably develops through a poorly replicating HIV-1_{Q151L}; however, it is also possible that it occurs through two concurrent base changes. The present data should explain the mechanism by which HIV-1_{Q151M} emerges after long-term chemotherapy with NRTI.

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Keywords: multi-dideoxynucleoside resistance, reverse transcriptase, viral fitness, anti-HIV agents

Introduction

Combination chemotherapy or highly active antiretroviral therapy using two or more reverse transcriptase inhibitors and protease inhibitors has dramatically improved the quality of life and survival of individuals infected with HIV-1 [1]. However, a growing body of literature has reported that 40–50% of those who initially achieve favourable viral suppression to undetectable levels have experienced treatment failure [2,3].

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Moreover, 10–40% of antiviral therapy-naive individuals infected with HIV-1 have persistent viral replication (plasma HIV RNA > 500 copies/ml) under highly active antiretroviral therapy [4], possibly due to transmission of drug-resistant HIV-1 variants [5]. The emergence of HIV-1 variants resistant to multiple antiretroviral drugs has become a significant problem among those who have received long-term antiviral therapy [6–8]. More problematic is that certain multidrug resistant HIV-1 can establish persistent infection as dominant quasispecies despite their impaired replicative competence following primary infection [9].

A set or subset of five mutations in the viral reverse transcriptase (RT), including Ala62→Val (A62V), V75I, F77L, F116Y, and Q151M, confers multi-dideoxynucleoside resistance (MDR) on HIV-1 [6,7,10–13]. Among the five mutations conferring MDR, an HIV-1 variant carrying the Q151M mutation often emerges first in HIV-1 isolated from patients receiving long-term combination chemotherapy using multiple nucleoside RT inhibitors (NRTI) [6,7,10–13]. Recent structural analysis of a stalled complex of HIV-1 RT with a DNA template:primer and a deoxynucleoside triphosphate (dNTP) has revealed that glutamine at position 151 (Q151) is located in the centre of the 3′-pocket which is thought to play a critical role in recognizing antiretroviral 2′,3′-dideoxynucleoside analogues (ddN) as substrates (in the form of triphosphates) as well as in the development of HIV-1 resistance against ddN [14].

Since two base changes (CAG→ATG) are required for the acquisition of the Q151M substitution, we generated two putative intermediate infectious clones, HIV-$1_{Q151L(CTG)}$ and HIV-$1_{Q151K(AAG)}$ [15]. In the present study, in an attempt to identify pathways for the emergence of HIV-1_{Q151M}, we compared the replication profiles of a variety of infectious HIV-1 clones, and examined their replicative fitness. We also examined another mutant HIV-1 clone carrying phenylalanine at position 151, the wild-type residue at the equivalent position in the polymerase of a plant retrovirus, cauliflower mosaic virus (CaMV) [16].

We found that HIV-1_{Q151L} replicated relatively poorly; however, when it was propagated further, three pathways of genetic changes were identified: (i) HIV-1_{Q151L} converted to HIV-1_{Q151M}; (ii) HIV-1_{Q151L} reverted to wild-type HIV-1; and (iii) HIV-1_{Q151L} acquired an additional mutation M230I. HIV-1_{Q151L} restored its replicative capacity by taking any of these pathways. To investigate the relative order for the replicative fitness of these related infectious clones, the competitive HIV-1 replication assay (CHRA) [15] was also performed. Together with the data of susceptibility of infectious HIV-1 clones to ddN and steady-state enzymatic assay data using a variety of recombinant RT preparations generated, it appears that HIV-1_{Q151M} probably develops through a poorly replicating intermediate, HIV-1_{Q151L}, although it is possible that it occurs through two concurrent base changes.

Materials and methods

Reagents and cells

Zidovudine and didanosine were from Sigma (St. Louis, Missouri, USA) and Calbiochem (San Diego, California, USA), respectively. Nevirapine was a kind gift from Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, Connecticut, USA). Saquinavir was provided by Roche Products (Welwyn Garden City, UK). H9 and MT-2 cells were grown in RPMI 1640-based culture medium supplemented with 10% foetal calf serum (FCS; Hyclone, Logan, Utah, USA), L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). Cos-7 cells were propagated in Dulbecco's Modified Eagle's medium supplemented with 10% FCS.

