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Upregulation of HTLV-1 and HTLV-2 Expression by HIV-1 In Vitro

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Abstract

Co-infections with HIV-1 and the human T leukemia virus types 1 and 2 (HTLV-1, HTLV-2) occur frequently, particularly in large metropolitan areas where injection drug use is a shared mode of transmission. Recent evidence suggests that HIV-HTLV co-infections are associated with upregulated HTLV-1/2 virus expression and disease. An in vitro model of HIV-1 and HTLV-1/2 co-infection was utilized to determine if cell free HIV-1 virions or recombinant HIV-1 Tat protein (200–1,000 ng/ml) upregulated HTLV-1/2 expression and infectivity. Exposure to HIV-1 increased the number of HTLV-1 antigen expressing cells, from 6% at baseline to 12% at 24 hr, and 20% at 120 hr ($P < 0.05$) post-exposure. A similar, although less robust response was observed in HTLV-2 infected cells. HIV-1 co-localized almost exclusively with HTLV-1/2 positive cells. Exposure to HIV-1 Tat protein (1,000 ng/ml) increased HTLV-1 p19 expression almost twofold by 48 hr, and cells co-stimulated with 10 nM phorbol myristate acetate (PMA) showed almost a fourfold increase over baseline. It is concluded that HIV-1 augments HTLV-1/2 infectivity in vitro. The findings also suggest a role for the HIV-1 Tat protein and PMA-inducible cellular factors, in HIV-1 induced HTLV-1/2 antigen expression.

Keywords

HIV-1; HTLV-1; HTLV-2; Tat protein; phorbol esters

INTRODUCTION

The human T-lymphotropic viruses type-1 and -2 (HTLV-1 and HTLV-2) are frequent co-pathogens among HIV-1 infected individuals, especially in large metropolitan areas where injection drug use is a common mode of viral transmission [Bartholomew et al., 1987; Khabbaz et al., 1992; Wiktor et al., 1992; Briggs et al., 1995; Figueroa et al., 1997; Carneiro-Proietti et al., 1998; Egan et al., 1999; Ciancianaini et al., 2001]. While HIV-1, HTLV-1, and HTLV-2 all share in common a preferential tropism for T-cells, the virologic manifestations of each of these three viruses differ significantly [Gallo, 2002]. HIV-1 is highly cytopathic for CD4+ T-cells, while HTLV-1 and HTLV-2 are non-cytopathic and have the potential to cause clonal proliferation and transformation of T-cells [Blattner, 1994; Gallo, 2002].

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Upregulated levels HTLV-1 and HTLV-2 messenger RNA expression in blood samples obtained from patients dually infected with HIV-1 and HTLV-1 or -2 was previously demonstrated [Beilke et al., 1997]. In addition, clinical evaluations of co-infected individuals suggest an increased expression of HTLV-1/-2-associated disease conditions, including HTLV-associated myelopathy and peripheral neuropathy [Beilke et al., 1994, 2004; Zehender et al., 1995; Nadler et al., 1996; Bessinger et al., 1997; Harrison et al., 1997]. Limited evidence suggests that highly active antiretroviral therapy (HAART) is ineffective in reducing HTLV-1 and HTLV-2 viral burden in dually infected individuals [Machuca and Soriano, 2000; Garcia-Lerma et al., 2001; Hill et al., 2003]. The increased incidence of retroviral co-infections and the limited efficacy of HAART suggest that an understanding of factors that dictate a productive co-infection will need to be delineated, and may suggest alternate treatment strategies in individuals manifesting productive co-infection with both HIV-1 and HTLV-1 and -2.

