Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 986–990, February 1991
Medical Sciences

Suppression of human immunodeficiency virus expression in chronically infected monocytic cells by glutathione, glutathione ester, and *N*-acetylcysteine

(tumor necrosis factor/interleukin 6/phorbol 12-myristate 13-acetate)

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Contributed by Alton Meister, November 20, 1990

ABSTRACT **The effects of glutathione (GSH), glutathione ester (GSE), and *N*-acetyl-L-cysteine (NAC) on the induction of human immunodeficiency virus (HIV) expression were investigated in the chronically infected monocytic U1 cell line, a previously described cellular model for HIV latency. U1 cells constitutively express low levels of virus, which can be increased by phorbol 12-myristate 13-acetate (PMA), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and other inducers. GSH, GSE, and NAC suppressed in a dose-dependent fashion the induction of HIV expression mediated by PMA, TNF-α, and IL-6, in the absence of cytotoxic or cytostatic effects. Reverse transcriptase activity, inducible by PMA, TNF-α, or IL-6, was decreased by 80–90% after pretreatment with GSH, GSE, or NAC. The induction of total HIV protein synthesis was also decreased appreciably after pretreatment with GSH, GSE, or NAC. The accumulation of HIV mRNA was substantially suppressed after pretreatment with NAC but to a lesser extent after pretreatment with GSH or GSE. Although PMA induces the expression of TNF-α in U1 cells, the suppressive effect of GSH, GSE, and NAC on PMA-induced HIV expression in U1 cells was not associated with the inhibition of TNF-α expression. The present findings, which elucidate relationships between cellular GSH and HIV expression, suggest that therapy with thiols may be of value in the treatment of HIV infection.**

The early stages of human immunodeficiency virus (HIV) infection can be separated from the later progressively symptomatic stage by several years of clinical latency (1, 2). Early infection is characterized by a low frequency of infected cells and a low level of viral expression (3). The progression to acquired immunodeficiency syndrome (AIDS) is characterized by increased levels of viremia and p24 antigenemia, activation of HIV expression in infected cells, an increased number of infected cells, and severe immune dysfunction (1–3). These findings suggest that the evolution of HIV infection and the progression of immunosuppression is associated with an increased activation of latent virus. A number of factors that induce the activation of latent HIV *in vitro* have been identified by use of the chronically infected promonocytic U1 cell line, which harbors HIV proviral DNA in its genome (4). In particular, phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor α (TNF-α) are potent transcriptional activators of HIV expression in this model system (4–6). TNF-α may play a critical role in the pathogenesis of AIDS, consistent with the markedly increased level of this cytokine in HIV-infected individuals (7, 8). In addition, elevated levels of interleukin 6 (IL-6) in the plasma (9) and cerebrospinal fluid (10) of HIV-infected individuals have been described, and it has been found that IL-6 can induce the expression of HIV in chronically infected U1 cells by acting predominantly at the posttranscriptional level (11).

The possibility that oxidative phenomena and thiols may be involved in the induction of HIV expression has not been extensively examined. It has been reported that HIV-seropositive individuals have decreased levels of total acid-soluble thiols and of glutathione (GSH, L-γ-glutamyl-L-cysteinyl glycine) in their plasma, peripheral blood monocytes, and lung epithelial lining fluid (12, 13). There is evidence that GSH is an important immunomodulator and that it is required, for example, for T-cell activation (14–17). GSH, which constitutes >90% of the cellular non-protein thiols and which is present at levels of 0.5–10 mM, provides cells with their reducing environment and serves as the major cellular antioxidant (18–20).