Generation of infectious HIV-1 clones

Infectious molecular HIV-1 clones with mutations of interest were generated using the pHXB2RIP7-based plasmid, pSUM9, as described previously [10,17]. An HIV-1 clone carrying the wild-type RT was designated HIV-1_{WT}. An HIV-1 clone carrying the amino acid substitution Q151M (CAG→ATG) in the RT-encoding region was generated and designated HIV-1_{Q151M}. Two putative intermediate infectious clones (HIV-1_{Q151L} and HIV-1_{Q151K}) for the generation of HIV-1_{Q151M}, carrying Q151L (CAG→CTG) and Q151K (CAG→AAG) substitutions, respectively, and an HIV-1 clone designated HIV-1_{Q151F}, carrying an amino acid substitution Q151F (CAG→TTC) which is observed at the homologous position in RT of CaMV, were also generated. An amino acid substitution M230I (ATG→ATA) was introduced to HIV-1_{WT}, HIV-1_{Q151L}, HIV-1_{Q151K} and HIV-1_{Q151F}, and the resultant HIV-1 clones were designated HIV-1_{M230I}, HIV-$1_{Q151L/M230I}$, HIV-$1_{Q151K/M230I}$, and HIV-$1_{Q151F/M230I}$, respectively. The substitution of Q at position 151 with F, K, L or M is biochemically nonconservative, while the substitution of M at position 230 with I is conservative. To obtain virus preparations, plasmid DNA (1.5 μg) for each molecular clone was transfected into Cos-7 cells. Determination of the nucleotide sequence of infectious clones obtained following transfection and propagation confirmed that each clone carried only the intended mutation(s) in the *pol* gene.

Determination of the profile of viral replication

Plasmid DNA (1.5 μg) for each molecular clone was transfected into Cos-7 cells in 6-well tissue culture plates using LipofectAmine as specified by the manu-

facturer (Life Technologies, Gaithersburg, Maryland, USA). On the following day, MT-2 cells (10^5) were added and the amounts of $p24^{gag}$ antigen production in the culture supernatant were determined at 1- or 2-day intervals using a commercially available radioimmunoassay kit (Dupont/NEN, Boston, Massachusetts, USA). Half of the medium was replenished every 2 or 3 days, and when significant cytopathic effect (CPE) by molecular clones was observed in MT-2 cells, the same number of newly prepared uninfected MT-2 cells were added at a proper interval so that the culture was not lost. The assays were conducted in triplicate and the geometric means and standard deviations (SD) were determined. The nucleotide sequence of the polymerase-encoding region of each molecular clone was determined on day 10 after transfection and propagation. In long-term propagation of HIV-1_{Q151L}, the nucleotide sequence of the polymerase-encoding region was determined every 7 days in culture. When an amino acid change was identified, the viral culture was maintained for at least an additional 7 days to obtain one more nucleotide sequence to confirm that the change remained.

Viral titration

To determine virus infectious titres, MT-2 cells (2000 cells/well) in 96-well flat-bottomed microtiter culture plates (Costar, Cambridge, Massachusetts, USA) were exposed to each infectious clone which had been prepared in H9 cells and serially diluted. MT-2 cell cultures were examined for HIV-1-induced CPE on day 7, and the 50% tissue culture infectious dose ($TCID_{50}$) was determined by the method of Reed and Muench [18]. All titration assays were performed in eight replicates.

CHRA

The relative order for replicative fitness of infectious molecular HIV-1 clones was determined by CHRA as described previously [15]. Briefly, a fixed amount (200 $TCID_{50}$) of an infectious clone was combined with three different amounts (100, 200, and 300 $TCID_{50}$) of the second infectious clone, and the mixture was added to freshly prepared H9 cells (3×10^5) in the presence or absence of various concentrations of zidovudine or didanosine. On the following day, one-third of infected H9 cells was harvested, washed twice with phosphate-buffered saline (PBS), and cellular DNA was purified with Instagene Matrix (Bio-Rad, Hercules, California, USA) according to the manufacturer's protocol. Purified DNA was subjected to nested PCR and sequencing as described below. The HIV-1 coculture which best approximated a 50:50 mixture on day 1 was further propagated. At periodic time points, cellular DNA samples were purified from infected H9 cells and a viral population change was determined by direct DNA sequencing. If the HIV-1 coculture was determined not to be dominated by either of the clones, the cell-free supernatant of virus coculture (1 ml) was transmitted to fresh uninfected H9 cells (1.5×10^5 in 1 ml) every 7–10 days and 8 ml fresh culture medium was added on the following day. The cells harvested at the end of each passage were subjected to DNA purification, PCR, and subsequently to direct DNA sequencing, and viral population changes were evaluated as described below. When an amino acid change was identified, the viral culture was maintained for another 7 days to confirm that the change remained the same and the absence of unintended amino acid substitutions was confirmed at the termination of the assays.

