Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 345-350

Evaluation of the effects of erythro-9(2-hydroxy-3-nonyl) adenine (EHNA) on HIV-1 production in vitro

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Received August 31, 1989

SUMMARY: Effects of erythro-9(2-hydroxy-3-nonyl) adenine (EHNA), an inhibitor of the common Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4.), on HIV-1 production was evaluated in vitro. Reverse transcriptase (RT) activity in the supernatant was inhibited by nearly 50% in EHNA-treated HIV-1 infected H9 cells, when compared with untreated but infected H9 cells. There was also a significant decrease in cell viability, but this was reversed following the addition of deoxycytidine (dC) to these cultures. The combined treatment was also effective in suppressing HIV-1 release from HIV-1-infected U937 cells. This combined EHNA plus dC treatment had no effect on RT activity in the cell lysates, suggesting that the inhibition of HIV-1 production may be due to the disturbance of virus release from infected cells.

Patients with the acquired immune deficiency syndrome (AIDS) have been reported to have higher levels of adenosine deaminase (ADA) activity in their serum as well as erythrocytes (1, 2), and this increase has been found to correlate closely with the HIV-1 infection. Since symptomatic as well as asymptomatic, but seropositive patients have high serum ADA activity, this increase may not be due to non-specific factors, such as opportunistic infections, malignancies or hepatitis (1). These results led us to propose that the HIV-1 production may require changes in the purine metabolic pathways. Alternatively, the inhibition of ADA or other enzymes related to purine metabolic pathways may affect HIV-1 production.

In the present study, we tested the effects of ADA inhibitor, erythro-9 (2-hydroxy-3-nonyl) adenine (EHNA), on the production of HIV-1 by HIV-1 infected cell lines. Our results indicate that the combined treatment of EHNA plus deoxycytidine (dC) resulted in the inhibition of HIV-1 production from HIV-1 infected T (H9) and promonocyte (U937) cell lines. Since there was no effect on RT activity in cell lysates, the results suggest that the inhibition of HIV-1 production may be due to an inhibition of HIV-1 release from infected cells.

0006-291X/89 $1.50

MATERIALS AND METHODS

Cell lines: H9 ($CD3^+$, $CD4^+$, $CD8^-$, and $HLA\text{-}DR^+$) and HIV-1(HTLV-IIIB) producing H9 (H9-IIIB) cell lines were kindly provided by Dr. Robert-Guroff (National Cancer Institute, MD). H9-IIIBN and H9-IIIBF were subclones from H9-IIIB. SupT1 ($CD3^+$, $CD4^+$, $CD8^+$, and $HLA\text{-}DR^+$), markedly sensitive to both syncytium induction and infection by HIV-1, were provided to us by Dr. Hoxie (Hospital of the University of Pennsylvania, PA). U937 (Leu $M3^+$, $HLA\text{-}DR^+$, and $CD4^+$) cell line was obtained from the American Type Culture Collection (Rockville, MD). U937-IIIB line was a single HIV-1 infected clone obtained from HIV-1 infected U937. Briefly, HIV-1 infected U937 cells and H9-IIIB cells were cloned by limiting dilution and cells which tested positive for both syncytium induction against SupT1 cells, and reverse transcriptase (RT) activity, were expanded and cryopreserved.

Materials: Erythro-9 (2-hydroxy-3-nonyl) adenine (EHNA) was a generous gift of Maruho Corporation (Japan). Deoxycytidine (dC) was purchased from Sigma (St. Louis, MO). Monoclonal antibody 2E12.1 (specific for envelope protein gp120; Epitope, Beaverton, OR) was a gift from Dr. Nakagawa (Kumamoto University, Japan).