It has recently been reported that the transcriptional activation by PMA or TNF-α of a transfected HIV long terminal repeat (LTR) construct in T lymphocytic cells is inhibited by *N*-acetyl-L-cysteine (NAC) (21). NAC, which has been used clinically to treat acetaminophen toxicity, is believed to act by increasing the intracellular level of GSH (22, 23). Cellular GSH levels may also be increased by administration of GSH monoester (GSE), a readily transported derivative of GSH, which also protects very effectively against acetaminophen toxicity. Although GSH is not transported intact into cells, it may be degraded into its constituent amino acids, which after transport are used for the synthesis of GSH. These findings suggested that cellular reducing systems may be involved in preventing HIV activation and thus in maintaining a state of viral latency. We therefore investigated the hypothesis that GSH, GSH derivatives, or NAC may inhibit the induction of virus expression in our model system of chronic HIV infection. We used the U1 cell line and the transcriptional activators PMA and TNF-α as well as the posttranscriptionally active cytokine IL-6.

MATERIALS AND METHODS

Cell Lines. The origin and characterization of the U1 cell line have been reported (4). Briefly, the U1 cell line was derived from infection of U-937 promonocytic cells with HIV and carries two copies of proviral DNA, as determined by restriction enzyme analysis. U1 cells show a minimal constitutive level of HIV expression, as determined by immu-

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Abbreviations: HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; PMA, phorbol 12-myristate 13-acetate; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; GSH, glutathione; GSE, glutathione monoester; NAC, *N*-acetyl-L-cysteine; RT, reverse transcriptase; LTR, long terminal repeat.

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nohistochemical staining for viral antigens and by measurement of the expression of the p24 gag antigen, reverse transcriptase (RT) activity, viral protein, and mRNA synthesis. Activation of HIV expression in U1 cells can be induced by a variety of stimuli including PMA, TNF-α, and IL-6 (4–6, 11). The cells are routinely maintained at a density of 2–5 × 10^5 per ml in RPMI 1640 (Whittaker Bioproducts), supplemented with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD).

Induction of HIV Expression. In order to study the effect of GSH, GSE, and NAC on PMA-, TNF-α-, or IL-6-mediated induction of HIV expression, U1 cells were pretreated with various concentrations of these agents for 6 hr at 37°C in 5% CO_2. Then, PMA (10 nM; Sigma), recombinant TNF-α (100 units/ml; Genzyme), or IL-6 (100 units/ml; Amgen Biologicals) were added to the culture and further incubated for various periods of time. To monitor HIV activity, the level of RT was measured in the supernatant and the levels of HIV mRNA and protein synthesis were determined. GSH and NAC were purchased from Sigma. GSE, a GSH derivative that is transported into cells without undergoing transmembrane degradation, was prepared as the semihydrosulfate and carefully neutralized as described (24, 25).

RT Assay. Levels of RT activity present in U1 culture supernatants were determined by the method of Willey *et al.* (26). Briefly, aliquots (10 μl) of supernatant were added to 50 μl of a mixture containing poly(A), oligo(dT) (Pharmacia), $MgCl_2$, and ^{32}P-labeled dTTP (Amersham) and were incubated at 37°C. After 2 hr of incubation, 6 μl of the reaction mixture was spotted onto DE81 filter paper (Whatman) and air-dried. The filters were then washed in 1× standard saline citrate (SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7) and 95% ethanol, dried, cut, and placed in a scintillation counter (LS 7000, Beckman) for measurement of radioactivity.

Western Blot Analysis. Cell lysates were prepared from U1 cells that had been pretreated for 6 hr with GSH, GSE, or NAC (15 mM) and then stimulated with PMA (10 nM), TNF-α (100 units/ml), or IL-6 (100 units/ml) for 40 hr. The amount of protein in each cell lysate was determined by using a Bio-Rad protein quantification kit. Samples containing equal amounts of proteins were loaded onto an SDS/10–20% gradient polyacrylamide gel (Integration Separation Sciences, Hyde Park, MA) and fractionated by electrophoresis. The proteins were then transferred for 8 hr onto nitrocellulose filters and fixed with methanol. Following saturation of specific binding sites with a 5% milk solution, filters were incubated with a 1:1000 dilution of a pool of AIDS patients' sera containing anti-HIV antibodies recognizing the major viral proteins. After three washes, filters were incubated with ^{125}I-labeled protein A (200,000 dpm/ml) for 90 min. Filters were washed, dried, and exposed to x-ray film (Eastman Kodak).

