N-Acetylcysteine: A New Approach to Anti-HIV Therapy

MARIO ROEDERER, STEPHEN W. ELA, FRANK J.T. STAAL, LEONORE A. HERZENBERG, and LEONARD A. HERZENBERG

ABSTRACT

Several investigators have implicated depletion of glutathione (GSH) and production of reactive oxygen intermediates (ROIs) in the regulation of the human immunodeficiency virus (HIV). We have shown directly that N-acetylcysteine (NAC) blocks HIV expression in chronic and acute infection models, and HIV replication in normal peripheral blood mononuclear cells. NAC is a cysteine prodrug which maintains intracellular thiol levels during oxidative stress and replenishes depleted GSH. The observed antiviral effect of NAC is due to inhibition of viral stimulation by ROIs, which are produced in response to inflammatory cytokines.

We have also shown that HIV-infected individuals have decreased intracellular GSH levels in their circulating T cells. Since GSH is the major protection against the production of ROIs, we hypothesize that the observed decrease is due to a chronic oxidative stress induced by continual exposure to elevated levels of inflammatory cytokines. Together, these results provide a rationale for clinical trials testing the efficacy of GSH-replenishing drugs such as NAC in the treatment of AIDS. NAC is different than many other antiviral drugs in that it inhibits host-mediated stimulation of viral replication arising in normal immune responses, and may thereby extend latency. In addition, it inhibits the action of inflammatory cytokines which may mediate cachexia, thereby raising the possibility that it may alleviate the deleterious wasting that accompanies late stage AIDS.

INTRODUCTION

Droge and colleagues first showed that human immunodeficiency virus-positive (HIV+) individuals have decreased levels of acid-soluble thiols, in particular cysteine and glutathione (GSH), in their plasma and leukocytes.1 This observation suggests that oxidative stress may play an important role in the progression of acquired immunodeficiency syndrome (AIDS), because GSH is the major intracellular defense against the production of reactive oxygen intermediates (ROIs), overproduction of such oxidants depletes GSH. In addition, they showed that glutamate levels were increased in plasma. Glutamate inhibits the cellular uptake of cysteine, used in GSH synthesis. Crystal and colleagues confirmed the depletion of plasma GSH, and further showed that there is a depression of the GSH levels in the bronchoalveolar lavage fluid of HIV+ individuals.2 We have shown that the decrease in intracellular GSH in leukocytes is confined primarily to the T-cell subsets.3-6 Finally, Smith et al. have shown that vertical transmission of HIV to children also results in decreased GSH levels.7

We suggested that the decline in GSH in HIV+ individuals might be due to chronic exposure to inflammatory cytokines.8 Fauci and colleagues suggested that inflammatory cytokines such as tumor necrosis factor α (TNF), interleukin (IL)-1, IL-6, and granulocyte-macrophage colony stimulating factor (GM-CSF) play central roles in the progression of HIV.9-10 Several laboratories have shown that HIV+ individuals have elevated production and levels of inflammatory cytokines.11-17 Besides directly stimulating transcription and replication of HIV, these cytokines cause an oxidative stress at both a systemic and cellular level. Thus, chronic exposure to elevated levels of cytokines may result in depletion of the major antioxidant defense, intracellular GSH.

These observations led to the suggestion that treatment of the HIV infection (and other syndromes in which inflammatory cytokines are overproduced) should include agents to restore systemic and intracellular GSH.18-21 Over the past two years, several studies showed that GSH-replenishing drugs, such as N-acetylcysteine (NAC), glutathione ethyl ester, and GSH itself, have profound effects on the regulation of inflammatory
cytokines: for instance, these compounds strongly inhibit the stimulation of HIV (in both acute infections and chronic infections) by TNF, IL-1, IL-6, (GM-CSF), and phosphatidyl inositol (PI) turnover.

In this report, we review the basic observations which have led to the realization that oxidative stress plays a critical role in AIDS, and that drugs which are GSH precursors can alleviate this stress. On the basis of these results, we strongly recommend that treatment of AIDS include therapies aimed at restoring depleted GSH levels.

