

## Glutathione Precursor and Antioxidant Activities of *N*-Acetylcysteine and Oxothiazolidine Carboxylate Compared in *in Vitro* Studies of HIV Replication

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### ABSTRACT

*N*-Acetyl-L-cysteine (NAC) and L-2-oxothiazolidine 4-carboxylate (OTC) are pro-GSH drugs that been proposed for AIDS therapy. In this article we compare the antiviral activities of these compounds in various *in vitro* HIV infection models. Although both compounds blocked cytokine induction of HIV in acute and chronic infection models, and in HIV-LTR reporter cell systems, NAC was far more effective than OTC, even at sub-optimal doses. To test whether this difference is due to GSH conversion efficacies of these compounds, we measured GSH restoration by NAC or OTC in GSH-depleted peripheral blood mononuclear cells (PBMCs), using flow cytometry. In isolated PBMCs, NAC fully replenishes depleted intracellular GSH whereas OTC only minimally replenishes GSH. This ability to replenish GSH *in vitro* and its ability to scavenge free radicals directly explain why NAC has more potent antiviral activities *in vitro*.

### INTRODUCTION

OXIDANTS ARISING from endogenous and exogenous sources are neutralized by glutathione (GSH), the main intracellular thiol of eukaryotic cells.<sup>1,2</sup> In the acquired immune deficiency syndrome (AIDS), chronic inflammation and elevated levels of cytokines such as tumor necrosis factor (TNF)<sup>3-5</sup> are associated with depletion of plasma<sup>6,7</sup> and intracellular GSH levels.<sup>8,9</sup> The resultant oxidative stress may play a role in the progression of human immunodeficiency virus (HIV) infection.<sup>10</sup> Therefore, the reduction of oxidative stress may be desirable for therapy of HIV in AIDS during the asymptomatic stage as well as later stages.

Drugs such as vitamin C or penicillamine alleviate oxidative stress by neutralizing oxidants but do not directly increase GSH levels. In contrast, nontoxic GSH prodrugs such as *N*-acetyl-L-cysteine (NAC) and L-2-oxothiazolidine 4-carboxylate (OTC) are readily taken up into cells and converted into cysteine by *N*-acetylase and 5-oxoprolinase, respectively. Normal levels of GSH are then regenerated from newly converted cysteine, the limiting precursor in GSH biosynthesis.

Both NAC and OTC have been shown to replenish GSH levels in mice<sup>2</sup> and to enhance both human and murine T cell acti-

vation and mitogenesis.<sup>11,12</sup> However, NAC is distinct from OTC in that it can scavenge free radicals, because its sulfur is in a reduced state whereas the OTC sulfur is masked. In addition to replenishing GSH, NAC acts as an antioxidant to block the activation of nuclear factor kappa-B (NF- $\kappa$ B),<sup>13</sup> the transcription element that activates HIV from latency.<sup>14</sup> Because NF- $\kappa$ B is activated by inflammatory cytokines<sup>15,16</sup> and oxidants,<sup>17</sup> blocking its activation is a major mechanism by which NAC inhibits HIV expression in various cell lines and primary cells.<sup>18-21</sup>

Unlike NAC, OTC is strictly a GSH precursor and does not have the dual ability of NAC to scavenge oxidants and replenish GSH. It requires intact GSH synthesis machinery to function as an antioxidant prodrug. For example, OTC fails to protect mice against oxidative toxicity due to acetaminophen poisoning when buthionine sulfoximine (BSO) is coadministered<sup>22</sup> to block *de novo* GSH synthesis.<sup>23</sup> Therefore, because both OTC and NAC have been proposed for AIDS therapy, we compared the ability of these pharmacologically distinct drugs to replenish intracellular GSH and inhibit induction of HIV.