Northern Blot Analysis. Total cellular RNA was isolated from U1 cells that had been pretreated for 6 hr with GSH, GSE, or NAC (15 mM) and then stimulated with PMA (10 nM) or TNF-α (100 units/ml) for 24 hr prior to extraction of RNA with an RNA isolation kit (Stratagene). Ten micrograms of RNA was loaded onto a 1% agarose/2.2 M formaldehyde gel, fractionated electrophoretically, blotted onto a nitrocellulose filter, and hybridized as described (27). To detect HIV mRNA, a ^{32}P-labeled HIV LTR (*Hin*dIII–Ava I) was used as a probe (28). To measure the level of TNF-α mRNA, total cellular RNA was hybridized with a full-length TNF-α cDNA probe (29).

RESULTS

Suppression of RT Activity by GSH, GSE, and NAC in U1 Cells. While U1 cells express only a minimal level of RT activity under normal growing conditions, the levels of RT increase remarkably in the presence of HIV inducers. PMA (10 nM) and TNF-α (100 units/ml) induced 10- to 30-fold increases of RT activity, while IL-6 (100 units/ml) produced a 3- to 5-fold increase in RT activity in U1 cells (Fig. 1). We observed that a single pretreatment with GSH (15 mM), GSE (15 mM), or NAC (15 mM) produced a long-lasting suppression of RT activity in U1 cells stimulated with each of these viral inducers (Fig. 1). The suppression of RT activity produced by GSH, GSE, or NAC was dose-dependent (Fig. 2). At a concentration of 15 mM, GSH, GSE, or NAC suppressed RT activity usually >90% in PMA- or TNF-α-stimulated cells. Treatment with GSH, GSE, or NAC at this concentration also produced a substantial suppression of RT activity in IL-6-stimulated cells. The degree of suppression of RT activity was dependent on the duration of treatment of cells with GSH, GSE, or NAC, prior to the addition of the viral inducers. A 6-hr pretreatment with GSH, GSE, or NAC produced more suppression of RT activity than did 3 hr of pretreatment or the simultaneous addition of GSH, GSE, or NAC with the viral inducers (data not shown).

Effect of GSH, GSE, and NAC on HIV Protein Synthesis. Consistent with the suppression of RT activity detected in the culture supernatant, a 6-hr pretreatment of cells with GSH, GSE, or NAC (15 mM) inhibited the induction of total HIV protein synthesis mediated by PMA (Fig. 3). Similarly, stim-