Individual nucleotide mixture ratios were determined based on relative peak heights in electropherograms produced from direct DNA sequencing of HIV-1 genome as previously described [12,15]. Briefly, the height of the major peak was categorized as equal to or 1.25-, 1.5-, 2-, 2.5-, 3-, or 4-fold higher than the other peak, and the representation of the major peak was defined as approximating 50, 56, 60, 67, 71, 75, or 80%, respectively. A minor population comprising less than 20% of the total population was not reliably determined due to background peaks and such data were considered to be of 0% minor population. Nucleotide mixtures were evaluated based on the average of the forward and reverse results. For cocultures involving clones which contained two or more mutations, we determined the ratios based on the average height of each peak. In certain assays, different initial ratios of the two test HIV-1 clones were also employed for confirmation. To avoid the PCR-related artefactual components which might potentially affect the data, the assays were performed on multiple time points (or passages) with all approximations of viral proportions made in a totally blinded and non-ordered manner.

Determination of nucleotide sequence of HIV-1

Nucleotide sequences of the RT-encoding region were determined as described previously [10,12]. In brief, each DNA purified from the cells was subjected to nested PCR. The first round of PCR consisted of 35 cycles with a 55°C annealing temperature and used primers SA009 (5′-TTTAAATTTTCCCATTAGCC CTAT-3′) and SA015 (5′-ACTCCATGTACTGGT TCTTTTAGA-3′), which generated a fragment including codons 1 through 316 of RT of HIV-1. First-round PCR products (1 μl) were used directly in the second round of 30 cycles at a 58°C annealing temperature, with primers 881MF (5′-TGTAAAACGAC GGCCAGTCCCGGGATGGATGGCCCAAAAGTT AAACAA-3′) and 891MR (5′-CAGGAAACAGCTA TGACCGCTAGCCCAATTCAATTTTCCCACTAA-3′), which included tailed M13 forward and M13 reverse standard primer sequences, respectively. This generated a fragment which spanned codons 16

through 266 of RT, containing an M13 tail at each end. Second-round PCR products were purified with PCR Select III columns (Eppendorf-5 Prime, Boulder, Colorado, USA) and sequenced directly, using both M13 forward and M13 reverse dye-labeled primers on Model 373 automated DNA sequencer (Perkin-Elmer, Foster City, California, USA). After electrophoresis, sample files were processed with Factura software (Perkin-Elmer). The forward and reverse electropherograms for each sample were aligned with Auto-Assembler software (Perkin-Elmer). At the relevant nucleotide positions, heterozygous base peaks were analysed as described above.

Determination of drug sensitivity of HIV-1

The sensitivity of infectious clones to various drugs was determined as previously described [10,19] with minor modifications. Briefly, MT-2 cells (2000 cells/well) in 96-well flat-bottomed microtiter culture plates were exposed to 100 $TCID_{50}$ of each infectious HIV-1 clone in the presence or absence of various concentrations of drugs in threefold serial dilutions, and incubated at 37°C for 7 days. Drug concentrations that suppressed HIV-1 replication by 50% (50% inhibitory concentration; IC_{50}) were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [20]. Briefly, after culture medium (100 µl) was removed from each well, MTT solution (7.5 mg/ml, 10 µl) in PBS was added. After incubation at 37°C for 1.5 h, acidified 2-propanol containing 4% (v/v) TritonX-100 (100 µl) was added to each well. The optical density (wavelength 570 nm) was measured in a microplate reader (Model 3550, Bio-Rad). All assays were performed in triplicate.