It has been speculated that HIV-1 may have either direct inductive effects on HTLV-1/2 viral replication, or that HIV-1 may activate HTLV-1/2 viral expression via interaction with host cellular genes [Moriuchi et al., 1998; Sun et al., 2006]. In addition, the HIV-1 trans-activating gene product, Tat, could potentially have inductive effects on HTLV-1/-2 in co-infected micro-environments [Ensoli et al., 1993; Mondal and Agrawal, 1998; Huigen et al., 2004]. In this study, evidence is presented suggesting that exposure to HIV-1 virions can increase levels of HTLV-1 and HTLV-2 viral antigen expression in vitro. Furthermore, the experiments indicate that recombinant Tat protein increases HTLV-1 gene expression, in concert with the phorbol ester, PMA, and PMA-induced cellular factors.

METHODS

Cell Cultures

HTLV-1 infected MT-2 cells, HTLV-2 infected MoT cells, and uninfected HUT-78 and HIV-1 (HTLV-III_B) infected H9 cells were used as positive and negative control cell lines (provided by R. Gallo, M.D.). MT2, MoT, Hut 78, and HTLV-III_B cell lines were grown in full growth medium [RPMI medium supplemented with 10% FCS, 2.05mM L-glutamine, 1% penicillin/streptomycin (v/v)]. Two additional clinical interleukin 2 (IL-2) dependent cell lines, designated NO-HTLV-1 and NO-HTLV-2, were established from known HTLV-1 and HTLV-2 infected individuals [Beilke, 1994, 1995]. NO-HTLV-1 and NO-HTLV-2 patient samples were maintained in full growth medium supplemented with 10% recombinant IL-2 (Hemagen, Columbia, MD). Cultures were sub-cultured (1:3) every 3–5 days and fresh full growth medium was added to cultures. The cultures exhibited a stable, continuous rate of doubling beyond 6 months, and exhibited persistent, stable levels of HTLV-1/2 mRNA expression by in situ hybridization, and HTLV-1/2 viral antigen expression as determined by indirect immunofluorescence microscopy (IFA) [Beilke, 1992, 1994]. All cells were grown in a humidified incubator at 37°C with 5% CO₂.

Reagents

For the IFA experiments, polyclonal antiserum was obtained from an HTLV-1 infected rabbit. This reagent was found in preliminary experiments to work efficiently for staining of HTLV-1 infected MT-2 and NO-HTLV-1 cells, and also HTLV-2 infected MoT cells and NO-HTLV-2 cells. For experiments using HIV-1 Tat protein, a mouse monoclonal antibody against HTLV-1 p19 antigen was used (Olympus, Lake Success, NY). Primary pooled sera for HIV staining were obtained from HIV-1 seropositive, HTLV-1/2 seronegative patients. Secondary antibodies included goat anti-human or anti-mouse fluorescein isothiocyanate (FITC) conjugated or goat anti-rabbit rhodamine (TRITC) conjugated antibodies (Boehringer, Indianapolis, IN).

Recombinant HIV-1 Tat protein (MN-strain; 101 amino acid) was obtained from ImmunoDiagnostics (Woburn, MA). The protein was re-suspended as previously described [Hui et al., 2006] in PBS containing 0.1% BSA and 0.1 mM DTT and adjusted to a concentration of 1.0 µg/µl and stored in individual aliquots at -80°C until use (for a maximum of 3 months). Phorbol-12-myristate-13-acetate (PMA) was obtained from SIGMA, USA, and was dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml concentration and stored at -80°C.

Treatments

The HTLV-IIIB infection of cells and incubation with recombinant Tat protein and PMA were conducted as follows: NO-HTLV-1 or NO-HTLV-2 cells (1×10^5 cells) were infected with cell-free, 0.2 µm filtered, high-titer stocks derived from HTLV-IIIB cultures (approximately 1×10^5 infectious particles/milliliter of virus stock). The virus was allowed to adsorb to the cells for 1 hr, and then cells were washed three times, resuspended in culture medium and placed into 24-well tissue culture plates. Infected cells were harvested at baseline, 4, 24, and 120 hr post-infection, washed, resuspended in phosphate buffer saline (PBS), and cytocentrifuged onto microscope slides for IFA staining.