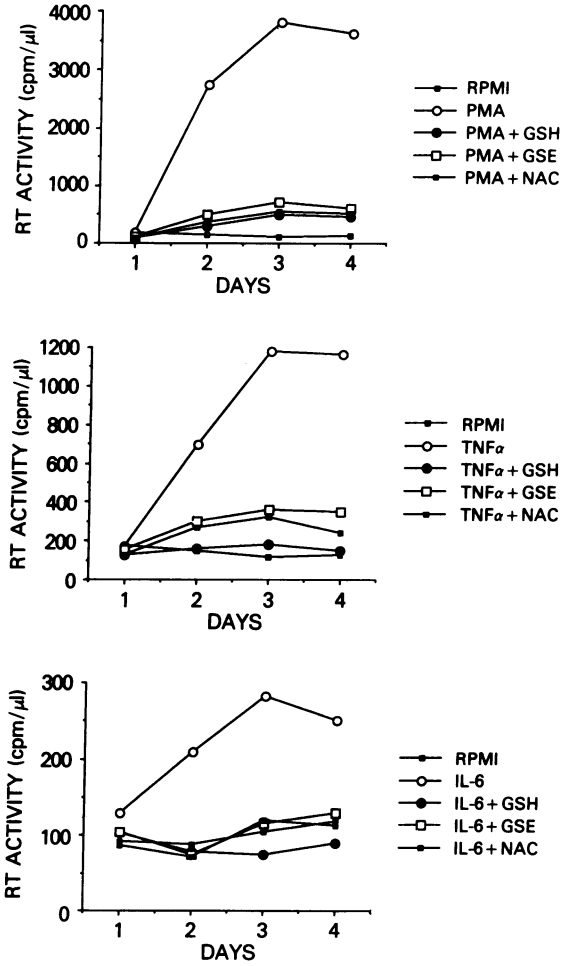

FIG. 1. Kinetics of inhibition of RT activity by GSH, GSE, and NAC in U1 cells stimulated with PMA, TNF-α, or IL-6. Cells plated at a density of 3 × 10^5 per ml in fresh growth medium (RPMI) were pretreated for 6 hr at 37°C with GSH (15 mM), GSE (15 mM), or NAC (15 mM) and then stimulated with PMA (10 nM), TNF-α (100 units/ml), or IL-6 (100 units/ml). Over a period of 4 days the supernatants were harvested at specific time intervals and RT activity was measured. A representative of three independent experiments performed in triplicate is shown. Variability between triplicate replicas was <15%.

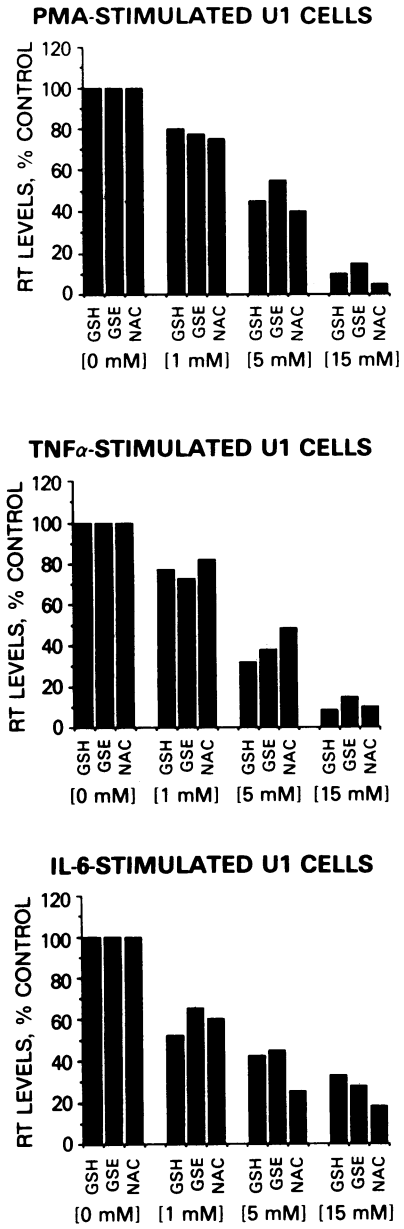

FIG. 2. Concentration-dependent suppression of RT activity by GSH, GSE, and NAC in U1 cells. Cells were pretreated for 6 hr with various concentrations of GSH, GSE, or NAC and then stimulated with PMA (10 nM), TNF-α (100 units/ml), or IL-6 (100 units/ml) for 48 hr. RT activity was determined in the culture supernatants. The average RT values determined in three independent experiments are shown as a percentage of control (given as 100%). Each experiment was performed in triplicate. Variability among triplicate replicas was <15%.

ulation of HIV protein synthesis by TNF-α and IL-6, was also inhibited by each of these agents (data not shown). We did not detect any significant qualitative or quantitative change in the pattern of total cellular protein as a result of treatment with GSH, GSE, or NAC in stimulated or unstimulated cells. At lower concentrations (1–5 mM), GSH, GSE, and NAC did not show a suppressive effect on the induction of total HIV protein synthesis (data not shown), although they did produce a decrease of RT levels (Fig. 2). Consistent with previous reports, we observed that unstimulated U1 cells did not express significant amounts of viral proteins (4, 11).

