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N-Acetylcysteine Inhibits Latent HIV Expression in Chronically Infected Cells

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ABSTRACT

The progression of the human immunodeficiency virus (HIV) infection from its early latent (asymptomatic) stage to active, late-stage acquired immunodeficiency syndrome (AIDS) apparently begins with the production of inflammatory cytokines that stimulate the expression and replication of the latent virus. We have shown that *N*-acetylcysteine, a cysteine precursor that is converted intracellularly into glutathione, blocks cytokine-stimulated HIV replication in an acutely infected T-cell line and in acutely infected peripheral blood mononuclear cells from normal individuals. In this report, we show that *N*-acetylcysteine also inhibits stimulated HIV expression in chronically infected monocyte and T-cell lines which are used as models for latent infection in AIDS. Furthermore, we show that *N*-acetylcysteine blocks viral production in monocyte cell lines more effectively than it blocks viral production in T cells. Since monocytes are a major reservoir for HIV in infected individuals, these results suggest that *N*-acetylcysteine may slow the change from latency to the later stages of AIDS in HIV-infected individuals.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS (HIV) infection begins with the introduction of the retroviral genome into T cells and macrophages. After an initial viremic stage, free virus is greatly diminished and the infection becomes latent. At this stage, most of the virions are integrated into the DNA of T cells. Fauci and colleagues have proposed that the virus remains in this latent state (and infected individuals remain asymptomatic) until tumor necrosis factor (TNF)-α or other inflammatory cytokines trigger viral replication and expression.[1,2] The observation that TNF-α also upregulates its own production[3–5] suggests that it may be responsible for the rapid escalation of the acquired immunodeficiency syndrome (AIDS) in its later stages. This hypothesis is supported by data demonstrating (1) that TNF-α levels are abnormally high in serum samples from asymptomatic HIV-infected individuals, (2) that TNF-α levels rise as opportunistic infections become frequent in patients with ARC and AIDS, and (3) that TNF-α levels are high in all individuals with CDC stage IV AIDS.[6–9]

TNF-α or phorbol myristate acetate diester (PMA) stimulate the expression of HIV in part by activating the transcription factor NF-κB, which in turn increases the promoter activity of the HIV long terminal repeat (LTR).[10–13] We have shown that *N*-acetylcysteine (NAC) inhibits these stimulations in a variety of cell lines as well as PHA-stimulated peripheral blood mononuclear cells (PBMC) from normal individuals.[14] Additionally, we have shown that NAC inhibits HIV replication by inhibiting the activation of NF-κB in cultured cells.[15]

We now show that NAC inhibits the production of virions from chronically infected cell lines. This inhibition is due to the inhibition of synthesis of RNA as directed by the HIV LTR. Since these cell lines serve as models for latent infection,[2,10] our results demonstrate that NAC may be useful as an adjunct in therapy of individuals who are HIV^+ but are still asymptomatic, in that it may retard or prevent the onset of viremia.

MATERIALS AND METHODS

All chemicals were obtained from Sigma (St. Louis, MO), unless otherwise noted. ACH2 and U1[2,10] cells were obtained from the AIDS Research and Reference Reagent Program (NIAID, Bethesda, MD).

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Monitoring HIV infections

ACH2 cells (10^6 per ml) were stimulated for 24 h with 0.5 ng/ml TNF-α. The supernatant was separately titered on MOLT-4 T cells;[16] it was then used to infect MOLT-4 at a multiplicity of infection (MOI) of 0.3, or the PBMC at an MOI of 2. PBMC from heparinized blood of HIV^- individuals were isolated by Ficoll-Hypaque density centrifugation and were stimulated for 3 days with 2 μg/ml phytohemagglutinin (PHA) in RPMI-1640 supplemented with 20% fetal calf serum (FCS), 290 μg/ml L-glutamine, 100 U/ml penicillin, 70 μg/ml streptomycin, and 5% purified human interleukin-2 (IL-2) (Pharmacia ENI). After PHA stimulation, 10^6 cells per sample were infected. For acute infections, cells were infected for 2 h, and then washed three times before continuing culturing. ACH2, MOLT-4, and U1 were grown in RPMI-1640 supplemented with 10% FCS, 290 μg/ml L-glutamine, 100 U/ml penicillin, and 70 μg/ml streptomycin. Stimulations were as follows: TNF-α, 0.5 ng/ml (U1, ACH2) or 10 ng/ml (MOLT-4, PBMC); PMA, 0.16 ng/ml (U1, ACH2, MOLT-4) or 20 ng/ml (PBMC). 1 M NAC stock solutions in RPMI (pH 7.0) were kept at −20°C. After 2 days of stimulation, 200 μl of culture supernatant (diluted appropriately) were used for p24 enzyme-linked immunosorbent assays (ELISA) (Abbott Labs).