Construction of expression vectors and enzyme preparation

Recombinant RT preparations were generated as previously described [21]. In brief, an amino acid substitution of interest (Q151M, Q151K, or Q151L) was introduced by oligonucleotide-directed mutagenesis using the plus strand of pTZNX1, the modified pTZ19R (Pharmacia Biotech, Piscataway, New Jersey, USA) [21], as a template. Mutagenesis was carried out by introducing one mutation in each mutagenesis reaction. Insertion of such a substitution was confirmed by determination of nucleotide sequence. To construct a wild-type RT expression vector, pKRT08, the *XmaI–NheI* fragment of pTZNX1 was inserted as described previously [21]. For the overproduction of the enzyme, *Escherichia coli* JM109 (Promega, Madison, Wisconsin, USA) was transformed with a wild-type or mutant RT expression vector [21]. Purity of RT-derived polypeptides was > 90% as assessed by polyacrylamide gel electrophoresis.

Steady-state kinetic analysis

Steady-state kinetic constants of RT_{wt} and mutant RT preparations were determined using p(rA) · $(dT)_{12}$ as a template-primer in processive modes as previously described [21]. The K_m and V_{max} values were determined from initial linear steady-state velocities with Lineweaver–Burk plot analyses, and the k_{cat} values were calculated by dividing V_{max} by active enzyme concentrations [21].

Results

Replication fitness of HIV-1 clones carrying a mutation at position 151

The Q151M(ATG) substitution represents a primary mutation known to confer MDR on HIV-1 [6,7,10]. We generated two putative intermediate infectious clones (HIV-$1_{Q151L(CTG)}$ and HIV-$1_{Q151K(AAG)}$) for the emergence of HIV-1_{Q151M}, using site-directed mutagenesis [15]. We also generated another mutant HIV-1 clone (HIV-1_{Q151F}) carrying phenylalanine at position 151, the homologous residue in the RT of a plant retrovirus, CaMV [16], hypothesizing that the Q151F substitution could support the enzymatic activity of HIV-1 RT.

First, we determined the replication kinetics of HIV-1_{WT}, HIV-1_{Q151M}, HIV-1_{Q151F}, HIV-1_{Q151K}, and HIV-1_{Q151L} in the absence of drugs (Fig. 1a). The replication profile of HIV-1_{Q151M} was comparable to that of HIV-1_{WT} as shown in Fig. 1a in agreement with our previous data [15]. In contrast, although marginal amounts of $p24^{gag}$ antigen were detected in the culture medium for HIV-1_{Q151K} and HIV-1_{Q151F} in the first 3 days of culture, by day 4 the amount reached a plateau and no further production of $p24^{gag}$ antigen was detected, suggesting that while marginal amounts of both virions were produced upon transfection, those virions either replicated poorly or failed to replicate at all. Throughout the 10-day culture period of HIV-1_{Q151K} and HIV-1_{Q151F}, no syncytium formation or CPE was observed. However, in the culture for HIV-1_{Q151L} propagation, a low but notable level of syncytium formation was observed in the 10-day culture period. When we further propagated HIV-1_{Q151L}, the virus started to replicate rapidly around day 12 and continued to do so (Fig. 1b). Around day 20 and beyond, the replication profile of HIV-1_{Q151L} became similar to that of HIV-1_{WT} and HIV-1_{Q151M} and the accumulated amounts of $p24^{gag}$ antigen produced in culture supernatants ultimately became comparable (Fig. 1b).

Amino acid substitutions in RT of HIV-1_{Q151L} as its propagation continued

Since the replicative fitness of HIV-1_{Q151L} seemed to improve in long-term culture without drugs (Fig. 1b), we asked whether HIV-1_{Q151L} underwent genetic

