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## The Interrelationship of Tumor Necrosis Factor, Glutathione, and AIDS

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The studies summarized here have evolved from observations made by other laboratories that thiols are chronically depressed in patients with HIV. Remarkably, our findings have produced some fundamental advances in understanding signal transduction and stimulation of gene expression, especially those of HIV. We now recognize that intracellular thiols and redox potential play important roles in many processes central to gene regulation.

Glutathione (GSH) is a cysteine-containing tripeptide, found in all eukaryotic cells at relatively high concentrations (ca. 1-10 mM), which regulates the cytosolic redox potential. It plays a number of different roles, all of which are crucial to cell survival and function. It serves as a cofactor for several enzymes; it is required for the synthesis of DNA precursors; it reduces protein disulfides and thus regulates sulfhydryl-dependent enzymes; and it reduces intracellular oxidants (free radicals, reactive metabolic intermediates) and thereby protects the cells from damage by these agents. Several laboratories have recently been exploring the role(s) GSH plays in HIV-1 infection and AIDS symptomatology. Droge and co-workers [1] demonstrated that HIV-infected individuals (even asymptomatic) have lower levels of serum acid-soluble thiols and intracellular GSH in their peripheral blood mononuclear cells (PBMC). Crystal and co-workers [2] confirmed these results and extended them by showing that the lung epithelial lining fluid is even more dramatically affected; again, even in asymptomatic HIV positive individuals.

These findings may be of considerable clinical significance. Lymphocyte function may be particularly dependent on GSH levels. Soluble thiols contribute to lymphocyte growth and activation (e.g. murine B and T cell lines grown in vitro often require addition of 2-mercaptoethanol to the culture medium). A number of studies have shown that T cell function can be

correlated with GSH levels directly, both in vitro and in vivo. For example, lymphocytes depleted of soluble GSH are strongly inhibited in their response to lectins (as mitogens) [3]. Furthermore, drugs which increase GSH levels act synergistically with concanavalin A to stimulate T cells [4]. Droge et al. [5] demonstrated that increasing previously lowered GSH levels in mice augments the activation of cytolytic T cells, demonstrating the importance of GSH levels in vivo.

The replenishment of intracellular GSH has been found to protect rats against lethal injections of tumor necrosis factor  $\alpha$  (TNF) [6]. TNF is not only toxic for whole organisms, it is also cytotoxic at the cell level. It exerts some of its toxic effects by stimulating production of reactive oxidative intermediates (ROI) [7, 8] in many or all cells, in addition to the 'respiratory burst' initiated in neutrophils in man. Intracellular GSH protects cells by scavenging ROI; however, the oxidant-buffering capacity of cellular GSH may be overcome by excessive stimulation with TNF.

Fauci and co-workers [9] have suggested that TNF plays a central role in the progression of AIDS. This inflammatory cytokine stimulates viral transcription and replication; furthermore, the stimulation is synergistic with a number of lymphokines including IL-1, IL-6, and GM-CSF. That TNF is of central importance in AIDS is further substantiated by the observations that serum levels of the cytokine increase during the progression of AIDS and are high in all individuals with full AIDS [10, 11]. These elevated TNF levels may well be responsible for depressed GSH levels; certainly, the lowered GSH levels contribute to an increased sensitivity to TNF [12].

We will describe the strong interrelationship between intracellular thiol levels, TNF, and the progression of AIDS. We have shown that intracellular GSH is significantly decreased in CD4 and CD8 T cells from HIV-infected individuals [13]. Furthermore, we have demonstrated a central interaction between intracellular thiols and the signal transduction from inflammatory agents such as TNF, in that addition of exogenous thiols can completely inhibit stimulations by TNF [14]. Together, these results point to the importance of thiol replacement therapy in HIV-infected individuals.