Studies presented here show that NAC is an effective antiviral agent *in vitro*, at markedly lower doses than OTC, probably because it is a direct antioxidant. Furthermore, only NAC is

able to replenish depleted GSH in isolated peripheral blood mononuclear cells (PBMCs). Nevertheless, we conclude that these *in vitro* findings do not necessarily predict the efficacy of either drug in replenishing GSH *in vivo*, or their relative therapeutic value in the treatment of AIDS.

## MATERIALS AND METHODS

### Standard conditions

Cells were cultured in a humidified 37°C incubator aerated with a 5% CO<sub>2</sub>/air mixture. Stock solutions of NAC (Sigma Chemical Company, St. Louis, MO), BSO (Sigma), and OTC (Clintec Nutrition, Deerfield, IL) were prepared in RPMI-1640 medium and the pH (7.2) adjusted. Medium was supplemented with 10% fetal calf serum (FCS; Gemini Bioproducts, Inc., Calabasas, CA), L-glutamine (290 µg/ml), penicillin (100 units/ml), and streptomycin (70 µg/ml). For PBMC cultures, medium was supplemented with an additional 10% FCS, and recombinant interleukin 2 (IL-2 [25 U/ml]; Chiron Corporation, Emeryville, CA). Cells were washed (when necessary) with un-supplemented RPMI-1640 medium.

### HIV long terminal repeat lacZ reporter cell assay

The development, properties, and application of the 293.27.2 reporter cell line have been published.<sup>18,24</sup> Support medium for experiments and maintenance of cells was Dulbecco's modified Eagles's medium (GIBCO Laboratories, Grand Island, NY) supplemented as above. For a typical experiment, 2000 cells/well were plated in individual wells of a 96-well microtiter plate 12 to 24 hr before the experiment. Cells were then treated with various combinations of phorbol myristate acetate (PMA; Sigma), TNF (Cetus Corp., Emeryville, CA), NAC, and OTC. β-Galactosidase activity was measured using 4-methylumbelliferyl-β-D-galactosidase (MUG) as previously described.<sup>24</sup>

### Chronic HIV infection

ACH-2 and U1 cells were grown in supplemented RPMI-1640 medium. Cells at a density of  $5 \times 10^5$ /ml were cultured with TNF (0.5 ng/ml), NAC, or OTC. After 24 hr, p24 antigen (HIV core protein) was quantitated using enzyme-linked immunoadsorbent assay (Abbott Diagnostics, Chicago, IL).

### Acute HIV infection

Peripheral blood mononuclear cells from HIV-seronegative blood were separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient centrifugation, washed, and enriched for T cells by culturing for 3 days in supplemented RPMI-1640 containing phytohemagglutinin (PHA, 3 µg/ml; Sigma) at a cell density of  $3 \times 10^6$ /ml. Cells were washed, resuspended at  $1 \times 10^6$  cells/ml in supplemented RPMI-1640 with or without 100 µM BSO (Sigma) and incubated for 3 days. HIV infection of PBMCs was carried out by resuspending washed cells in the supernatant of TNF-stimulated ACH-2 cells as a source of virus, together with Polybrene (3 µg/ml; Sigma). (Virus was typically obtained by culturing ACH-2 cells at  $0.5 \times 10^6$ /ml with TNF [0.5 ng/ml] for 18 hr. Results from experiments with ACH-2-derived virus

gave identical results as when frozen stocks of HTLV-IIIb were used at a multiplicity of infection of 3.) After 45 min, cells were pelleted, washed three times, and cultured at  $1 \times 10^6$ /ml in supplemented RPMI-1640 medium containing various combinations of TNF (10 ng/ml), PMA (20 ng/ml), BSO (100 µM), NAC, and OTC. Cells were pelleted after 48 hr, their concentration readjusted to  $1 \times 10^6$  cells/ml, and recultured with fresh medium and supplements. Supernatants were collected after 24 hr and quantitated for p24 antigen.