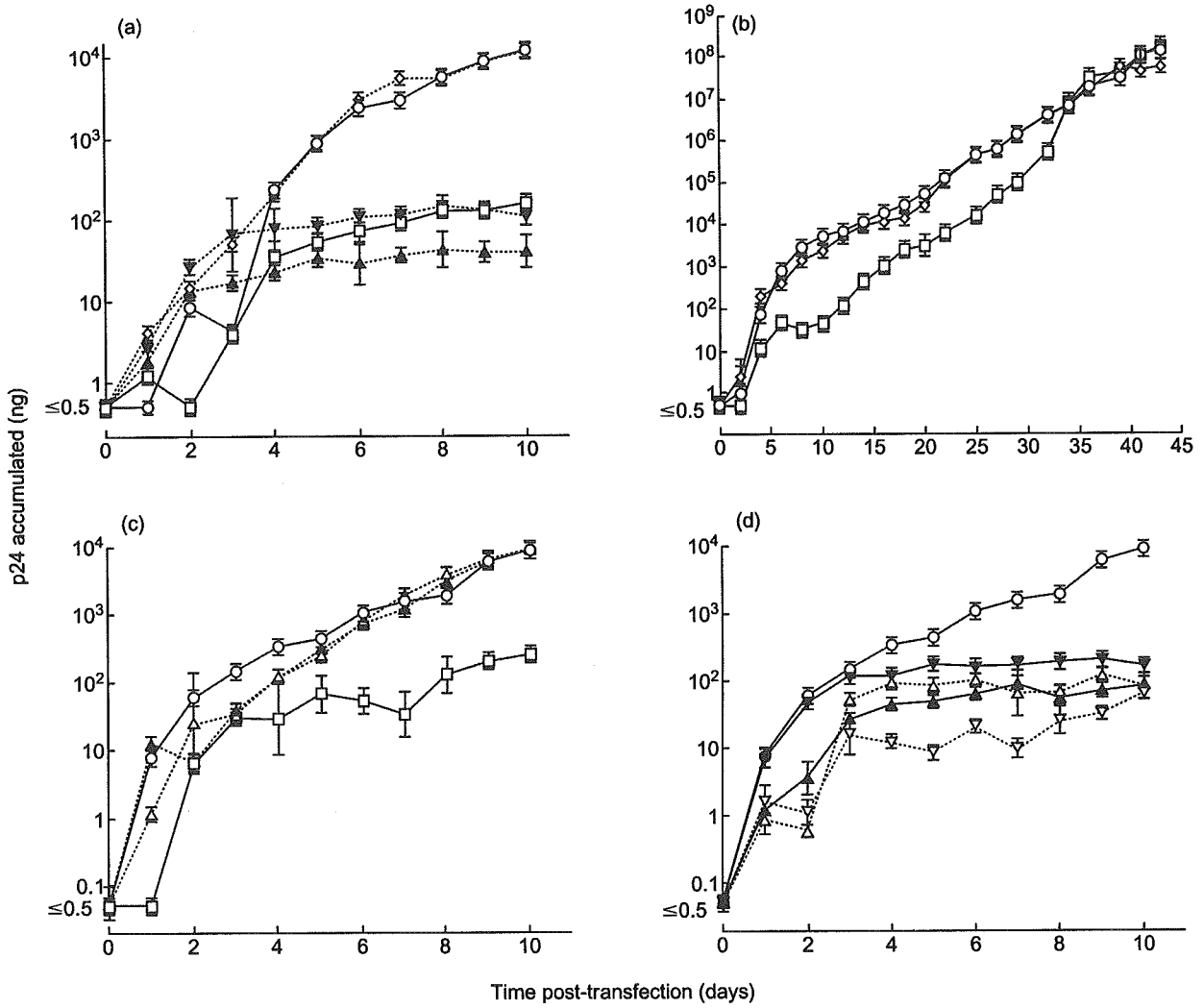

Fig. 1. Replication kinetics of various HIV-1 infectious clones. Cos-7 cells were transfected with plasmid DNA for a designated molecular clone. On the following day, MT-2 cells were added and the supernatants were collected. Note that the accumulated p24 Gag protein amounts are shown. (a) For HIV-1_{WT} (○), HIV-1_{Q151M} (◇), HIV-1_{Q151K} (▲), and HIV-1_{Q151L} (□) the values represent the geometric means (± SD) of three independent experiments, and for HIV-1_{Q151F} (▼) those of two independent experiments. (b) Replication kinetics of HIV-1_{Q151L}, which converted to HIV-1_{Q151M} on day 32. The values represent the geometric means (± SD) of two independent experiments. (c) Replication kinetics of HIV-$1_{Q151L/M230I}$ (△) compared to HIV-1_{WT}, HIV-1_{Q151L}, and HIV-1_{M230I} (▲) (the values represent the geometric means (± SD) of three independent experiments). (d) Replication profiles of HIV-1_{WT}, HIV-1_{Q151F}, HIV-$1_{Q151F/M230I}$ (▽), HIV-1_{Q151K}, and HIV-$1_{Q151K/M230I}$ (△) [the values represent the geometric means (± SD) of three independent experiments].