**INFLAMMATORY CYTOKINES DIRECTLY STIMULATE HIV**

HIV has taken advantage of signaling pathways by inflammatory cytokines to regulate its own transcription and replication. The HIV-1 long terminal repeat (LTR), the promoter and enhancer region for viral transcription, has two binding sites for the enhancer protein NF-kB, and the HIV-2 LTR has one such site. NF-κB is a cytokine complex which, upon cellular stimulation, translocates to the nucleus in an active form. Activation is sufficient for stimulation of the HIV LTR by TNF and phosphatidyl inositol (PI) turnover. Once active and in the nucleus, it can increase transcription of the viral genome tenfold.

Through the stimulation of transcription of HIV, TNF can activate other latent virus. Fauci and colleagues have developed two cyclically infected cell lines, ACH-2 (T cell) and U1 (monocyte/macrophage), which serve as models for latency. These cell lines do not produce virions unless stimulated; agents which can induce virus in these lines include TNF, PMA, GM-CSF, and IL-6.

Many cytokines will synergize with each other to give significantly greater induction than occurs with only one. This synergism occurs at the level of induction of NF-κB and mRNPs, and synergism is important from the standpoint that low production of several cytokines (at levels detectable in blood sera) may result in an acute stimulation of HIV as high production of a single cytokine.

**INFLAMMATORY CYTOKINES CAUSE OXIDATIVE STRESS THAT CAN BE COUNTERED BY INTRACELLULAR GSH**

Production of ROS is a normal outcome of stimulation of many types of cells. The most dramatic is that of neutrophils after stimulation by, for example, TNF. This "respiratory burst" includes the production of a variety of oxygen radicals, such as superoxide anion and hydroxyl radical. However, other cell types also produce ROS in response to stimulation (reviewed by Cross and Jones), including B lymphocytes, T lymphocytes, endothelial cells, and fibroblasts. PMA also causes the production of ROS. While TNF by itself can induce oxidant production, it is far more potent in combination with certain other agents (synergistic stimulation). In combination with interferon-γ (IFN-γ) or IFNα, a significantly higher production of ROS is possible.

The main defense against the potentially damaging ROS is intracellular GSH. This cysteine-containing tripeptide is found in virtually all eukaryotic organisms in millimolar concentrations in the cytoplasm and mitochondria. It is the largest source of free thiol inside cells, and thereby regulates the redox potential within the cell. The enzyme-catalyzed reduction (neutralization) of ROS consumes GSH (which is converted to GSSG). Subsequently, GSSG is either exported from the cell or enzymatically reduced to GSH by glutathione reductase.

Stimulation of polymorphonuclear cells with PMA, inducing a respiratory burst, can consume as much as 50% of the intracellular GSH. Bizer and Lauterburg showed that this was also true for stimulation with TNF; furthermore, they showed that the continued resynthesis of GSH is critical in maintaining the intracellular GSH levels after stimulation.

Stimulation of cells may overcome the protective effects of intracellular GSH. However, addition of an exogenous thiol source can restore the intracellular reducing capability. Zimmerman et al. have shown that NAC, which can restore intracellular GSH and directly scavenge ROS, neutralizes the toxic effects of TNF both on tumor cell lines and in vivo, in rats injected with lethal doses of TNF. By virtue of its ability to replace GSH, NAC is the antidote to acetaminophen overdose in humans, a condition which leads to fatal depletion of GSH in the liver.

**INTRACELLULAR THIOL LEVELS REGULATE LYMPHOCYTE FUNCTION**

While the observations summarized above suggest that intracellular GSH levels directly modulate stimulation by TNF (and other agents), it is difficult to separate the effects of increased ROS from decreased GSH (and vice versa). Since production of ROS leads to consumption of GSH, and a depletion of GSH could lead to increased levels of intracellular ROS, assignment of specific roles for one or the other must be made with caution. Nevertheless, a considerable amount is known about the roles that these reducing and oxidizing species play in cellular functions.

Feedback mechanisms maintain intracellular GSH levels. Thus, the most effective way to lower GSH levels is to incubate cells in the presence of buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase. Incubation of dividing cells with BSO for several days can deplete as much as 90% of intracellular GSH. While maintenance of GSH levels by exogenously added NAC protects cells against TNF cytotoxicity, depletion of GSH by BSO potentiates TNF cytotoxicity.

Wedner and colleagues first showed that depletion of GSH inhibited T-cell proliferation. In several studies, Fidelis et al. also showed the critical need for adequate intracellular GSH levels for T-cell proliferation. Reductions of GSH by 10-40% in T cells completely inhibited T-cell activation.