Next, NO-HTLV-1 cells were incubated with recombinant Tat protein (200 ng/ml) and cells were harvested on days 1, 3, 5, and 7, perform IFA analysis for HTLV-1 p19 expression. In preliminary IFA experiments, it was shown that uptake of HIV-1 Tat protein was observed in >65% of NO-HTLV-1 cells within 24 hr (data not shown). The NO-HTLV-1 cells were also stimulated with Tat alone (200 and 1,000 ng/ml) or in combination with PMA (16 nM) and cells and supernatants were harvested after 48 hr for determination of HTLV-1/2 viral p19 antigen levels, using an antigen capture ELISA kit (Zeptometrix, Buffalo, NY) as described below.

Indirect Immunofluorescence (IFA) Assays

The IFA assay was optimized used MT-2, MoT, and HTLV-IIIB cells cultured both individually and in combination to produce consistent positive signals [Beilke, 1992]. NO-HTLV-1 and NO-HTLV-2 cells were then analyzed by IFA with omission of either primary or secondary antibody to verify staining specificity. Briefly, cells were washed three times with phosphate buffered saline (PBS) and cytocentrifuged onto microscope slides, fixed in 95% ethanol, and then air-dried. Non-specific antibody binding was inhibited by incubation with a blocking buffer (PBS supplemented with 10% normal goat serum, 0.01% TritonX-100) for 16 hr at 4°C. The slides were placed into a humidified chamber and primary antibodies, diluted in blocking buffer, were added and incubated for 60 min at appropriate dilutions. After incubation with primary antibody, slides were washed repeatedly in PBS-T (PBS plus 0.01% Triton X-100) followed by incubation with either goat anti-human FITC conjugated or goat anti-rabbit TRITC conjugated antibodies (diluted 1:100). Slides were then washed several times with PBS-T and one final brief wash with de-ionized H₂O, air dried, mounted with clover slips and then examined on an Olympus BH-2 microscope at 400× magnification. Images were captured on Kodak Elite Chrome ISO 100 film. Double exposures of fields were obtained to co-localize FITC (HIV-1) and TRITC (HTLV-1 or HTLV-2) conjugates.

HTLV-1 p19 Antigen Capture Assay

The effect of Tat (200 and 1,000 ng/ml) in the presence or absence of PMA (16 nM) on expression of p19 antigen in NO-HTLV-1 cells was quantified in both culture supernatants and cell lysates. Samples were collected from duplicate wells at 48 hr post-treatment. Cell culture supernatants were centrifuged to remove any cells and debris, and both cell free supernatants and cell pellets were saved in duplicate aliquots at -80°C for ELISA studies. The p19 ELISA was performed using a standard panel with known quantities of HTLV p19 antigen, as per the manufacture's protocol (ZeptoMetrix Corp.). The protein contents in supernatants

and lysates were determined by using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Results obtained for p19 concentrations (pg/ml) were normalized to cellular protein contents (µg/ml) in each sample. ELISA experiments were performed in triplicate with values obtained from two replicate samples averaged in each experiment.

Statistical Evaluation

All statistical analysis was carried out using the INSTAT Graph Pad-2 software (Graph Pad, San Diego, CA). The results are expressed as standard error of means ($\pm$ SEM). The significance of changes from control values was determined by using a two-tailed Student *t*-test and comparison between three or more groups were carried by one-way analysis of variance (ANOVA). The *P* values of <0.05 were considered to be significant. For IFA experiments viewer bias was eliminated by double-blinded studies. In order to determine the numbers of cells which must be counted to assure a power of greater than 80%, a formula to determine the difference between two proportions was used to determine an appropriate sample size [Rosner, 1995].