Differential Effect of GSH, GSE, and NAC on HIV mRNA Accumulation. Having determined that GSH, GSE, or NAC at 15 mM suppressed HIV protein synthesis, we investigated

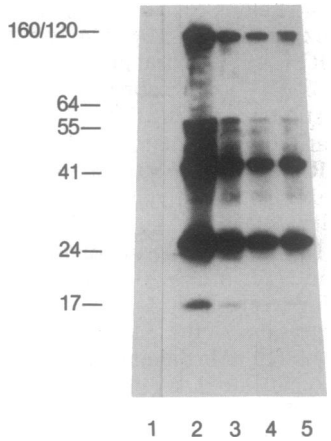

FIG. 3. Suppression of HIV protein level by GSH, GSE, and NAC (15 mM) in U1 cells stimulated with PMA. Western blot analysis of total cellular lysates (100 μg of protein per lane) from unstimulated cells (lane 1), PMA-stimulated cells (lane 2), PMA-stimulated cells pretreated with GSH (lane 3), PMA-stimulated cells pretreated with NAC (lane 4), and PMA-stimulated cells pretreated with GSE (lane 5). Molecular mass (kDa) is indicated at left. Results are representative of five independent experiments.

whether these three agents had any effect on mRNA accumulation in U1 cells. It had been shown that PMA and TNF-α increase HIV expression in U1 (5) and other infected cells (30–33) by inducing the cellular transcription factor NF-κB, whereas IL-6 induced HIV expression via a posttranscriptional mechanism without affecting the level of HIV mRNA in unstimulated or stimulated U1 cells (11). We found that a 6-hr pretreatment of cells with NAC (15 mM) completely blocked PMA induction of HIV mRNA. The level of mRNA in cells pretreated with NAC and stimulated with PMA was similar to the level of mRNA in unstimulated control cells (Fig. 4). Traces of mRNA from NAC-pretreated cells were detected by autoradiography only after several days of exposure. In contrast, pretreatment of U1 cells with GSH (15 mM) or GSE (15 mM) caused only a moderate reduction of mRNA accumulation in PMA-stimulated cells, despite the fact that GSH and GSE showed similar potency as compared with NAC in the suppression of RT activity and total HIV protein synthesis. Similarly, the increase of HIV mRNA induced by TNF-α was also inhibited markedly by NAC (15 mM), whereas pretreatment with GSH and GSE yielded only a 10% reduction.

GSH, GSE, and NAC Do Not Inhibit TNF-α Expression in Unstimulated or PMA-Stimulated U1 Cells. It was previously demonstrated that PMA induced HIV expression in U1 cells in part by TNF-α-dependent mechanisms (6). In addition, it was shown that the constitutive expression of HIV in certain cell lines is dependent on the endogenous and autocrine production of TNF-α (6). In this study we determined by Northern blot analysis that GSH (15 mM) or NAC (15 mM) had no suppressive effect on the PMA-induced increase of TNF-α expression (Fig. 5). This finding suggests that suppression of HIV activity by GSH and NAC is not due to the suppression of endogenous TNF-α production. In addition, these agents did not alter the expression of TNF-α in unstimulated U1 cells.

DISCUSSION

In the present study, we have used the chronically HIV-infected U1 cell line to investigate the potential antiviral effects of GSH, GSE, and NAC. Treatment of U1 cells with PMA, TNF-α, or IL-6 leads to an increased production of HIV virions and corresponding increases in the levels of RT,