Assay for HIV-1 production: HIV-1 infected clones were washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium (GIBCO, Grand Island,NY) supplemented with 10 % fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (50 µg/ml). Cells ($2x10^5$/ml) were added to 25 cm^2 tissue culture flasks (Corning, NY) with or without varying concentrations of EHNA and dC. The cultures were incubated at 37° C in 5% CO_2 atmosphere for three days. Following incubation the cell viability was determined using trypan blue as the vital stain. Virus production was determined by RT activity assay described below.

Reverse transcriptase (RT) Assay: For RT activity in supernatant (RT^S), 5 ml of the supernatant was mixed with 30% polyethylene glycol (M.W. 8000) and 4M NaCl (10 : 5 : 1). This mixture was kept cold overnight. After centrifugation (3000 rpm, 30 min), the pellet was resuspended in buffer C (50mM Tris-HCl, pH 7.5, 0.25 M KCl, and 0.25% Triton X-100). For RT activity in cell lysates (RT^{CL}), $1x10^6$ cells were washed three times with PBS and resuspended in buffer C. Cells were freeze-thawed three times with vigorous vortex each time. The completion of cell lysis by this method was comfirmed under the microscope. RT assay was carried out in a reaction mixture as previously reported (3). This reaction mixture contained 50 mM Tris-HCl (pH 7.5), 5mM Dithiothreitol (DTT), 100mM KCl, 0.01% Triton X-100, 10 µg/ml $(dT)_{15}$ $(A)_n$ (Pharmacia, Piscataway, NJ) as template primer, and [^{3}H] deoxythymidine triphosphate (New England Nuclear, Boston, Mass). The reaction mixture was incubated with RT samples for 1 hr and the reaction stopped by adding ice-cold 5 % TCA containing 1 mM sodium pyrophosphate. Samples were then filtered on millipore filters (0.45 µm), washed first with 5% TCA solution (5 times) and then with 2 ml of 70% ethanol. Filters were then dried in an oven. Scintillation fluid was added and the

radioactivity counted in Beckman ß-scintillation counter (LS5801, Beckman Instruments).

Detection of envelope protein (gp120) expression: Flow cytofluorometric analysis was carried out using FACSCAN (Becton Dickinson). Cells were incubated with optimal concentration of 2E12.1 for 30 min. After washing twice, cells were stained with fluorescein isothiocyanate (FITC) - conjugated goat anti-mouse IgG (Becton Dickinson).

RESULTS AND DISCUSSION

HIV-1 (HTLV-III_B) infected cell lines were treated with varying concentrations of EHNA. Several different concentrations of dC were also added to the cell cultures. Figure 1 shows the effects of either EHNA alone or combination of EHNA + dC on cell viability and RT^S activity. Both a decrease in cell viability as well as a loss of RT^S activity were observed with increasing concentrations of EHNA. 50 µM of EHNA treatment resulted in about 50% decrease in cell viability as well as 50% loss in RT^S activity. However, the addition of dC reversed this cell viability loss. The combined treatment with EHNA(50 µM) + dC(500µM) resulted in only 15% loss of cell viability but nearly 70% decrease in RT^S activity. Addition of dC alone decreased RT^S activity by approximately 5-10% (data not shown). Since the addition of EHNA + dC directly to