*GSH Levels in PBMC from HIV-Infected Individuals  
Decrease with the Progression of the Disease*

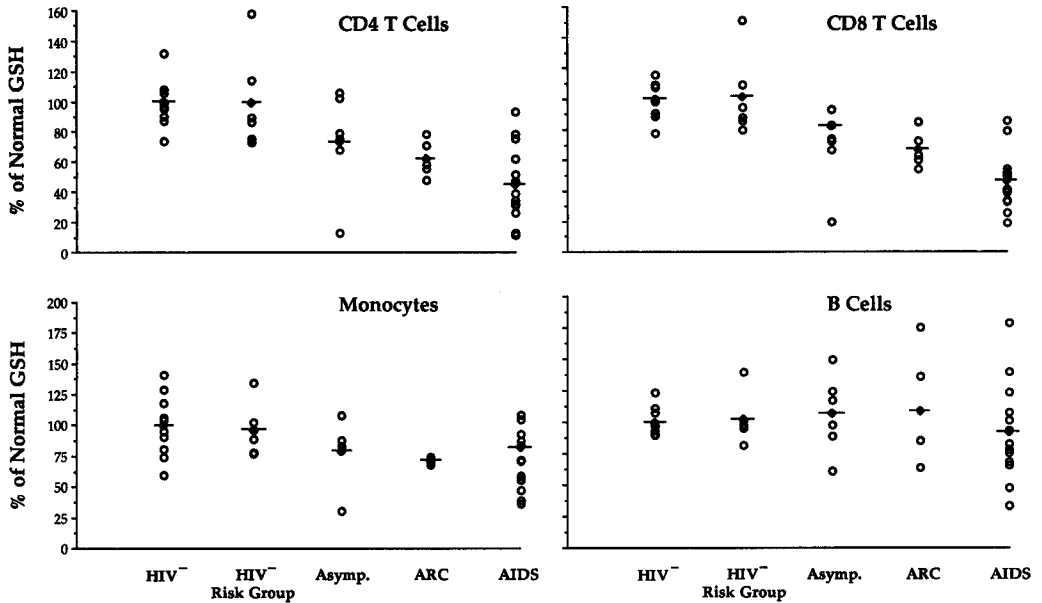
Rice et al. [15] have developed the use of monochlorobimane (MCB) as a FACS-based measurement for intracellular GSH levels. MCB, which is

nonfluorescent and readily crosses the cell membrane, is coupled intracellularly to GSH by GSH-S-transferase. The resulting adduct is fluorescent and trapped within cells. Under appropriate conditions, the intracellular fluorescence is proportional to the GSH content within the cells [15-17]; thus, the FACS can be easily used to quantitate intracellular GSH levels. We have extended this assay for use with surface immunophenotyping, in order to determine the precise levels of GSH within defined subsets of PBMC.

Surprisingly, these measurements showed that there are distinct types of human CD4 and CD8 T cells based on intracellular GSH levels [13]. Unlike in the mouse, human T cells can be divided into high-GSH cells and low-GSH cells. The high-GSH cells have roughly three times as much GSH as the low-GSH cells (but are not significantly larger in cell size). While the relative proportion of these two classes varies considerably from individual to individual, the absolute GSH levels within the cells is highly conserved among individuals [13]. The B cells, monocytes, and NK cells do not show a similar heterogeneity in intracellular GSH levels.

Examination of the GSH levels in PBMC from HIV-infected individuals demonstrates that the high-GSH T cells are selectively lost early in the progression of the disease, before the CD4 T cells are substantially lost [13]. The loss of these cells can be explained by a number of hypotheses, none of which has been ruled out: (1) high-GSH cells are sensitive to other conditions of the HIV infection (e.g. high TNF levels) and selectively die; (2) the high GSH levels are more difficult to maintain, especially under the oxidative stresses of opportunistic infections or high TNF levels, and the cells are 'converted' into low-GSH cells; (3) the de novo generation of these cells is altered by some aspect of the HIV infection.

Data in figure 1 demonstrate that the relative levels of intracellular GSH in T cell subsets in HIV-infected individuals decrease according to CDC stage (single factor analysis of variance: CD4<sup>+</sup> T cells,  $p < 0.0001$ ; CD8<sup>+</sup> T cells,  $p < 0.0001$ ). Individuals in the two uninfected control groups (randomly selected healthy individuals, and individuals classified as 'at risk') have similar GSH levels. In contrast, most of the HIV-infected individuals have lower than normal GSH levels. Furthermore, these levels decrease with the progression from the asymptomatic stage to ARC and finally AIDS. The importance of the loss of the high-GSH T cells to the overall decrease in mean GSH levels observed in HIV-infected individuals is underscored by data from patients in the ARC stage of the disease. Although ARC patients have lost relatively few CD4 T cells, they tend to have lost the majority of their