### Glutathione replenishment experiments

Peripheral blood mononuclear cells from HIV-seronegative blood were isolated as described above. Mononuclear cells were removed by adherence by culturing for 1 hr under standard conditions. Cells were cultured for 3 days in medium with or without 1 mM BSO. Cells were washed and then allowed to resume GSH synthesis in medium or media containing NAC or OTC. After 18 hr, cells were washed and stained with monochlorobimane (MCB; Molecular Probes, Portland, OR) for the measurement of intracellular GSH by fluorescence-activated cell sorting (FACS).<sup>25</sup>

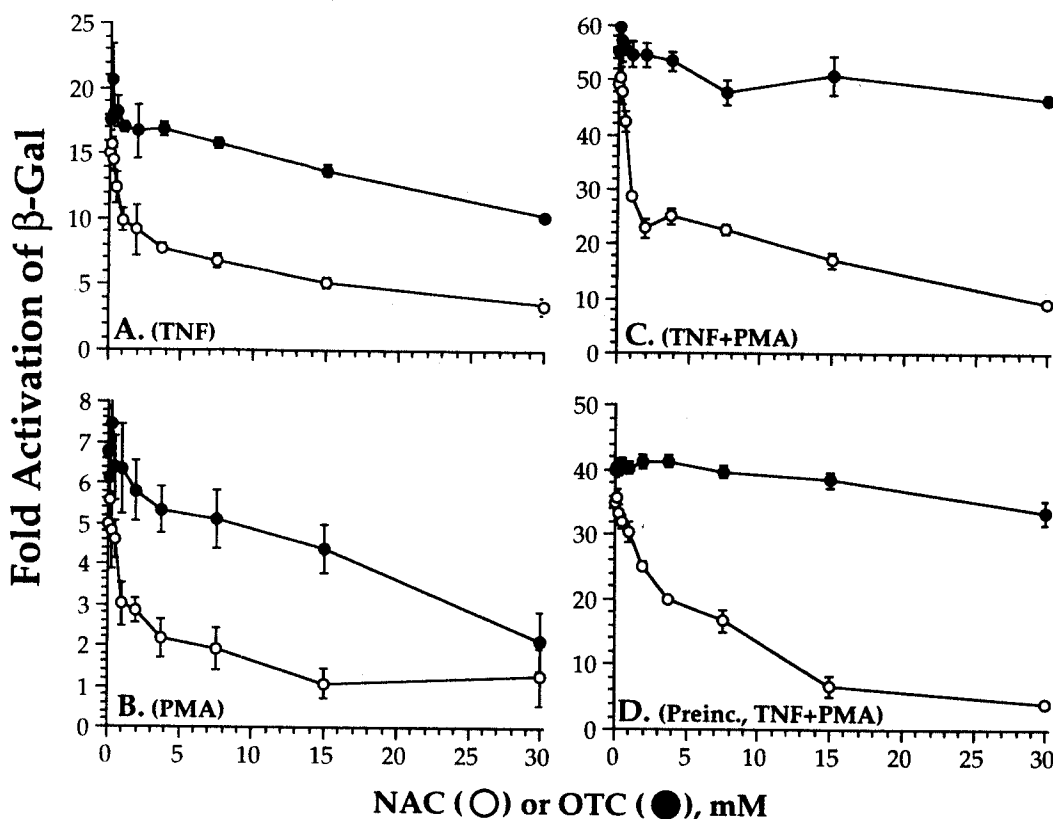
## RESULTS

### Cytokine-induced transcription initiated in the HIV long terminal repeat is inhibited by N-acetylcysteine and oxathiazolidine carboxylate

Numerous regulatory signals that activate cellular genes also induce viral expression through the HIV long terminal repeat (LTR), a critical region for transcription in the integrated virus.<sup>26</sup> To study HIV regulation we had previously developed a reporter cell system that can rapidly detect and quantitate HIV LTR activity.<sup>18</sup> Using this reporter system we have established that NAC effectively inhibits TNF- or PMA-generated intracellular signaling that activates the HIV LTR.