Messina and Lawrence showed that a 40% decrease in intracellular GSH inhibited cell cycle progression in PBMC. However, even a 90% decrease in intracellular GSH did not inhibit the stimulated expression of IL-2 receptor or secretion of IL-2. This is not surprising in view of the dependence of IL-2 receptor expression on activation of NF-κB. This activation is more likely under conditions of GSH depletion—see below.
Gmünder et al. confirmed this result, and further suggested that some processes are GSH dependent, while others are cysteine dependent. There seemed to be no linear correlation between the degree of GSH depletion and the inhibition observed.

Wong et al. used a novel approach to investigate the interrelationship of ROS and stimulation: cloning and overexpression of either manganese superoxide dismutase (MnSOD) to try to reduce ROI levels, or overexpression of the MnSOD antisense mRNA to increase ROI levels. Indeed, overexpression of the enzyme leads to inhibition of the effects of TNF, while overexpression of the antisense RNA leads to increased sensitivity to TNF (potentiation). There is also a crucial involvement of HIV in this in vitro system. In normal cells, TNF activates expression of MnSOD; however, in HIV-infected cells, this activation is suppressed. Furthermore, HIV infection sensitizes cells to stresses such as heat and radiation.

While depletion of GSH leads to inhibition of some T-cell functions (but increased sensitivity to inflammatory cytokines), supplementation can augment other functions, both in vitro and in vivo. Exogenously added GSH augments lymphocyte proliferation in response to lectin. Oxothiazolidine 4-carboxylate (OTC), a cysteine precursor which increases GSH levels, acts synergistically with concanavalin A to stimulate T cells.

Finally, Droge et al. showed that increasing previously lowered GSH levels in mice augments the activation of cytolytic T cells, demonstrating the importance of GSH levels in vivo.

We have shown that GSH supplementation through addition of NAC can completely inhibit inflammatory stimuli of HIV replication. NAC inhibits the stimulated expression of the HIV LTR through the inhibition of activation of NF-κB.

This inhibition results in blocking of viral replication in acutely infected cells and chronically infected cells. NAC not only inhibited stimulation of viral replication by TNF and PMA, but also by IL-6 and GM-CSF (Poli and Fauci, personal communication).

Recently, Baeruele’s laboratory has confirmed and extended our observations. They have shown that a wide variety of agents which induce NF-κB through distinct pathways (e.g., cycloheximide, double-stranded RNA, calcium ionophore, TNF, PMA, IL-1, LPS, and lectin) are all inhibited by addition of NAC.

This suggests that these stimulatory agents might all activate NF-κB by the same mechanism involving a NAC-sensitive signaling step.

Perhaps most significantly, Baeruele’s group showed that hydrogen peroxide (H₂O₂) can directly and specifically activate NF-κB in a Jurkat T-cell subclone. This activation leads to stimulation of HIV replication in latently infected Jurkat T cells. H₂O₂ also stimulated viral production in latently infected U1 promonocytes. These results suggest that activation of HIV in vivo might be accomplished not only by the stimulation of production of ROIs in infected cells, but also by production of ROIs in uninfected cells (e.g., granulocytes) which then diffuse into neighboring infected cells. While Frei et al. found an upper limit of 0.25 μM for H₂O₂ in the blood plasma of healthy subjects, it is probable that this value is higher under conditions of inflammatory stress, especially in local cellular environments.

These results confirm the observations of Fidelus, who showed that the production of oxygen radicals is not only a positive signal in T-cell activation, it is necessary for some signaling pathways (e.g., PMA). Thus, ROIs may be commonly used second messengers for inflammatory stimulations as induced by, for example, TNF and IL-1. Since H₂O₂ can activate HIV in the absence of other agents, and synergizes with some stimulatory agents, ROIs must be assigned an important stimulatory role in the progression of AIDS.