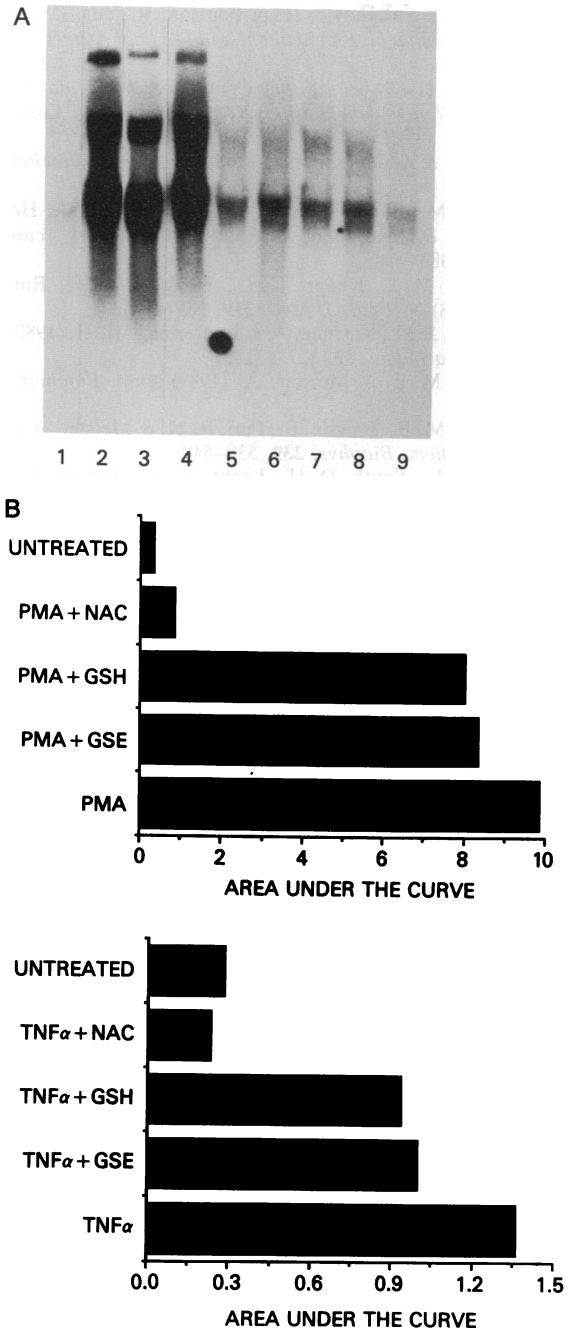

FIG. 4. Suppression of HIV mRNA accumulation by GSH, GSE, and NAC in U1 cells stimulated with PMA or TNF-α. (*A*) Northern blot analysis of total cellular RNA extracted from U1 cells pretreated for 6 hr with GSH (15 mM), GSE (15 mM), or NAC (15 mM) before stimulation with PMA or TNF-α. A ^{32}P-labeled HIV LTR was used as a probe. Lanes: 1, unstimulated cells; 2, PMA-stimulated cells; 3, PMA-stimulated cells pretreated with GSH; 4, PMA-stimulated cells pretreated with GSE; 5, PMA-stimulated cells pretreated with NAC; 6, TNF-α-stimulated cells; 7, TNF-α-stimulated cells pretreated with GSH; 8, TNF-α-stimulated cells pretreated with GSE; 9, TNF-α-stimulated cells pretreated with NAC. (*B*) Densitometric quantification of HIV mRNA from GSH-, GSE-, and NAC-pretreated U1 cells stimulated with PMA or TNF-α performed by scanning the autoradiogram from the same experiment shown in *A*. An LKB-222210 densitometer was used to perform the scanning.

cell-associated viral proteins, and mRNA (4–6, 11). In this model system, IL-6 stimulates HIV expression at a posttranscriptional level, while PMA and TNF-α stimulate HIV expression at a transcriptional level through an NF-κB-dependent mechanism (5, 30–33). Our experiments demonstrated that GSH, GSE, and NAC suppressed the induction of HIV expression in chronically infected cells stimulated with viral inducers that utilize different molecular mechanisms.

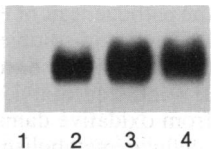

FIG. 5. Levels of TNF-α mRNA in U1 cells. Total cellular RNA was extracted from U1 cells pretreated for 6 hr with GSH or NAC (15 mM) and stimulated with PMA (10 nM) for 24 hr. Northern blot analysis was performed using a TNF-α cDNA probe. Lanes: 1, unstimulated cells; 2, PMA-stimulated cells; 3, PMA-stimulated cells pretreated with NAC; 4, PMA-stimulated cells pretreated with GSH. Actin gene expression was also unaffected in both untreated and PMA-treated U1 cells (data not shown).