Both NAC and OTC inhibit this activation in the 293.27.2 reporter cells (Fig. 1); NAC did so at much lower concentrations than OTC. To ascertain the possibility that OTC requires a lag period before exerting its full effect, we preincubated cells for up to 40 hr in NAC or OTC and then stimulated with TNF and PMA. Preincubation did not improve inhibition of either drug (Fig. 1D). Shorter preincubation times similarly did not enhance inhibition (data not shown). Inhibition of stimulated β-galactosidase expression in these reporter cells by NAC or OTC is specific.<sup>27</sup> Drug toxicity was assessed by ethidium bromide/acridine orange exclusion/uptake; cells maintained greater than 90% viability in all cultures. In separate experiments (performed on the 293.27.2 cells and Jurkat T cells), growth curves were performed at different concentrations of NAC or OTC; the doubling time was unaffected at doses up to 20 mM. However, at 30 mM, growth was somewhat slower, with no evidence of cytotoxicity (data not shown).

Cellular transcription factors such as NF-κB regulate HIV expression by binding within the HIV LTR.<sup>26</sup> N-Acetylcysteine inhibits HIV LTR-directed β-galactosidase by blocking NF-κB activation.<sup>13</sup> Nuclear protein extracts made from cells stimulated with PMA in the presence of NAC or OTC (both at 30 mM) were tested for the presence of NF-κB by electrophoretic



**FIG. 1.** *N*-Acetylcysteine and OTC inhibit TNF and PMA stimulation of the HIV LTR. *lacZ* is transcriptionally fused to the HIV LTR and stably transfected in 293.27.2 cells.  $\beta$ -Galactosidase activity directly reflects the HIV LTR promoter activity. Cells were incubated in medium containing TNF (10 ng/ml) (A), PMA (20 ng/ml) (B), or both (C), and different concentrations of NAC or OTC (0 to 30 mM) for 6 hr. (D) Cells were preincubated with NAC or OTC for 40 hr, after which TNF and PMA were added for 6 hr. For all conditions, 293.27.2 cells are cultured, stimulated, and assayed for activity in 96-well plates; eight independent replicate wells are used for each condition. Error bars represent  $\pm 1$  SD of the mean of eight wells.

mobility shift assay. *N*-Acetylcysteine completely inhibited PMA-induced NF- $\kappa$ B activation, whereas OTC had no effect (data not shown). Because OTC inhibited PMA-induced gene expression (although less effectively than NAC), the mechanism(s) by which OTC inhibits may be a subset of those by which NAC inhibits.

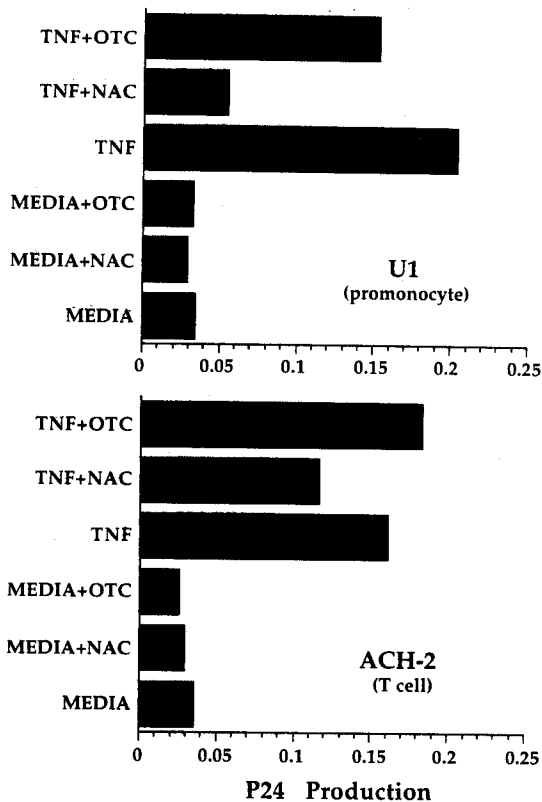
#### *N*-Acetylcysteine and oxothiazolidine carboxylate inhibit production of virions from chronically infected cell lines