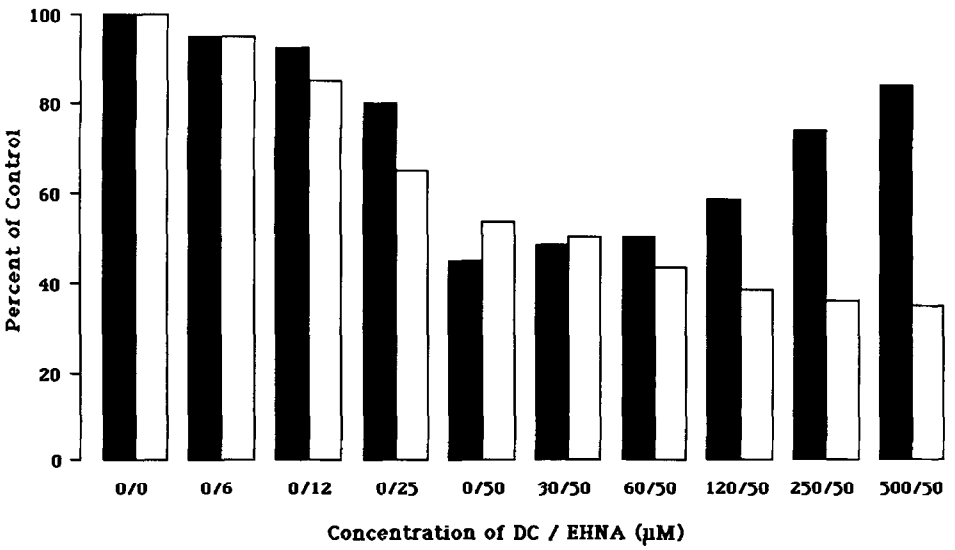

Figure 1. Effects of EHNA (0-50µM)+ dC (0-500µM) on HIV-1 production from HIV-1 infected cell line (H9-III_B). H9-III_B cells were incubated for 3 days in the presence of different concentrations EHNA plus increasing concentrations of dC (the number below the bar graph represents the concentrations [dC/ EHNA]). The data presented represents the mean percent of untreated controls performed in triplicate. ■ Cell viability; □ RT activity in the culture supernatant.

cell-free RT reactive mixture did not affect the reaction, these results would suggest that the combined treatment inhibited HIV-1 production from the infected cell line.

We also examined the inhibitory effects of combined EHNA + dC treatment on RT activity using other HIV-1 infected cell lines, such as U937-III_B, H9-III_BN and H9-III_BF (Figure 2). Although these lines showed different sensitivities with respect to cell viability and inhibition of RT^S activity following treatment with EHNA+dC, RT^{CL} activity was not affected in any of these cell lines. Since EHNA + dC treatment did not inhibit RT^{CL} activity whereas this combined treatment markedly inhibited RT^S activity, suggests that there was an inhibition of intact virion release from cells. Whether this inhibition was due to lack of formation of intact virion constructs within cells or defects in intact virion release from infected cells needs further investigation. Also, whether the decreased expression of viral envelope protein gp120 on cells, as seen following EHNA+dC treatment (Fig. 3), is related to inhibition of intact virions from infected cells needs further studies .

There are several reports which suggest that the inhibition of ADA induces the accumulation of deoxynucleosides triphosphates (dATP or dGTP) within cells resulting in cell toxicity especially in cells of the T cell lineage having relatively strong dC kinase (4 - 6). However, saturation of available dC kinase by addition of dC reduces such cell toxicity (7 - 9). Thus, in our studies the combined treatment of cells with EHNA + dC may have lead to an inhibition of ADA and less cell toxicity. Further, the inhibition of