RNA quantitation

The 293.27.2 cells were maintained and stimulated as previously described.[14] Approximately 5×10^7 cells for each condition were harvested after stimulations. Total cytoplasmic RNA was isolated essentially as described.[17] The final RNA concentration was determined spectrophotometrically. Aliquots of 7.5 μg of total RNA was loaded into the first lane of a slot-blot apparatus, followed by serial twofold dilutions, and transferred to nitrocellulose filters. The RNA was baked onto the filter and hybridized with a probe specific to *lacZ* or β-actin, to which an autoradiograph was exposed to 6 days. Hybridized probe was quantitated by direct counting of the filter for 24 h using a radioanalytic imaging system (Ambis Systems Inc); the amount of *lacZ*-specific signal was normalized by the amount of β-actin-specific signal. There was no systematic variation in the amount of β-actin RNA under the different stimulation conditions.

RESULTS

HIV expression in chronically infected cell lines is stimulated by TNF-α and PMA. Two of these cell lines, a chronically infected promonocytic cell line (U1) and a chronically infected T-cell line (ACH2),[2,10] are useful models for examining the mechanisms that control latent HIV expression. Data in Figure 1 show that NAC interferes with the activation of the integrated provirus in both ACH2 and U1, and thus that NAC can maintain these cell lines in their relatively latent state despite the presence of stimulatory levels of inflammatory cytokines.

Comparisons of the amount of NAC required to inhibit stimulation of viral replication in the various model systems and in cultured PBMC support the idea that NAC may be useful for slowing the change from HIV latency to active viral production in patients. The NAC inhibition of viral replication in chronically infected ACH2 T cells is comparable in extent and sensitivity to that in acutely infected MOLT-4 T cells (Fig. 1). The inhibition in U1 promonocytes, in contrast, is more extensive and is complete at a much lower NAC concentration.

Surprisingly, although short-term cultures of normal PBMC contain mainly T cells, the concentration of NAC which inhibits HIV replication falls in the range of concentrations required to inhibit viral replication in a monocyte line (Fig. 1), i.e., roughly ten times lower than that required to inhibit replication in T-cell lines (MOLT-4 and ACH2). Thus, it is likely that viral replication in these PBMC cultures occurs mainly in monocytes and/or in a subset of T cells that is much more sensitive than most T cells to NAC. In any event, the effectiveness of low concentrations of NAC (<1 mM) with PBMC is encouraging in terms of the doses of NAC that might be required in vivo to maintain latency and prevent the spread of the infection in HIV^+ individuals.

The ability to block TNF-α- and PMA-stimulated HIV production in chronically infected cell lines demonstrates that NAC can inhibit the expression of HIV after it has integrated into the genome (Fig. 1). This inhibition is due (at least in part) to interference with stimulation of LTR-directed mRNA production, since NAC inhibits the stimulation of transcription of a gene fusion of the HIV LTR with the bacterial reporter gene *lacZ* (Fig. 2). These findings are consistent with evidence from previous studies with this construct in which NAC inhibited the cytokine stimulation of β-galactosidase expression[14] and confirm that NAC inhibits cytokine-stimulated transcription of genes directed by the HIV LTR.

Quantitation of the LTR-directed RNA levels shows the same trends as that observed for the protein product (Table 1). Interestingly, the synergistic stimulation with TNF-α and PMA results in only slightly increased levels of mRNA (compared with PMA alone), whereas the protein level is substantially increased. This suggests that costimulation may result in a posttranscriptional enhancement of β-galactosidase production. Furthermore, the costimulation is considerably less sensitive to NAC than either stimulation alone, thus indicating that the posttranscriptional enhancement may not be NAC sensitive. NAC does inhibit the posttranscriptional stimulation of HIV production induced by IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF)[18] (A.S. Fauci and G. Poli, personal communication).