Severe immunodeficiency is the hallmark of AIDS and occurs after a period of chronic (latent) infection following initial exposure to the virus. Maintenance of viral latency and productive infection is controlled by viral regulatory elements (positive and negative) and host transcription proteins.<sup>26</sup> U1 and ACH-2 cells were developed from promonocyte (U937) and T cell (A3.01) lines, respectively, for modeling chronic infection.<sup>28,29</sup> HIV is upregulated in both of these cell types by TNF, PMA, and other cytokines. *N*-Acetylcysteine inhibits induction of HIV in promonocytes effectively, and partially in T cells (Fig. 2). On the other hand, OTC is partially effective in promonocytes, and ineffective in T cells. These results suggest

that effective inhibition of HIV expression by these drugs is complex and may involve factors other than GSH replenishment or direct antioxidant properties, such as efficiency of drug transport and optimal prodrug conversion into GSH itself. It is probable that monocytes are more effective than T cells in both these functions.

#### *Inhibition of HIV replication in peripheral blood mononuclear cells occurs at lower doses with N-acetylcysteine compared to oxothiazolidine carboxylate*

A PBMC culture may be more representative than cell lines as an *in vitro* model for conditions that exist *in vivo*; thus it is important to evaluate potential antivirals for AIDS therapy in such a model. Some significant aspects of a PBMC culture are as follows: the cells involved are not transformed, PBMCs contain many of the cell types that orchestrate immunity *in vivo*, and this model may maintain some elements of the dynamic network of cellular interactions, which include cytokine and cell contact signaling. For these experiments, PBMCs from healthy individuals were stimulated with PHA and then infected with HIV. Tumor necrosis factor and PMA induced a

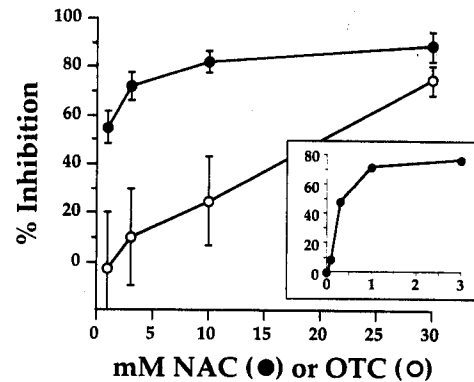


**FIG. 2.** *N*-Acetylcysteine and OTC inhibit stimulated HIV production in chronically infected cell lines. ACH-2 (T cells) or U1 (promonocytes) were cultivated with or without TNF (1 ng/ml), PMA (2 ng/ml), NAC (30 mM), or OTC (30 mM) for 24 hr. Virus in the supernatant was quantitated by p24 antigen ELISA. This experiment is representative of two independent experiments; the results with NAC are comparable to several previously published experiments.<sup>19,20,30</sup>

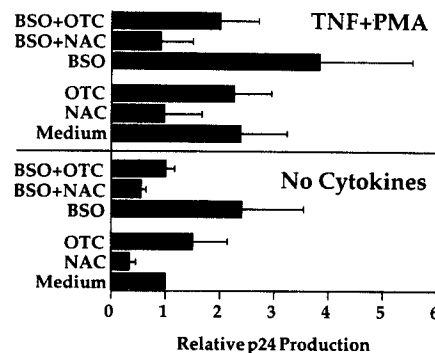
threefold increase in virus production from infected cells. As shown in Fig. 3, a 30-fold higher dose of OTC is required to achieve the same level of inhibition as NAC. Both NAC and OTC have much stronger antiviral effects in PBMCs than in 293.27.2 cells or in the chronically infected cell lines. Cell viability of PBMCs was not affected by these drugs, even at 30 mM, as assessed by trypan blue dye exclusion.