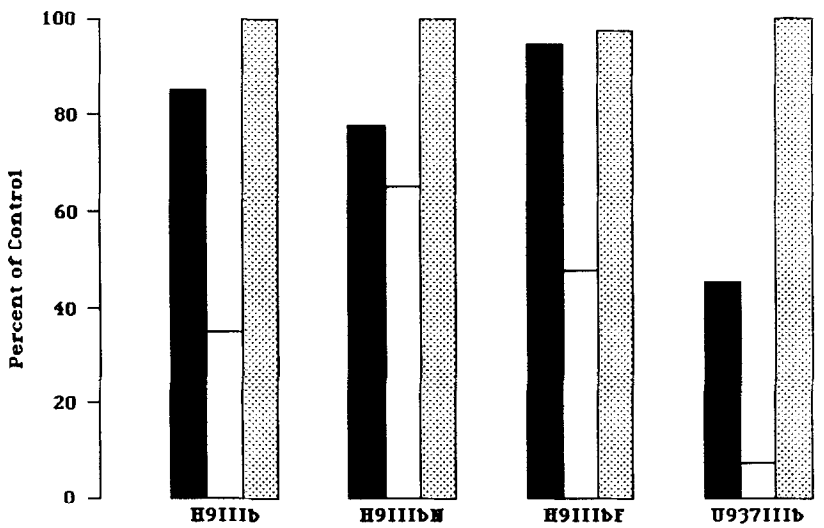

Figure 2. Effects of combined EHNA + dC treatment on HIV-1 production from U937-III_B and subclones of H9-III_B. The cells were incubated for 3 days in the presence of EHNA (50μM) + dC (500μM). The data presented represents the mean± SEM of percent of untreated control cultures. ■ Cell viability; □ RT activity in the culture supernatant; ⊠ RT activity in the cell lysates.

viral release by this combined treatment would suggest that HIV-1 release from infected cells may be regulated by purine metabolic pathways.

Recent studies by Guy et al (10) indicate that HIV-1 *nef* gene product, 27-kDa nef protein, has GTPase activity, and that it is a protein kinase capable of autophosphorylation. Their results suggested that the *nef* protein may act as an analogue of signal-transducing proteins probably by modifying cellular metabolic pathways. Since the regulation of ADA mRNAs is also related to protein kinase C activation (11), there may be a synchronous regulation of *nef* protein and ADA by signal transduction. It is possible that certain cellular factors, such as ADA, terminal deoxynucleotidyl transferase (TdT; EC 2.7.7.31) and purine nucleoside phosphorylase (PNP; EC 2.4.2.1), that change markedly during the course of T-cell development, affect HIV-1 replication and CD4 down-modulation. Thus sensitivity of cells to infectivity, syncytium formation and virion production may vary among CD4+ cell lines. The differences in purine metabolism among cell types may explain why HIV-1 replicates more actively in some $CD4^+$ T cells than other $CD4^+$ non-T cells. This approach from cellular metabolic pathways may help in understanding characteristics of HIV-1 infection in such cells.

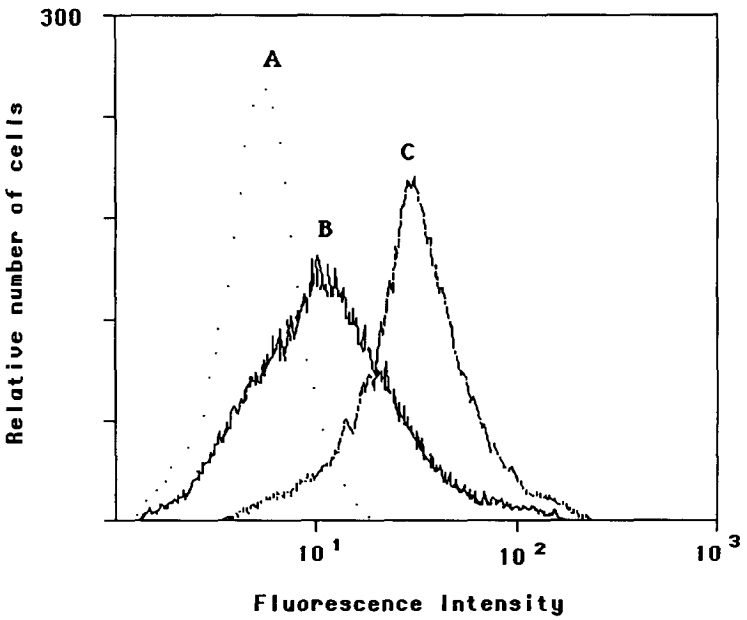

Figure 3. Effect of EHNA (50μM) + dC (500μM) on gp120 expression. Treated or untreated H9-III_B cells were stained with anti-gp120 monoclonal antibody (2E12.1). Results indicate relative number of cells and log fluorescence intensity. A, H9-III_B cells stained with FITC-conjugated goat anti-mouse antibody; B, Treated cells stained with 2E12.1; C, Untreated cells stained with 2E12.1.

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