DISCUSSION

Findings presented here and in two previous studies demonstrate that NAC primarily inhibits viral replication in acutely or chronically infected cells by inhibiting the activation of NF-κB (which has been shown earlier to be required for the cytokine-induced activation of the HIV LTR[11,12]). The data presented here suggest that the same is true for chronically infected cells. In essence, we have now shown that when cells are stimulated with cytokines in the presence of NAC: (1) activated NF-κB fails to appear in the nucleus, (2) transcription from the HIV LTR remains at the basal level, and (3) production of viral proteins (p24) or other gene products (β-galactosidase) under the control of the HIV LTR also fail to increase above basal level.

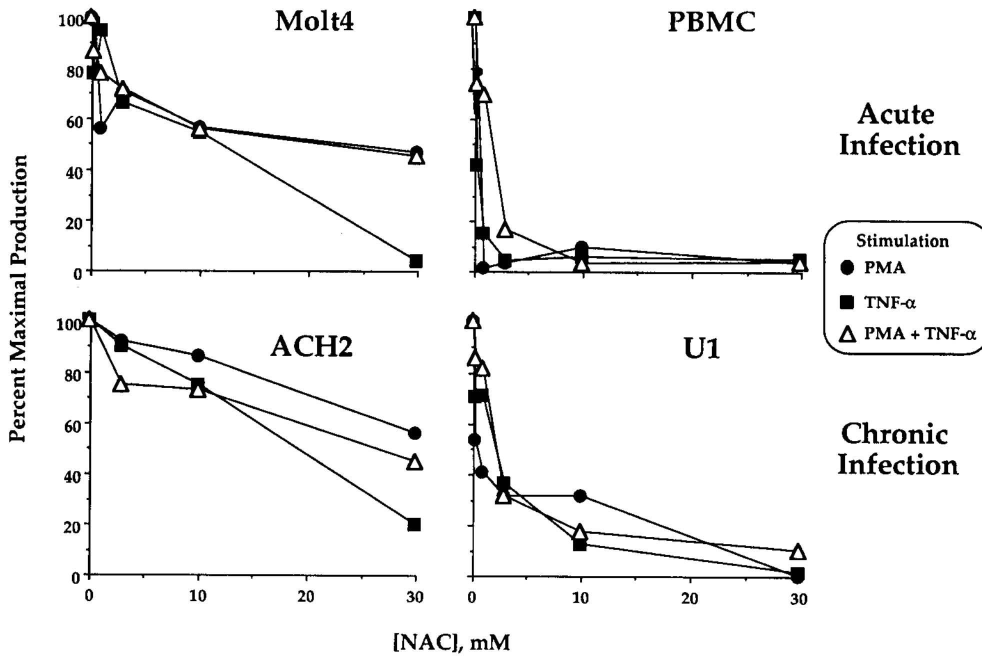

FIG. 1. NAC inhibits TNF-α- and PMA-induced stimulation of HIV replication. Results are shown as percent of maximal p24 core protein production for each stimulation condition. Top panels: MOLT-4 T cells or PHA-stimulated PBMC from normal, HIV^- individuals were infected with an ACH2 supernatant. After infection, cultures were maintained for 2 days in the presence or absence of TNF-α, PMA, and various concentrations of NAC. Bottom panels: ACH2 T cells or U1 promonocytes were cultured for 2 days in the presence or absence of TNF-α, PMA, and NAC. p24 core protein levels in the supernatant were determined by ELISA; these correlated well with the amount of infectious virus in the supernatant (determined in the MOLT-4 experiment). Stimulations were as follows: TNF-α, 0.5 ng/ml (U1, ACH2) or 10 ng/ml (MOLT-4, PBMC); PMA, 0.16 ng/ml (U1, ACH2, MOLT-4), or 20 ng/ml (PBMC). Concentrations of TNF-α and PMA used were those which give maximal stimulation of HIV replication without themselves being toxic.