In unstimulated PBMC cultures, NAC at 1 mM strongly inhibited virus production, whereas OTC had no effect even at 30 mM (Fig. 4). The "unstimulated" cultures are supplemented with IL-2 and may contain many secreted autocrine factors that positively regulate HIV production; NAC inhibits this regulation whereas OTC does not. Therefore, the inhibitory effect of GSH replenishment (e.g., by OTC or NAC) is on inflammatory-type stimulations such as that produced by TNF.

Peripheral T cells in HIV-infected individuals have decreased levels of intracellular GSH. We asked what effect this potentially altered redox status might have on the regulation of HIV replication. To lower GSH levels *in vitro*, cells are cultured with BSO, a specific inhibitor of  $\gamma$ -glutamylcysteine synthetase.<sup>23</sup> Phytohemagglutinin-blasted PBMCs incubated in 100  $\mu$ M BSO had a 50% loss in intracellular GSH with no ef-



**FIG. 3.** Lower doses of NAC (compared to OTC) effectively inhibit TNF and PMA stimulation of viral replication in HIV-infected normal PBMCs. Tumor necrosis factor (10 ng/ml) and PMA (20 ng/ml) and various concentrations of NAC or OTC were added to HIV-infected PBMCs. Cultures were washed and refed after 2 days; supernatants were collected on day 3 and p24 levels were measured. Tumor necrosis factor plus PMA gave a threefold induction of virus. Percent inhibition was calculated by the following formula:  $[(\text{control} - \text{experimental}) / \text{control}] \times 100$ . *N*-Acetylcysteine at 0.3 mM inhibited 50% of the virus generated by TNF plus PMA stimulation; this amount of inhibition was attained only with 10 mM OTC. The error bars represent  $\pm 1$  SD of three separate, independent experiments; two other experiments using different concentrations of inhibitors showed similar results. *Inset*: Results from one of these experiments, in which NAC was used at low concentrations.

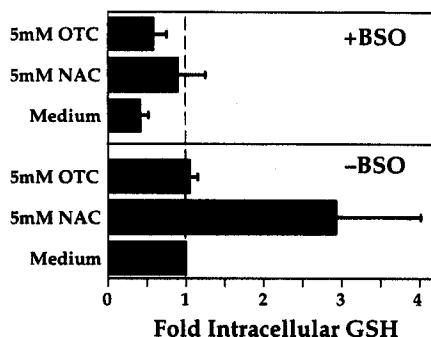


**FIG. 4.** *N*-Acetylcysteine and OTC at 1 mM concentrations block BSO-elevated HIV replication in PBMCs. Buthionine sulfoximine (100  $\mu$ M) pretreatment, which lowers intracellular GSH levels, dramatically increased virus production in infected, unstimulated PBMCs. In addition, this nonoxidative decrease of GSH exacerbated TNF and PMA stimulation of viral production. *N*-Acetylcysteine inhibits both the stimulation by TNF and PMA, and the elevated viral replication due to BSO. On the other hand, OTC did not inhibit the stimulation by TNF and PMA, but overcame the exacerbating effects of BSO on viral replication. The data are presented as mean  $\pm$  SD for three different experiments on three different donor PBMC samples. A fourth donor showed similar results, but was not included because different concentrations of inhibitors were used. A fifth donor, not included, showed similar results except for the requirement for higher concentrations of OTC (10 mM) to overcome the effects of BSO.

fect on viability (data not shown). Infection of GSH-depleted PBMCs yielded a greater production of HIV than in control cultures (Fig. 4). Unlike undepleted cultures, OTC was somewhat effective in blocking viral replication in the presence of BSO. On the other hand, NAC completely blocked HIV production in the presence of BSO, as it did in undepleted cultures. Furthermore, stimulation of HIV with TNF and PMA was exacerbated by the depletion of GSH. These results show that maintenance of GSH levels is important in keeping HIV replication to a minimum, and suggest that the low GSH levels *in vivo* may contribute to increased viral production.