INFLAMMATORY AND OXIDATIVE STRESSES ACCOMPANY AIDS

In apparent contradiction to the immunosuppressed state of individuals infected with HIV are the observations that their sera have elevated levels of several cytokines. While some studies showed significantly elevated levels of serum TNF, other studies did not show significant elevations (e.g., Fuchs et al. found no significant elevation in a retrospective study, but pointed out that instability of TNF in serum samples may lead to this discrepancy). Volf et al. showed that TNF mRNA in the PBMC from HIV+ individuals have a longer half-life than that in uninfected individuals; this may account for increased TNF production by resting alveolar macrophages from these individuals. Other than TNF, elevated levels of IL-1α, IL-1β, IL-2, IL-6, IFN-α, IFN-γ, and IFN-γ were measured in sera from HIV+ individuals. Furthermore, these individuals have hypertrophic cardiomyopathy, probably due to elevated cytokine levels. These results suggest that concomitant with the HIV infection is chronic stimulation by a variety of inflammatory agents.

A strong indication that an inflammatory stress accompanies AIDS is the elevated level of plasma neopterin. Macrophages stimulate neopterin principally in response to IFN-γ stimulation; thus, neopterin levels serve as a marker of the status of cell-mediated immunity. Not only is the level of neopterin correlated with the progression of the disease, but it is also a strong predictor for subsequent progression to AIDS.

Another indication that inflammatory stress accompanies AIDS is the chronic depletion of GSH (and other low-molecular weight thiols). HIV+ individuals have depleted levels of acid-soluble thiols in the plasma, and depleted levels of GSH in plasma and lung epithelial lining fluid. The chronic depletion of GSH is consistent with the observations of increased cytokine levels and that GSH levels (in T cells) decrease after stimulation in vitro.

Recently, Droge et al. have followed up on their earlier studies by showing that thiol depletion also occurs in a primate model for AIDS, that of simian immunodeficiency virus (SIV) infection of macaques. This last report is extremely important from several standpoints: it suggests that the chronic thiol depletion is a general result of retroviral infections; it shows that a decrease in acid-soluble thiols can occur within a week of infection (we have seen decreased levels of GSH in a patient 3 weeks after infection), and it suggests that the macaque SIV model may be useful in testing drugs targeted at restoring GSH.

Using a flow cytometric assay for intracellular GSH simultaneously with cell identification by membrane immunofluorescence, we have quantitated the GSH levels in subsets of PBMC. Our results are summarized as follows: (1) Intracellular GSH levels fall within a narrow range for each PBMC subset from normal individuals; (2) HIV+ individuals have 30–40% decreased GSH levels in both CD4 and CD8 T cells;
(3) this decrease is due primarily to the specific removal from circulation of a class of T cells with high GSH, and GSH levels in B cells and monocytes, while not significantly different from HIV individuals, vary from the mean considerably more than in normals.

One aspect of the inflammatory stress in AIDS, noted as early as 1983, is polyclonal B cell activation. Several observations confirm the activated state of B cells in HIV-infected individuals, including hypergammaglobulinemia, elevated expression of B-cell activation markers, increased frequency of B lymphomas, and elevated levels of plasma IL-6. In contrast to the B cells, T cells and monocytes appear to be less responsive if not anergic in HIV-infected individuals. Defects of immune function both in vivo and in vitro were observed early in AIDS research (e.g., Fauci et al. 102). This deficiency might be brought about by excessive stimulation with cytokines (i.e., induced anergy). Voh et al. found that PBMC from HIV-infected individuals produce lower levels of IFN-α and IFN-γ (but increased levels of TNF) in response to stimulation. Fuchs et al. found that IFN-γ production by PBMC from HIV-infected individuals inversely correlates with the serum levels of IFN-γ, as is true for IL-2 production and serum levels of neopterin. They predict that the diminished PBMC responsiveness will be found not only for AIDS, but also for other diseases in which immune activation is present.

In addition to cytokine-induced anergy may be a low-GSH-induced anergy. The progressive loss of T-cell function in AIDS-related complex (ARC) and AIDS patients and the polyclonal B-cell activation correlate with the observed decrease in intracellular GSH in T cells but not B cells. In view of the sensitivity of T-cell (and other lymphocyte) function to GSH levels, the loss of GSH may partially explain the immuno
deficiency in these HIV-infected individuals. As discussed above, depletions of intracellular GSH can lead to complete inhibition of mitogen-induced T-cell proliferation.