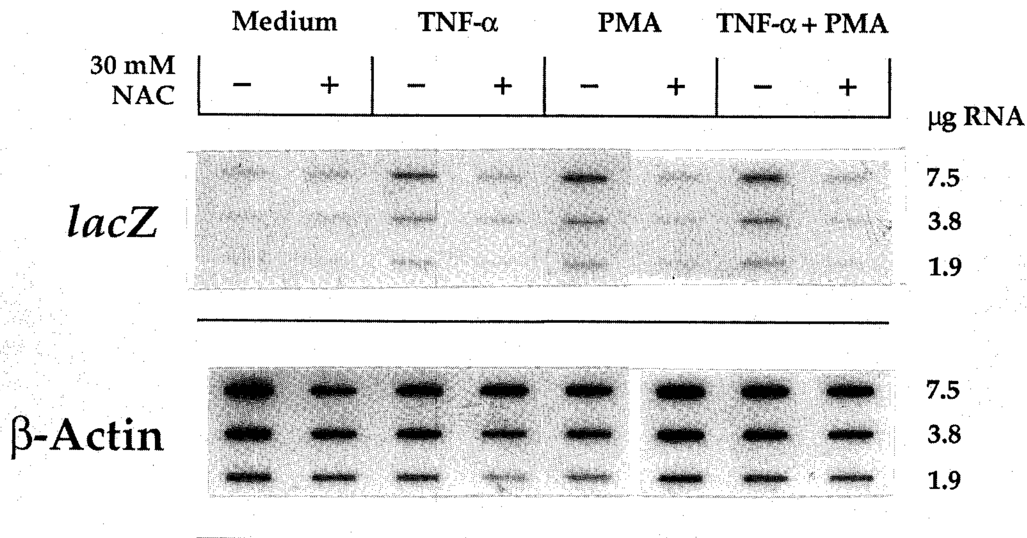

FIG. 2. NAC inhibits stimulation of transcription at the HIV LTR. 293.27.2 cells[14] were cultured with 10 ng/ml TNF-α, 20 ng/ml PMA, and/or 30 mM NAC for 6 h. Both *lacZ* mRNA (derived from the stably transfected HIV LTR-*lacZ* gene fusion) and β-actin mRNA were quantitated. Both TNF-α and PMA stimulate synthesis of *lacZ* mRNA; NAC completely inhibits the stimulation. Quantitation of RNAse protection assays confirmed these results (data not shown).

TABLE 1. NAC INHIBITS STIMULATED RNA AND PROTEIN PRODUCTION DIRECTED BY THE HIV LTR

Stimulus	*β-Gal activity*		lac*Z RNA*	
	−NAC	*+NAC*	*−NAC*	*+NAC*
TNF-α	5.2	1.8	2.6	0.9
PMA	9.2	1.2	4.6	1.2
TNF-α + PMA	19.3	7.4	4.9	1.5

Values are normalized to amount of β-Gal activity or *lacZ* RNA in unstimulated cultures of 293.27.2 cells without NAC (defined as 1.0). Incubation conditions: TNF-α, 10 ng/ml; PMA, 20 ng/ml; NAC, 30 mM; 6 h stimulation.

These findings point to the importance of intracellular glutathione levels in modulating signal transduction and the cytokine-stimulated expression of genes controlled by the HIV LTR. Reduced glutathione (GSH), the principal intracellular antioxidant, is oxidized when it reduces reactive oxidative intermediates generated during oxidative stress. Stimulation with TNF-α or PMA induces oxidative stress in certain kinds of cells[19–21] and causes a rapid decrease in GSH levels in the cell lines used here.[15] Simply decreasing GSH levels (e.g., by intracellular oxidation using diamide) is not sufficient to activate the HIV LTR; however, such decreases significantly augment stimulation by TNF-α.[15] Since replenishing intracellular glutathione (i.e., total glutathione, GSH plus the oxidized form, GSSG) with NAC inhibits stimulation with TNF-α,[14,15] these data indicate that the decrease in glutathione levels induced by TNF-α (or other inflammatory cytokines) is necessary to permit signal transduction.[15]

This observation is particularly important in terms of maintaining latency in HIV-infected individuals, since GSH levels in these individuals are consistently below normal.[22–24] Restoring depleted glutathione (and consequently GSH) in persons with latent HIV infection (e.g., by treatment with NAC) could be expected to inhibit signal transduction from inflammatory cytokines, and hence prevent cytokine-stimulation of HIV replication in latently-infected cells (as it does in vitro). Additionally, NAC could be expected to sustain normal sensitivity to TNF-α, rather than the increased sensitivity observed in GSH-depleted cells. Finally, since the TNF-α promoter has four NF-κB or NF-κB-related binding sites,[25] maintenance of normal glutathione levels in HIV-infected individuals could decrease further production of TNF-α through autocrine stimulation. Thus, treatment with NAC aimed at restoring glutathione and/or GSH may well extend HIV latency and prevent progression to clinical AIDS.

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NOTE

Since submission of this manuscript, similar results for U1 cells were published by Fauci and colleagues.[26] These results, like those reported here, confirm that NAC inhibits the stimulation of HIV replication; they further show that the stimulation of HIV replication is inhibited by other thiol-containing compounds.

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