RESULTS

Exposure to HIV-1 Showed Temporal Increase in the Number of HTLV-1 and HTLV-2 Infected Lymphocytes

Basal expression of HTLV-1/2 viral antigens in NO-HTLV-1 and NO-HTLV-2 cell lines remained constant while maintained in culture (6.2% and 3.9% of cells, respectively). Following exposure to cell-free stocks of HIV the number of infected cells was analyzed at 0, 4, 24, and 120 hr post-infection. At 0 and 4 hr post-infection, only HTLV-1 infection was detected, and the quantity of HTLV-1 positive cells was similar to untreated cells. Beginning at 24 hr post-infection, a small amount of HIV-1 specific staining was observed along with a significant increase in the number of HTLV-1 positive cells which continued to increase until day 5 when the experiment was terminated. Similarly, in NO-HTLV-2 infected cells, the first detectable level of HIV infection was observed at 24 hr. At 120 hr, a large increase in the number of HIV-1 infected cells was observed along with a statistically significant increase in the number of HTLV-1 and HTLV-2 cells (Fig. 1).

HIV-1 and HTLV-1 or HTLV-2 Were Co-Localized by Dual Label IFA and Merged Image Analysis

Merged image analysis was employed to co-localize HIV-1 and HTLV-1/2 viral antigens in the same cell following HIV-1 infection, using dual FITC (fluorescein) and TRITC (rhodamine) filters. Low levels of non-specific staining were observed under FITC, TRITC, and dual filters (Fig. 2a–c). Low levels of background staining facilitated the differentiation of positive and negative, non-stained cells in later experiments. Several cultures were analyzed and the overall rate of mono-infection of NO-HTLV-1 cells was observed to be approximately 6%. Analysis of cells infected with only HTLV-1 or HIV-1 and small population of dual-infected cells were documented by merged image analysis (Fig. 2d–f). The representative IFA shows a HIV infected cell (Fig. 2d) which was also positive for HTLV-1 (Fig. 2e). The merged image (Fig. 2f) clearly shows co-infection of the same lymphocyte with HIV and HTLV-1. It was common to find mono-infected NO-HTLV-1 cells at all time points. Interestingly, greater than 95% of HIV-positive cells also contained HTLV-1, but HIV-1 mono-infection were rarely observed (Fig. 2a–f).

Exposure to Either Infectious HIV-1 or Recombinant Tat Protein Rapidly Increased HTLV-1 p19 Expressing Cells

To determine whether HIV-1 Tat protein alone had inductive effects on HTLV-1/2 expression, the immunofluorescence experiments were repeated following exposure to HIV-1 Tat protein (200 ng/ml). In the initial experiments, exposure to HIV-1 virions resulted in an increase in HTLV-p19 expressing cells at approximately 72 hr. In contrast, the effects of HIV-1 Tat protein were observed within 24 hr and persisted until third day, after which HTLV-1 antigen expression fell to levels seen in controls (Fig. 3).

Synergistic Effects of Tat in Increasing HTLV-1 p19 Expression in the Presence of PMA

To further explore the possible mechanism for the inductive effects of Tat protein, preliminary experiments were performed to determine whether Tat works in conjunction with different second messenger signaling pathways and cellular transcription factors. The protein kinase C (PKC) pathway, frequently induced in HIV-1 infected cells, is essential for optimal Tat function. Therefore, experiments of Tat-mediated HTLV-1/2 viral expression were extended to include the addition of the phorbol myristate acetate (PMA), a known PKC-inducer. HTLV-1 p19 antigen capture ELISA assays were performed for the detection of both intracellular and extracellular levels of HTLV-1 viral antigen. Cells were treated with Tat (200–1,000 ng/ml) alone and in combination with PMA (16 nM), and culture supernatants and cell lysates were obtained at 48 hr post-treatment. Significant ($P < 0.05$) effects were observed upon co-treatment with both Tat and PMA. A detectable increase in p19 levels in the supernatants was evident at higher concentrations of Tat (1,000 ng/ml). A more pronounced increase in the intracellular p19 levels was observed at lower concentrations (200 ng/ml), especially on co-stimulation with PMA (Fig. 4).