TREATMENT OF HIV INFECTION SHOULD INCLUDE GSH REPLACEMENT THERAPY

It is clear now that there is an intricate and large set of interactions between GSH levels, the production of oxidants, inflammatory cytokines, and the progression of HIV infection. These interactions are depicted in Figure 1. From these interactions, we predict that there are several loops in the network which contribute to progressive deterioration in the health of HIV-infected individuals. First, there is the autoimmune stimulation of cytokines: for example, TNF stimulates its own production. Second, the stimulated production of ROS can lead to decreased GSH levels, which lead to increased TNF sensitivity. And third, both cytokines and ROS directly stimulate HIV; the HIV infection leads to chronically elevated levels of cytokines in vivo.

Increasing GSH levels can not only decrease ROS, but also inhibit stimulation by inflammatory cytokines. Thus, this part of the network represents an excellent point for therapeutic intervention. Therapies designed to increase GSH levels in the blood could have many positive effects: restoration of general GSH levels, alleviating the observed radiation sensitivity of HIV individuals; restoration of GSH levels in T cells, thereby possibly alleviating the suppressed state of these cells; inhibition of further inflammatory stimulation and production of ROS, thereby preventing direct stimulation of virus and further production of cytokines; and reduction of TNF ( cachectin) levels, thereby potentially alleviating the distressing wasting (cachexia) that often accompanies late-stage AIDS.

Many antioxidants are of potential use in this regard. Some compounds, which do not replenish GSH, may have beneficial effects by inhibiting ROS production and sparing extant GSH. For instance, penicillamine inhibits HIV production effectively in vitro, and its effectiveness as an anti-inflammatory is correlated with increasing GSH levels. It has antiviral activity in HIV-infected individuals; however, penicillamine induced transient depressions in CD4 T-cell numbers and lymphoproliferative capacity in these individuals. Hyaluronic acid, an anticoagulant agent used in cancer trials, inhibits TNF in vitro, and it inhibits both the TNF and PMA stimulation of viral transcription and viral replication (Roederer, unpublished). Auranofin, a gold-based antioxidant which quenches singlet oxygen and is used as an antiarthritis, specifically inhibits PMA but not TNF-stimulated ROS production (Roederer, unpublished). Finally, vitamin E effectively inhibits HIV replication.

However, we feel that most effective compounds will be GSH prodrugs; NAC, OTC, GSH itself, and glutathione ester (GSE). NAC has a long history of use in humans, dating back 25 years. Its pharmacokinetics and safety are well-established. OTC is similar to NAC in that it is a cysteine prodrug requiring intracellular enzymatic conversion; its pharmacokinetics have also been described. GSH and GSE also are possible candidates: they have antiviral activity and can increase plasma GSH. As Halliwell and Cross suggest, antioxidant
N-ACETYLCYSTEINE AS ANTI-HIV THERAPY

HIV Infection

AZT \textit{inhibits} Reverse Transcriptase

Provirus

DNA Integration

Latency

Cytokines

NAC \textit{inhibits} NF-\kappa B Activation

ROIs, H$_2$O$_2$

Expression

FIG. 2. NAC is a novel approach to anti-HIV therapy. NAC inhibits the host-mediated stimulation of viral transcription and production. Current therapies (AZT, DDI, DDC) are directed at inhibition of the reverse transcriptase and are ineffective in the inhibition of viral production after latency has been established.

therapy for patients is merited for drugs like vitamin C and NAC, which have extremely limited toxic effects.\textsuperscript{21} NAC, GSH, and GSE are effective in blocking the stimulation of acute HIV replication and the stimulation of latently infected cell lines. As of now, there are few therapies which are designed to inhibit HIV \textit{after} it has integrated into the genome; i.e., AZT, DDI, and DDC all work by inhibiting the reverse transcriptase necessary for integration of the virus. Furthermore, owing to the mutability of HIV, resistant variants of the virus can arise during long-term treatments with these drugs. GSH replenishment therapy should be effective in extending latency by inhibiting the host-based stimulation of HIV replication (Fig. 2). Furthermore, this type of therapy may have many positive effects on the symptomology of the HIV infection, e.g., against lymphocyte anergy, immunosuppression, development of opportunistic infections, and wasting.

ACKNOWLEDGMENTS

We thank Drs. Poli, Fauci, and Basuerle for sharing unpublished data. MR is a Senior Fellow of the Leukemia Society of America; FITS was supported by Department of Genetics funds, and LAH was supported in part by NIH Grant CA42509.

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Address reprint requests to:
Leonard A. Herzenberg
Department of Genetics
Stanford University
Stanford, CA 94305