*Fig. 1.* Intracellular GSH levels decline with the progression of AIDS. PBMC from 16 uninfected individuals (including 6 homosexual males, grouped in the 'Risk group') and 42 HIV-infected individuals (8 asymptomatic, 9 classified with ARC, and 25 with AIDS) were analyzed for intracellular GSH and surface immunophenotype by FACS. Each circle represents an individual; horizontal bars represent the mean level for each group. Cell types are defined as follows: CD4 cells = CD4<sup>+</sup> lymphocytes; CD8 cells = CD8<sup>+</sup> lymphocytes; monocytes = CD14<sup>+</sup> monocytes; B cells = CD20<sup>+</sup> lymphocytes. The data for CD4 and CD8 T cells have been presented elsewhere [13].

high-GSH T cells. Consequently, the mean GSH levels in their CD4 and CD8 T cell subsets is clearly decreased.

As the HIV infection progresses from ARC to AIDS, the mean GSH levels in the remaining T cells in many patients continue to decline. This decline does not appear to be directly related to the loss of CD4 (or CD8) T cells, since there is no correlation between the absolute number of cells remaining in either of these subsets with the intracellular level of GSH per cell. In contrast, there is a strong correlation ( $r = 0.75$ ) between the levels of GSH in each person's CD4 and CD8 cells in HIV-infected individuals, as there is in uninfected individuals. This suggests that if there is a mechanism that coordinately regulates intracellular GSH among CD4 and CD8 T cell subsets [13], it is unaffected by the HIV infection.

While the T cell GSH levels are strongly affected by the HIV disease, the levels in monocytes and B cells are less affected (fig. 1). The average GSH levels for these cell types do not decrease significantly from normal in HIV-infected individuals; however, there is a much greater variation in the absolute levels from individual to individual than in normals. As is seen in figure 1, some individuals with AIDS have a very marked decrease in GSH levels in their B cells, as in their T cells; however, there are several AIDS individuals with high levels of GSH in only the B cell subset. These results emphasize the need to measure intracellular GSH levels in defined subsets of PBMC, as bulk measurements would not reveal the clear differences among subsets that are evident after FACS analyses.

### *TNF Stimulates HIV Activity*

Fauci and co-workers [18] have demonstrated that TNF stimulates HIV transcription via activation of the DNA transcription factor, nuclear factor  $\kappa$ B (NF- $\kappa$ B). Other inflammatory agents, such as phorbol esters (e.g. PMA) and IL-1 also stimulate HIV. In order to study the effects of cytokines, drugs, and other potential modulators of HIV-directed gene expression, we measure several parameters in several cell systems to model various aspects of the HIV infection, e.g. transcription, acute infection, and chronic (latent) infection.

We constructed a gene fusion of the HIV long terminal repeat (LTR), which contains the promoter and several enhancer-binding sequences of the HIV genome, with the coding sequence for the bacterial  $\beta$ -galactosidase ( $\beta$ -gal, from the *Escherichia coli lacZ* gene). This construct has been stably integrated into a human epithelial cell line, 293.27.2. The reporter construct is activated by incubation of the cells with TNF and/or PMA [14], and is induced by the presence of intracellular TAT protein [M. Montano and M. Roederer, unpubl. observations]. Thus, this cell line serves as a model for HIV transcription.

To study acute infection of HIV in vitro, we use either the CD4 T cell line Molt4, or phytohemagglutinin (PHA)-stimulated PBMC from uninfected individuals. These cells are infected with the infectious supernatant from ACH2 cells (see below), and are assayed over a period of days for either the production of infectious virions or for the presence of the viral core protein p24 in the culture supernatant.

Finally, the chronic infection models we use are the cell lines U1 and ACH2, developed in Fauci's laboratory by Folks et al. U1 is a promonocytic

Table 1. Activation of HIV model systems by inflammatory agents

Model system		Stimulation (fold activation) <sup>1</sup>			
		TNF	PMA	TNF + PMA	
Activation of HIV LTR- <i>lacZ</i>	RNA transcription <sup>2</sup>	2.6	4.6	4.9	
	$\beta$ -gal activity <sup>3</sup>	5.2	9.2	19.3	
Activation of viral replication	chronic infection <sup>4</sup>	U1	130	150	250
		ACH2	120	230	460
	acute infection <sup>4</sup>	Molt4	5.4	17	27
		PBMC	70	100	190

<sup>1</sup> Fold activation is as compared to an unstimulated culture under identical conditions; the following indicators were measured:

<sup>2</sup> Cytoplasmic *lacZ* mRNA levels, normalized by  $\beta$ -actin mRNA levels.