DISCUSSION

Limited data exist regarding the viral dynamics of HIV-1 and HTLV-1/2 viral co-infection in vitro [Lin et al., 1995; Cheng et al., 1998; Moriuchi et al., 1998; Nekhai et al., 2007]. One study suggested that a factor or factors secreted by HTLV-1 could enhance replication of HIV-1 in vitro [Moriuchi et al., 1998]. Sun et al. [2006] demonstrated that HIV-1-mediated syncytium formation promoted cell-to-cell transfer of HTLV-1 Tax protein with subsequent activation of the HTLV-1 transcription. De Rossi et al. [1991] conducted experiments suggesting that superinfection of HTLV-1 infected cells with live HIV-1 activated HTLV-1 viral expression but not the tat protein, whereas the current study has suggested that tat protein and PMA has a combined effect on HTLV p19 expression.

In this study, exposure to either HIV-1 or the HIV-1 Tat protein increased the number of both HTLV-1 and HTLV-2 infected cells (Fig. 1) which is a result of both increased infectivity and increased productivity, as evident from the number of positive cells (Fig. 3) and the total level of p19 expression (Fig. 4). There was a significant increase in HTLV-1 positive cells at 24 hr post-infection and the amount of both IFA positivity (Fig. 2) and subjective staining intensity were shown to peak at 120 hr (5 days) post-infection.

The IFA experiments demonstrated a preferential replication of HIV-1 within HTLV-1/-2 infected cells. This suggests the possibility that HIV-1 and HTLV-1/-2 interact dynamically within the same cells in vivo. Co-localization of HIV-1 and HTLV-1 or -2 within the same cells would support the contention that HIV-1 itself and/or associated HIV-1 gene products are responsible for the observed increase in HTLV-1/-2 expression. Interestingly, the cells that were determined to be HIV-1 positive were nearly always HTLV-1 positive as well, and only in rare cases were HIV mono-infected cells observed.

In clinical samples, previous experiments documented higher levels of HTLV-1/2 *tax/rex* mRNA in PBMC samples from HTLV-1/2 and in subjects dually infected with HIV [Beilke et al., 1997]. The current experiments corroborate this finding, and suggest the possibility that HIV-1 Tat protein, released by the infected cells, may play a dominant role in increasing HTLV-1 infectivity and p19 expression. A number of previous studies have documented the possible role of other transcription factors and second messenger signaling pathways activated in different cell types following exposure to HIV-1 Tat protein [Bohan et al., 1992; Zauli et al., 1992; Hui et al., 2006]. Exogenously added Tat protein can have similar inductive effect as compared to the infectious HIV-1, suggest that Tat may activate HTLV-1 proviral activation. This is clearly indicative of the time dependent increase in HTLV-1 p19 expression by both HIV-1 virions and recombinant Tat protein. Since activation of HIV-1 and HTLV-1/2 provirus is under the control of both viral regulatory genes as well as cellular transcription factors [Ensoli et al., 1993; Lin et al., 1995; Hui et al., 2006], the possibility exists that HIV-1 Tat can activate the HTLV-1 long terminal repeat (LTR), either directly or via cooperation with cellular co-factors [Nakhai et al., 2007]. The rapid increase in p19 expression, which occurred within 24 hr following exogenous addition of Tat protein, in contrast to the peak observed at 3–5 days with HIV virions, also indicates that the delayed effect may be due to the time required to establish HIV infection and Tat protein expression. The presence of HIV virions only in productively HTLV-1/2 infected cells suggests that such an augmented effect may occur predominantly in co-infected cells. Nonetheless, the current experiments cannot exclude the possibility that HIV-1 induction of HTLV-1 expression is due to a paracrine effect of HIV-1 (or HIV-Tat) on neighboring cells.

The observation that PMA-stimulation of cultures enabled a significant increase in Tat mediated increase in p19 expression also suggests that activated cellular factors may be necessary for optimal initiation and propagation of the co-infection cycle. The PKC pathway is potently stimulated by PMA, and specific PKC isozymes are activated during a number of inflammatory stimuli [Aksoy et al., 2004; Hayashi et al., 2007] occurring in co-infected reservoirs, due to immune activation and cytokine expression. HIV-1 co-infection may further cause activation of inflammatory signaling via PKC, so it is possible that in addition to Tat, PKC-inducible cellular factors could mediate activation of HTLV-1/2 infectivity.