#### *N-Acetylcysteine replenishes glutathione in buthionine sulfoximine-treated peripheral blood mononuclear cells*

Low intracellular GSH renders a cell sensitive to oxidative damage, and supplementation of GSH with exogenous pro-GSH compounds has been shown to be protective.<sup>31,32</sup> Many factors can influence the conversion of the pro-GSH form to the GSH form, including transportation of the compound into cells and the levels of enzymes needed to convert the drugs. In these experiments, we measured the effect of NAC or OTC on intracellular GSH in normal and GSH-depleted PBMCs. Unstimulated PBMCs cultured in 1 mM BSO for 3 days resulted in a 60% loss of GSH (Fig. 5, top). At this point, BSO was removed by extensive washing of the cells. Subsequent addition of 5 mM NAC for 18 hr replenished GSH, whereas OTC only minimally replenished GSH. In PBMCs not treated with BSO, NAC treatment resulted in higher levels of intracellular GSH than with equivalent concentrations of OTC (Fig. 5, bottom). These results show that in isolated PBMCs, NAC is a better GSH prodrug than OTC. Taken together with the property of



**FIG. 5.** *N-Acetylcysteine* replenishes GSH more effectively than OTC in BSO-treated PBMCs. For BSO treatment, PBMCs were cultured in 1 mM BSO for 3 days. Cells were washed to remove BSO, and then cultured with NAC, OTC, or neither, for 18 hr. Intracellular GSH was measured by flow cytometry; the median GSH level in the cultures is shown. Buthionine sulfoximine-untreated cells were cultured identically except that BSO was not included. *N-Acetylcysteine* increased intracellular GSH in both the GSH-depleted and the undepleted cultures, whereas OTC increased GSH only in the depleted cultures. (Monochlorobimane, the fluorogenic reactant for measuring GSH, does not react with cysteine, NAC, or OTC, under these conditions.) Data are presented as mean  $\pm$  SD for four independent experiments; measurements were normalized to untreated cultures, arbitrarily set to 1.

NAC as an antioxidant, these results may explain why much lower doses of NAC inhibit HIV replication *in vitro* than OTC.

## DISCUSSION

Studies here demonstrate that cytokine activation of HIV is inhibited by NAC or OTC in three different experimental models: acute infection, chronic infection, and an HIV LTR-*lacZ* reporter assay. Inhibition by both drugs is dose dependent, specific,<sup>27</sup> and nontoxic. Most importantly, however, nonoxidative depletion of intracellular GSH in PBMCs exacerbates inflammatory stimulation of HIV and is in itself sufficient to increase viral replication almost as much as stimulation with TNF and PMA. Both NAC and OTC inhibit this increased replication in GSH-depleted cells. These findings confirm the importance of intracellular GSH levels in the modulation of inflammatory stimulation of HIV transcription.

*N-Acetylcysteine* and OTC are more potent inhibitors of HIV in U1 cells than in ACH-2 cells, perhaps because monocytes internalize and convert the GSH prodrugs more efficiently than do T cells. Monocytes and macrophages (M/M $\phi$ ) have powerful antioxidant systems that should be supported by rapid and efficient uptake of cysteine. For example, the metabolic integrity of the postphagocytic respiratory process, which includes hexose-monophosphate shunt activation, oxygen consumption, and superoxide radical generation, is vital for the phagocytic process. In addition, M/M $\phi$  contribute substantially to the restoration of depleted GSH in T cells simply by being present in the culture (P. A. Raju and M. Roederer, unpublished observations, 1993), perhaps because they secrete cysteine or cystine. Thus, the more efficient conversion of GSH prodrugs that we observe in M/M $\phi$  leads both to a decrease in inflammatory stimulation and better antioxidant support for other cells.