<sup>3</sup>  $\beta$ -gal measured by the MUG assay.

<sup>4</sup> p24 levels in the culture supernatant, measured by ELISA.

cell line, derived from infection of U937, and produces noninfectious virions upon stimulation. ACH2 is a CD4 T cell line which, when stimulated, produces infectious virions. Both cell lines have been extensively characterized by Fauci and co-workers [9, 19], and serve as useful models for studying latency in the AIDS disease.

As shown in table 1, all of these model systems respond similarly to stimulation by TNF and/or PMA. In general, PMA gives a somewhat stronger stimulation than TNF. Using both agents together gives a synergistic stimulation, in that a much larger response is obtained than with the maximum effective dose of either agent alone.

Levels of TNF as high as several hundred picograms per milliliter of blood have been observed in sera from HIV-infected individuals [10]. These levels of TNF are more than sufficient to stimulate the virus, as the concentration for half-maximal stimulation is approximately 300 pg/ml (data not shown). Furthermore, in view of the synergistic stimulation that is observed between TNF and other inflammatory agents [9], substantial stimulation of HIV transcription and replication is likely to occur at even lower levels of TNF.

*The Interaction of Inflammatory Stimulation and Intracellular Thiols*

While TNF stimulates HIV through the activation of NF- $\kappa$ B, it has long been known to have a wide variety of effects on different cell types. It exerts some of its toxic effects by stimulating production of ROI [7, 8] in many or all cells, in addition to the 'respiratory burst' initiated in neutrophils in man. The main defense against oxidative stresses within cells is intracellular GSH, which acts by scavenging the produced ROI. However, the oxidant-buffering capacity of GSH can be overcome by excessive stimulation with TNF [20]. Thus, under these conditions, it is likely that providing an exogenous source of thiols will increase the resistance of cells to the damaging effects of TNF.

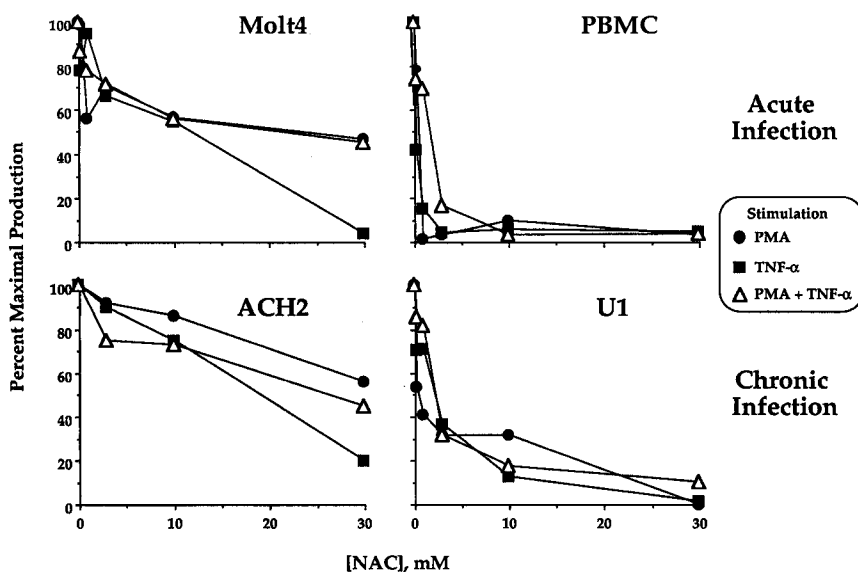
In fact, Zimmerman et al. [20, 21] have demonstrated that addition of N-acetylcysteine (NAC), a nontoxic cysteine prodrug, can protect both cells *in vitro* and animals against the toxic effects of high levels of TNF. NAC is also commonly used as an antidote to acetaminophen poisoning, which is lethal through an oxidative mechanism.

We hypothesized that the production of ROI is a necessary event in the stimulation pathway of TNF (and other agents, such as PMA), and that inhibiting or at least reducing the production of ROI following TNF stimulation would inhibit the resulting stimulation of viral activity. Since NAC is nontoxic, enters cells readily, and can be converted to GSH, we used it in our models of HIV regulation to study the effect of a thiol antioxidant on inflammatory stimulations.