While the current investigations provide evidence that HIV-1 and Tat protein may upregulate HTLV-1/2 expression, the study does not fully address the prior observation that HIV disease progression is delayed in patients with HIV/HTLV co-infections [Beilke et al., 1994, 1997]. The proliferative effects of HTLV-1/2 infection may prevent the lytic HIV cycle from depleting the host lymphocytes. As seen with severe HTLV-1 disease presented as adult T cell leukemia/lymphoma or HTLV-2 disease presented as TSP/HAM, promotion of cell division is a prominent feature of HTLV-1/2 pathogenesis [Blattner, 1994; Gallo, 2002]. Established HTLV-1/2 models account for this proliferative effect in the pathogenesis of HTLV-1/2 [Cheng et al., 1998; Gallo, 2002]. In addition to proliferation, another potential mechanism focuses on similar gene homology of HTLV-1/2 Tax and HIV Tat proteins [Hui et al., 2006]. These gene products may interact at similar molecular pathways and sequester specific transcription factors, thus enabling a competition between both viruses. A productive infection by one virus may also alter disease progression and potentially enable HTLV-1/2 or HIV to replicate at altered rates. However, further work on viral dynamics, both in vivo and in vitro will be needed to test these hypotheses. The findings from these viral dynamics studies may have probable significance in generating alternative strategies to suppress productive co-infection and regulate disease progression by either viruses.

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Fig. 1.

Inductive effects of HIV-1 on HTLV-1/2 viral antigen expression. The NO-HTLV-1 (a) and NO-HTLV-2 (b) cell lines were exposed to cell free HIV-1, and the number of cells infected with either viruses were quantified by immunofluorescence assays (IFA) at 0, 4, 24, 120 hr post-infection. The experiments were repeated twice ($n = 2$) and five images were captured at each time point. The number of mono-infected cells was enumerated in each culture and percent positive cells are shown in the bar graphs. The error bars represent $\pm$ SEM of values and as compared to the initial levels, significant differences in positive cells observed at each time point are represented as P values ($*P < 0.05$).

Fig. 2.

Co-localization of HTLV-1 and HIV-1 by merged image IFA analysis. The NO-HTLV-1 cultures were exposed to cell free HIV-1 were monitored for dually-infected cells at 5 days post-infection. Dual label IFA analysis was then performed to co-localize HIV-1 (FITCfilter) and HTLV-1 (rhodamine TRITC filter). The negative controls (a–c) were determined by staining in the absence of virus specific antibodies and labeling with either FITC (a), TRITC (b), and dual labeling (c). Co-infection was demonstrated in cultures incubated with the respective antibodies and by microscopic inspection of cells in the same field (d–f).

Fig. 3.

Temporal effects of HIV-1 or Tat stimulation on HTLV-1 activation. Cell free HIV virions or recombinant HIV-Tat protein were added to NO-HTLV-1 cells. Cells were harvested and washed on days 1, 3, 5, and 7, and then cytocentrifuged onto microscope slides. The cells were then stained with a monoclonal antibody to HTLV-I p19 antigen and the number of p19 expressing cells was examined by IFA. The significant differences in p19 expressions are represented as *P* values (***P* < 0.05).

Fig. 4.

Synergistic effects HIV Tat protein (200 and 1,000 ng/ml) in combination with PMA (16 nM) on HTLV-1 p19 expression. The data obtained at 48 hr are shown in the bar graphs. Both intracellular (cell lysate) and extracellular (supernatant) p19 production were analyzed. The p19 levels were normalized to total protein contents and represented as pg of p19/μg of protein. The error bars represent $\pm$ SEM SEM of values and significant differences at each time point, as compared to the initial time point, are represented as *P* values (**P* < 0.05; ***P* < 0.01).