These findings have important implications for AIDS. Monocyte-macrophages are tumoricidal-microbiocidal cells and play a central role in cell-mediated immunity by processing and presenting antigen to T cells. Furthermore, M/M $\phi$  are a major reservoir of HIV in AIDS patients and contain much of virus in the brains of AIDS encephalopathy patients. Thus, low levels of NAC and OTC *in vivo* may be sufficient to supply significant amounts of cysteine to M/M $\phi$  and thereby influence the course of the HIV infection.

We and others have suggested that GSH replenishment therapy should precede or accompany other treatments for HIV infection. This suggestion is based partly on the antiviral properties of NAC and OTC. The latent phase of HIV infection, prior to activation by cytokines or other stimulants, is a strategic point for antiviral therapy,<sup>28,29</sup> because blocking HIV transcription prevents spread of the virus to other cells. But NAC and OTC may have at least two additional significant benefits. First, both drugs have been shown to augment T cell function and proliferation,<sup>11,12</sup> thereby improving normal immune responses. Second, NAC and OTC, given in combination with other antivirals, may improve the effectiveness of those antivirals. For example, *in vitro* studies show that NAC and AZT (zidovudine) synergize to inhibit viral spread through a PBMC culture.<sup>18</sup>

Although NAC is widely used to restore GSH in the treatment of acetaminophen overdose, some questions have been raised about the potential availability of orally administered

NAC in HIV-infected individuals. de Quay *et al.* observed increased GSH levels in HIV-infected individuals after a single dose of NAC.<sup>33</sup> We have previously demonstrated that HIV-infected individuals taking NAC daily showed increased GSH levels in CD4 and CD8 T cells, as early as 1 week after starting NAC and continuing for several weeks.<sup>25</sup> Nevertheless, a phase I clinical trial at the National Institutes of Health<sup>34</sup> indicated that NAC is safe for HIV-infected individuals, and further indicated that "bioavailability" is low. Bioavailability is strictly defined as the ratio of free, unmetabolized drug in plasma after oral administration versus intravenous administration.

The findings of low bioavailability for NAC are, in fact, consistent with the observed increases in GSH following NAC administration and the observed efficacy of NAC as an antidote for acetaminophen poisoning. Orally administered NAC is rapidly converted to cysteine, cystine, or GSH in the gut and liver before reaching the circulation.<sup>35</sup> Low blood levels of NAC are actually indicative of its efficient conversion to functional compounds such as GSH. As we have indicated, NAC can restore GSH levels in plasma and in CD4 and CD8 T cells in AIDS patients. Therefore, NAC must be functionally available at readily supported doses in HIV-infected individuals.

In any event, the goal in GSH replenishment therapy of HIV-infected individuals should not be to achieve concentrations of prodrugs in the blood that exhibit antiviral activity *in vitro*. Because HIV-infected individuals have low GSH levels in a variety of tissues, including T cells, and because depletion of GSH levels is sufficient to increase viral replication, the primary therapeutic objective should be to restore overall GSH levels *in vivo*. Such restoration can be expected to dampen viral replication, antagonize inflammatory cytokine activity, block TNF production (and thus cachexia), and potentially restore immune responsiveness.

Although we have shown that NAC is markedly more effective in blocking HIV expression than OTC *in vitro*, both drugs could prove equally effective in a clinical setting. AIDS is initiated by HIV and advanced by a number of cofactors including oxidative stress. *N*-Acetylcysteine and OTC, as sources of cysteine, and NAC as a direct antioxidant, can facilitate replenishment of GSH, block oxidative damage to cells, and perhaps slow the progression of the HIV infection.

## ACKNOWLEDGMENTS

We thank Dr. Charles E. Myers of the National Cancer Institute for helpful discussions. M. Roederer is a Senior Fellow of the Leukemia Society of America. This work was supported in part by NIH Grants LM-04836 and AI-31770 to Leonard A. Herzenberg and by NIH Grant CA-42509 to Leonard A. Herzenberg.

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