Figure 2 shows that NAC interferes with the activation of viral replication from either acutely infected (Molt4, PBMC) or chronically infected (U1, ACH2) cells of either monocytic or T cell lineages. The inhibition of this stimulation is especially important in the chronically infected cells, since it suggests that NAC can maintain these cell lines in their relatively latent state despite the presence of high levels of stimulatory cytokines.

Interestingly, the dose-response curves of the stimulations for NAC are distinctly different for the T cell lines (ACH2, Molt4) than for the monocyte line or PBMC. In the T cell lines, even 30 mM NAC inhibited PMA stimulation only about 50%, whereas TNF stimulation was inhibited somewhat more effectively. In the monocytes, PMA was somewhat more sensitive to NAC than TNF, with 50% inhibition occurring at 1 and 3 mM, respectively. Finally, PBMC were exquisitely sensitive to NAC, with virtually complete inhibition of stimulation occurring at 1 mM NAC.

Although short-term cultures of normal PBMC contain mainly T cells, the amount of NAC which inhibits replication falls within the range of

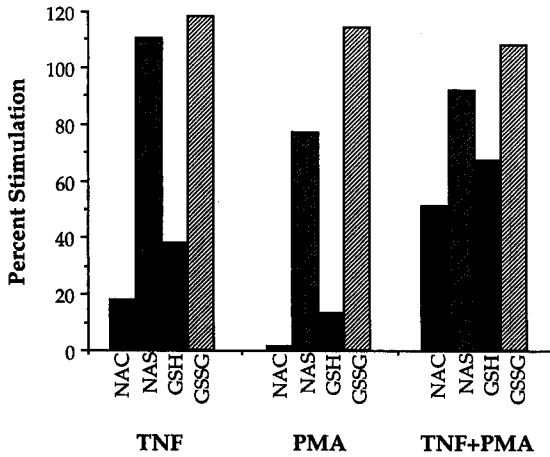


*Fig. 2.* NAC inhibits replication of HIV in both acute and chronic infection models. Results are shown as percent of maximal p24 core protein production for each stimulation condition. Top panels: Molt4 T cells or PHA-stimulated PBMC from normal, HIV<sup>-</sup> individuals were infected with an ACH2 supernatant. After infection, cultures were maintained for 2 days in the presence or absence of TNF, 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, 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 Molt4 experiment). These data have been presented elsewhere [34].

concentrations which inhibits monocytes, i.e. roughly tenfold lower than that required to inhibit replication in T cells. Thus, it is likely that viral replication in the PBMC either occurs mainly in the monocytic cells and/or in a subset of T cells that is particularly sensitive to NAC (unlike the cultured lines).

The ability to block lymphokine-stimulated HIV production in chronically-infected cell lines demonstrates that NAC can inhibit the expression of HIV after it has integrated into the genome. This inhibition could occur at any of several levels, e.g. transcription, translation, or viral assembly and budding. Using the 293.27.2 cell line as a model for viral transcription, we demonstrated that at least a part of the inhibition of NAC was through the inhibition of the stimulation of transcription.





**Fig. 3.** Stimulation of the HIV LTR by TNF and PMA is specifically inhibited by thiols. 293.27.2 cells [14] were incubated for 6 h with 10 ng/ml TNF and/or 20 ng/ml PMA, and with or without 30 mM N-acetylcysteine (NAC), 30 mM N-acetylserine (NAS), 30 mM reduced glutathione (GSH), or 30 mM oxidized glutathione (GSSG). Inhibition is seen only by thiol-containing compounds (NAC, GSH).

Figure 3 shows that the addition of exogenous thiols (either NAC or GSH) results in potent inhibition of the TNF or PMA-induced stimulations of the production of  $\beta$ -gal as directed by the HIV LTR. While GSH and NAC were both very effective at inhibiting the stimulation of the LTR, neither GSSG (oxidized glutathione, in which the thiol is not functional), nor N-acetylserine (identical to NAC, with the exception of an oxygen atom in place of the sulfur atom) inhibited the stimulation of  $\beta$ -gal activity. Thus, the inhibition depends on the presence of the reducing thiol.