alterations during this time. Direct nucleotide sequence analyses of cellular DNA samples from infected MT-2 cells revealed three genetic alteration pathways: (i) HIV-1_{Q151L} changed to HIV-1_{Q151M} in eight of 16 independently executed experiments; (ii) HIV-1_{Q151L} reverted to wild-type HIV-1_{Q151} in four of 16 experiments; and (iii) HIV-1_{Q151L} acquired another mutation M230I, which apparently improved the viral fitness, in four of 16 experiments (Table 1). Fig. 1b illustrates a representative replication profile among eight independent cultures in which HIV-1_{Q151L} ultimately converted to HIV-1_{Q151M}. The predominance of the resulting HIV-1_{Q151M} was seen on day 38 of culture (the mean in eight independent propagation experiments). The revertant HIV-1_{WT} was seen to be dominant on day 31 of culture (the mean in four independent propagation experiments). In contrast, in the four propagation experiments where the acquisition of the M230I substitution by HIV-1_{Q151L} was seen, the resultant HIV-$1_{Q151L/M230I}$ was predominantly seen on day 22 of culture (the mean in four independent propagation experiments). It was noted that all of the resulting HIV-1 in these cultures (i.e., HIV-1_{WT}, HIV-1_{Q151M}, and HIV-$1_{Q151L/M230I}$) had a robust and reproducible replicative capability comparable to HIV-1_{WT}. Using the same conditions, we also attempted to

Table 1. Amino acid substitutions in reverse transcriptase which occurred at position 151 as HIV-1_{Q151L} was continuously propagated. Direct nucleotide sequence was determined every 7 days in culture. When an amino acid change was identified, the viral culture was maintained for at least an additional 7 days to confirm that the change remained the same.

Genotype of starting virus	Predominant virus genotype identified in culture	Frequency in experiments [n (%)]
HIV-1_{Q151L}	HIV-1_{wt}	4/16 (25)
	HIV-1_{Q151M}	8/16 (50)
	HIV-$1_{Q151L/M230I}$	4/16 (25)
HIV-1_{Q151K}	Failed to replicate	3/3 (100)
HIV-1_{Q151F}	Failed to replicate	2/2 (100)

propagate two other clones, HIV-1_{Q151K} and HIV-1_{Q151F}, for longer periods of time in three and two independent experiments, respectively. However, all such attempts failed and no progeny virions were produced (Table 1).

Effects of M230I substitution on the replication of HIV-1 clones

As illustrated in Table 1, HIV-1_{Q151L} acquired the M230I substitution during long-term culture, which restored the replicative fitness of HIV-1_{Q151L}. This M230I substitution has been previously reported when HIV-1 with a Y115W substitution (HIV-1_{Y115W}) was propagated in the absence of drug, and this substitution was shown to mitigate the polymerase dysfunction of HIV-1_{Y115W} by increasing the dNTP binding affinity of the enzyme [22]. In order to determine the effect of M230I substitution on the poorly replicating HIV-1_{Q151L}, we constructed an infectious clone carrying both Q151L and M230I substitutions, designated HIV-$1_{Q151L/M230I}$. As shown in Fig. 1c, HIV-1_{Q151L} replicated relatively poorly; however, HIV-$1_{Q151L/M230I}$ replicated as efficiently as HIV-1_{WT} over a 10-day period of propagation. HIV-1 carrying only M230I (HIV-1_{M230I}) also replicated comparably to HIV-1_{WT} (Fig. 1c). These data suggest that the M230I substitution *per se* does not significantly alter the enzymatic activity of wild type RT (RT_{WT}), while it restores the compromised replicative fitness of HIV-1_{Q151L}. However, as shown in Fig. 1d, the introduction of M230I substitution to HIV-1_{Q151K} and HIV-1_{Q151F}, generating HIV-$1_{Q151K/M230I}$ and HIV-$1_{Q151F/M230I}$, respectively, did not restore their compromised replicative abilities.