We found that GSH, GSE, and NAC produced a comparable suppressive effect on RT activity in PMA-, TNF-α-, and IL-6-stimulated cells. Although GSH, GSE, and NAC produced a similar level of suppression of RT activity in the supernatant of U1 cells stimulated with these various stimuli, which corresponded to a decreased level of extracellular viral particles, these agents had a differential effect on HIV mRNA accumulation. NAC produced >90% suppression of the HIV mRNA level in both PMA- and TNF-α-stimulated U1 cells. Furthermore, consistent with previous reports (21), pretreatment with NAC decreased the transcriptional activity of the HIV LTR in U1 and U-937 cells transfected with an HIV LTR construct and stimulated with PMA or TNF-α (data not shown). In contrast, GSH and GSE reduced the HIV mRNA level only 10–15%. Although it has been assumed that NAC exerts some of its effects by increasing intracellular GSH (19, 23), our findings suggest that the mechanisms of NAC-mediated inhibition of HIV expression may be complex and involve, for example, a more efficient rate of internalization, an extracellular effect, or an effect on intracellular proteins. It was recently reported that NAC suppresses TNF-α-dependent, acute HIV infection *in vitro* and exerts a cooperative *in vitro* antiviral effect with 3′-azido-3′-deoxythymidine (AZT) (21). In addition, NAC was found to directly inhibit HIV LTR transcription (21) and to exert an inhibitory effect on NF-κB binding activity (34). Other thiols, which are not directly used for GSH synthesis, have been reported to suppress HIV replication in acute systems. Such thiols [including penicillamine (35, 36), and 2,3-dimercapto-1-propanol (37)], as well as ascorbate (vitamin C) (38) could act to spare cellular GSH; the thiols might liberate GSH moieties from mixed disulfide linkages with proteins.

Our data thus demonstrate that these three thiols affect HIV expression at multiple stages of the HIV activation process. At 15 mM, GSH, GSE, and NAC suppressed both HIV production and total viral protein synthesis. At lower concentrations (1–5 mM), however, GSH, GSE, and NAC did not affect HIV protein synthesis or mRNA accumulation but appeared to suppress predominantly posttranslational events of HIV expression, such as viral assembly or budding from the plasma membrane. Unlike interferon α-treated cells (39), RT levels measured after cell disruption were not different from those detected in the supernatants of intact cells (data not shown), and ultrastructural analysis failed to detect increased aggregation of viral particles on the plasma membrane (Jan Orenstein, personal communication), indicating that low concentrations of NAC, GSH, and GSE affected earlier posttranslational steps of viral expression than did interferon α (39).

The observation that GSH, GSE, and NAC downregulate the expression of HIV in chronically infected cells may further

our understanding of the pathogenesis of HIV infection *in vivo*. Decreased levels of GSH and acid-soluble thiols were found in the plasma, lung epithelial lining fluid, and peripheral blood monocytic cells of HIV-infected individuals (12, 13). GSH, which protects cells from oxidative damage and which plays an important role in cellular metabolism and function, also plays a critical role in immunoregulation (14–20). GSH deficiency in association with increased levels of TNF-α (40–43) and IL-6 (43, 44) may play an important role in the progression of HIV infection. A decreased level of GSH potentiates sensitivity to TNF-α (45), which has a direct stimulatory effect on HIV expression (5, 6, 11, 31–33, 43) and also increases susceptibility to oxidative effects (46, 47). An increase in the serum level of TNF-α has been found in AIDS patients (7, 8). In addition, increased levels of IL-6 have been reported in the plasma (9) and cerebrospinal fluid (10) of HIV-infected individuals. Moreover, IL-6 can activate superoxide generation in mononuclear phagocytes (48), leading to a further increase in reactive oxygen intermediates and a consequent consumption of GSH. Our observation that GSH, GSE, and NAC can suppress the induction of HIV expression *in vitro* by TNF-α and IL-6 leads to the hypothesis that these agents may have similar effects *in vivo* and therefore that the administration of these or similar agents may have therapeutic value in HIV-infected patients. Such therapy may be effective in limiting progression of the disease process, which, by a presently unknown mechanism, produces a striking decrease in the level of a major cellular antioxidant.

This research was supported in part by National Institutes of Health Grant 2 R37 DK-12034 to A.M.

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