Since this result still does not rule out a posttranscriptional inhibition by NAC, we examined the levels of *lacZ* RNA (as transcribed from the HIV LTR) in the 293.27.2 cells before and after treatments with TNF, PMA, and/or NAC. Consistent with the enzymatic measurements, *lacZ* RNA levels increased with TNF or PMA stimulation: this increase was abolished if NAC was present. Quantitation of the RNA levels show the same trends as observed for  $\beta$ -gal activity; that is, costimulation with TNF and PMA resulted in higher levels than either agent alone. Interestingly, this synergistic stimulation resulted in only slightly increased levels of mRNA (table 1), whereas the protein level increases substantially. This suggests that costimu-

lation may result in a posttranscriptional enhancement of  $\beta$ -gal production. Furthermore, the synergistic stimulation is less sensitive to NAC than either stimulation alone, indicating that the posttranscriptional enhancement may not be NAC-sensitive. (Interestingly, NAC does inhibit the posttranscriptional stimulation of HIV production induced by IL-6 and GM-CSF [A.S. Fauci and G. Poli, pers. commun.] )

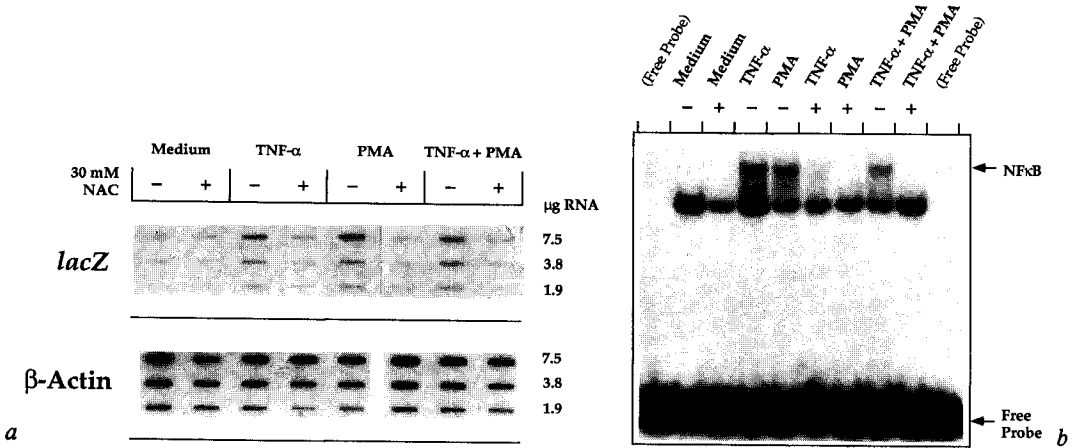
Since NF- $\kappa$ B has been shown to be directly involved in the stimulation of the HIV LTR induced by TNF and/or PMA [18], we examined the levels of the activated factor in the nucleus of cells stimulated in the presence or absence of NAC. NF- $\kappa$ B is present in the cytoplasm of unstimulated cells in a complex with an inhibitor, I $\kappa$ B [22, 23]. When cells are stimulated with TNF or PMA, I $\kappa$ B is phosphorylated and subsequently dissociates from NF- $\kappa$ B. This factor is transported into the nucleus, where it binds to specific sites in enhancer elements and stimulates the transcriptional activities of many promoters. The levels of the factor in the nucleus can be determined by electrophoretic mobility shift assays (EMSA) of nuclear protein extracts binding to specific oligonucleotides containing the binding site.

Such assays demonstrate that the NAC inhibition of stimulated mRNA synthesis can be explained in terms of an inhibition of NF- $\kappa$ B (fig. 4b). While PMA and TNF substantially increase the levels of activated NF- $\kappa$ B in the nucleus, inclusion of NAC results in no increase above basal activity. This inhibition could be at any stage prior to the appearance of active NF- $\kappa$ B in the nucleus; e.g. inhibitions of phosphorylation of I $\kappa$ B, transport of NF- $\kappa$ B to the nucleus, or binding of NF- $\kappa$ B to DNA.

### *Discussion*

TNF, which stimulates HIV replication, is also known as cachectin, a molecule at least partially responsible for cachexia or wasting, a condition characterized by anorexia, loss of muscle strength, and dramatic weight loss [24]. Continuous low-level administration of TNF has been shown to induce cachexia in mice [25] similar to that seen in the terminal stages of AIDS [26]. High TNF levels are frequently found in AIDS patients [10, 11]. In fact, increases in TNF levels [10] and decreases in GSH levels [1] clearly correlate with the progression of AIDS.