Sensitivity of infectious HIV-1 clones against drugs

We next determined the drug susceptibility of various infectious HIV-1 clones examined in this study, considering that all drug-resistant HIV-1 variants emerge as the result of the interplay of viral drug resistance levels and viral replicative fitness [15,17]. HIV-1_{Q151M} showed reduced sensitivity to zidovudine (14-fold) and didanosine (13-fold) compared with HIV-1_{WT} as assessed with the MTT assay using MT-2 cells as targets (Table 2). HIV-$1_{Q151L/M230I}$ and HIV-1_{M230I} were found to be more susceptible to zidovudine than HIV-1_{WT} (Table 2). HIV-1_{M230I} was also more sensitive to didanosine relative to HIV-1_{WT}. It was also noted that HIV-$1_{Q151L/M230I}$ and HIV-1_{M230I} showed substantial levels of resistance to a non-nucleoside RT inhibitor (NNRTI) nevirapine with 13.7- and 10-fold greater IC_{50} values, respectively, than HIV-1_{WT}. As anticipated, there was no difference in the sensitivity to a protease inhibitor (saquinavir) among the infectious clones examined.

CHRA with infectious HIV-1 clones in the absence of drugs

To further investigate the possible role of the M230I substitution in viral fitness, we examined the replicative fitness of various infectious clones using CHRA. When we examined the replicative profile of HIV-$1_{Q151L/M230I}$ using $p24^{gag}$ antigen production as an endpoint, the M230I substitution appeared to virtually completely restore the compromised replicative fitness of HIV-1_{Q151L} (Fig. 1c). However, when HIV-$1_{Q151L/M230I}$ was co-propagated with HIV-1_{WT} or HIV-1_{Q151M}, HIV-$1_{Q151L/M230I}$ was rapidly outgrown (Fig. 2a and c). In the replication assay shown in Fig. 1c, HIV-1_{M230I} was seen to replicate comparably to HIV-1_{WT}, however, HIV-1_{M230I} was readily outgrown by HIV-1_{WT} and HIV-1_{Q151M} as assessed in CHRA (Fig. 2b and d). Even when the CHRA was initiated with a greater ratio for HIV-1_{M230I}, HIV-1_{WT} and HIV-1_{Q151M} readily

Table 2. Sensitivity of infectious HIV-1 clones to zidovudine, didanosine, nevirapine or saquinavir. MT-2 cells (2×10^3) were exposed to 100 $TCID_{50}$ of HIV-1_{WT}, HIV-1_{Q151M}, HIV-$1_{Q151L/M230I}$, or HIV-1_{M230I} and were cultured in the presence of various concentrations of zidovudine, didanosine, nevirapine, or saquinavir. The 50% inhibitory concentration (IC_{50}) values were determined using the MTT assay on day 7 of culture. All assays were conducted in triplicate. Mean IC_{50} values (± SD) are shown. The numbers in parentheses represent fold changes compared to the 50% inhibitory concentration against HIV-1_{WT}.

	IC_{50} (μM)			
Infectious clone	Zidovudine	Didanosine	Nevirapine	Saquinavir
HIV-1_{WT}	0.012 ± 0.002 (1 ×)	3.2 ± 1.0 (1 ×)	0.019 ± 0.001 (1 ×)	0.012 ± 0.002 (1 ×)
HIV-1_{Q151M}	0.17 ± 0.06 (14 ×)	41.0 ± 4.0 (13 ×)	0.068 ± 0.004 (3.6 ×)	0.014 ± 0.003 (1.2 ×)
HIV-$1_{Q151L/M230I}$	0.0053 ± 0.0006 (0.44 ×)	3.7 ± 0.9 (1.2 ×)	0.26 ± 0.02 (13.7 ×)	0.017 ± 0.004 (1.4 ×)
HIV-1_{M230I}	0.0054 ± 0.0012 (0.45 ×)	0.83 ± 0.12 (0.26 ×)	0.19 ± 0.03 (10 ×)	0.014 ± 0.004 (1.2 ×)