We suspect that these changes are tightly coupled. That is, the declining GSH levels in AIDS patients may contribute to the increasing TNF levels and vice versa, since a decline in intracellular GSH increases sensitivity to



**Fig. 4.** NAC inhibits stimulation of mRNA production and activation of NF-κB. *a* mRNA was prepared from 293.27.2 cells stimulated with TNF or PMA in the presence or absence of NAC as in figure 3. Dilutions of RNA were slot-blotted onto a nitrocellulose filter, baked and hybridized with a probe specific for *lacZ*. These data have been presented elsewhere [34]. *b* Electrophoretic mobility shift assays were performed on nuclear extracts prepared from 293.27.2 cells stimulated as in *a*. The upper arrow points to the band specific for NF-κB; the lower to the free probe. The first and last lanes contain free probe without nuclear extracts added. In the same extracts, there was no change in the amount of the constitutive OCT-1 enhancer-binding protein under any of these conditions (data not shown). Similar results were obtained with the Jurkat T cell line [M. Anderson and F. Staal, unpubl. observations]. These data have been presented elsewhere [12].

TNF [12, 20] and TNF decreases intracellular GSH. Furthermore, since TNF production is stimulated by bacterial and parasitic infections, this positive feedback loop between TNF and GSH may explain the rapid progression of the disease once opportunistic infections begin.

The interaction of TNF and GSH in the progression of AIDS is depicted in figure 5. After the initial HIV infection, opportunistic infections arise. These cause an inflammatory response (and thus oxidative stress). Part of the inflammatory response is the release of cytokines such as TNF, which directly stimulates both HIV replication and the production of more TNF [27–29]; the oxidative stress consumes intracellular GSH stores. The reduction of available GSH has pleiotropic effects, including increased sensitivity to further oxidative stress, increased sensitivity to inflammatory cytokines, and decreased immunological effector function. Thus, a cycle develops in

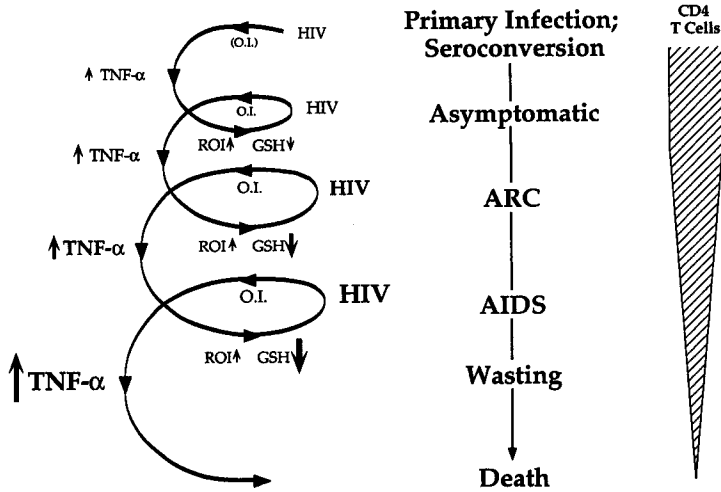


Fig. 5. Interaction of TNF, GSH, and HIV in the AIDS disease. O.I. = Opportunistic infection; GSH = glutathione; ROI = reactive oxygen intermediates; TNF = tumor necrosis factor. Arrows pointing up denote increasing levels; arrows pointing down denote decreasing levels; the arrow size symbolizes the quantity.

which TNF levels steadily rise, GSH levels steadily drop, and the disease worsens progressively.

We believe that NAC may provide a safe and effective defense against the detrimental depletions of thiol stores and increases in TNF levels. This cysteine derivative has been orally administered for more than 20 years for fluidizing mucus in the treatment of pulmonary disorders. Its pharmacokinetics and safety are well established [30–33]. Treatment of HIV-infected individuals with NAC may serve many functions, including the restoration of depleted GSH levels, inhibition of TNF toxicity (and thus, for example, wasting), and inhibition of the stimulation of viral replication induced by the inflammatory cytokines. Such treatment could be valuable for maintaining latency in asymptomatic patients and for stopping or slowing the progression of the disease in viremic patients.

In summary, we have shown that there is a critical interaction between intracellular thiols, inflammatory cytokines, and the regulation of expression of HIV. These results are important both at a basic level, in that they show that ROI production and intracellular thiol levels play a central role in gene regulation, and at a clinical level, in that they suggest that NAC may be a

useful adjunct in AIDS therapy by replenishing depleted GSH and blocking HIV stimulation by lymphokines produced during leukocyte activation.

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