

Disturbed Glutathione Metabolism and Decreased Antioxidant Levels in Human Immunodeficiency Virus–Infected Patients during Highly Active Antiretroviral Therapy—Potential Immunomodulatory Effects of Antioxidants

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Oxidative stress has been implicated in the pathogenesis of human immunodeficiency virus (HIV) infection. We examined the effect of highly active antiretroviral therapy (HAART) on plasma levels of several antioxidants and intracellular glutathione-redox status in CD4⁺ T cells, in 20 HIV-infected patients. HAART was accompanied by both an improvement of glutathione-redox status and an increase in levels of antioxidant vitamins, without full normalization. Glutathione supplementation in vitro increases T cell proliferation and suppresses the spontaneous release of tumor necrosis factor- α from peripheral blood mononuclear cells, in HIV-infected patients receiving HAART. Our findings suggest that therapeutic intervention aimed at normalization of oxidative disturbances in HIV infection could be of interest, in addition to HAART.

Highly reactive oxygen species are formed during a variety of biochemical reactions, and this steady-state formation of pro-oxidants is normally balanced by a similar rate of consumption by antioxidants. Oxidative stress results from an imbalance between formation and neutralization of pro-oxidants [1]. Enhanced oxidative stress has been implicated in the pathogenesis of several disorders—such as ischemic diseases of the heart and

brain, neurodegenerative disorders, and cancers—and several lines of evidence suggest that enhanced oxidative stress also plays a pathogenic role in human immunodeficiency virus (HIV) infection [1–4]. Thus, many studies have found impaired antioxidant defense and, in particular, disturbed glutathione metabolism, in HIV-infected patients [3–7]. Moreover, enhanced oxidative stress may be involved in the development of both impaired responsiveness and enhanced apoptosis, in T cells from HIV-infected patients, and may possibly involve tumor necrosis factor (TNF)- α -related mechanisms [8, 9]. The clinical significance of the HIV-associated glutathione disturbances is reflected by a strong association between decreased survival of HIV-infected patients and low thiol levels in both plasma [10] and CD4⁺ T cells [11].

Highly active antiretroviral therapy (HAART) often leads to dramatic improvement in clinical, viral, and immunologic parameters in HIV-infected patients [12, 13]. However, a favorable response is not achieved in all patients, and the emergence of drug-resistant strains and serious long-term side effects of HAART have increasingly been reported [12, 13]. The HAART-induced changes in the plasma-lipid profile have received particular attention [14]. Furthermore, although most of the patients experience a rise in circulating CD4⁺ T cells, HIV-associated immunodeficiency seems to persist, to a variable degree, during HAART [15]. Finally, HAART does not eradicate the virus, and a pool of chronically infected T cells persists even after several years of HAART, with undetectable HIV-RNA levels in plasma, resulting in rapid virologic rebound after withdrawal of HAART [13, 16]. Thus, there clearly is a need for additional treatment modalities in HIV infection, and, if such treatment also could counteract side effects of HAART, it would be of particular importance. To further elucidate the possibly pathogenic roles that enhanced oxidative stress and disturbed glutathione metabolism play in HIV infection, we examined the effect of HAART on plasma levels of several antioxidants and parameters of lipid peroxidation, as well as on intracellular glutathione-redox status in CD4⁺ T cells.

Patients, materials, and methods. Twenty patients (15 men and 5 women; median age, 39 years; age range, 25–55 years) were included in the study; 9 patients were classified as asymptomatic HIV-infected patients (Centers for Disease Control and Prevention [CDC] group A), 5 were classified as symptomatic non-AIDS HIV-infected patients (CDC group B), and 6 were classified as patients with AIDS (CDC group C). All patients received 1 HIV protease inhibitor (either indinavir [800 mg thrice daily; $n = 15$] or zidovudine [600 mg twice daily;

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$n = 5$) in combination with 2 nucleoside analogs (either zidovudine [250 mg] plus lamivudine [150 mg] [twice daily] [$n = 16$] or lamivudine [150 mg] plus stavudine [40 mg] [twice daily] [$n = 4$]). Plasma samples were taken after 8, 26, 52, and 78 weeks of HAART. For glutathione-redox status in CD4⁺ T cells, cells were isolated at baseline and after 8, 26, and 52 weeks of HAART. Blood samples were also collected from 10 healthy, HIV-seronegative blood donors (7 men and 3 women; median age, 38 years; age range, 25–59 years). Informed consent for blood sampling was obtained from all patients and control subjects.

Blood was drawn into pyrogen-free blood-collection EDTA vacuum tubes that were immediately immersed in melting ice and were centrifuged at 600 *g* for 10 min within 30 min. Plasma was stored at -80°C until analysis, and samples were thawed only once.

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep; Nycomed) gradient centrifugation within 30 min after collection of blood. Negative selection of CD3⁺ T cells (functional studies) and positive selection of CD4⁺ T cells (glutathione analyses), from PBMCs, were performed by use of monodispersed immunomagnetic beads (Dynal), as described elsewhere [5, 17]. Purified CD4⁺ T cells were immediately transferred to liquid nitrogen and were stored until further analysis. The purity of the CD4⁺ T cells was $>98\%$ (by staining of cytospin, using an alkaline phosphatase–anti-alkaline phosphatase procedure [5]), and that of the CD3⁺ T cells was $>95\%$ (by flow cytometry).

Glutathione analysis was performed as described elsewhere [5]. In brief, ice-cold 5% sulfosalicylic acid (Merck) containing dithioerythritol (Sigma) ($50\ \mu\text{mol/L}$) was added to the frozen cell pellets, to prevent *ex vivo* oxidation of the thiol groups. After being thawed, precipitated proteins and immunomagnetic beads were immediately removed by centrifugation. Total free glutathione (reduced glutathione plus glutathione disulfide plus soluble glutathione-mixed disulfide; for simplicity, referred to as “total glutathione”) and reduced glutathione were determined in the acid extract by a chromatographic procedure [18]. The fraction of oxidized glutathione (glutathione disulfide plus soluble glutathione-mixed disulfide) was calculated by subtracting the amount of reduced glutathione from the total amount of glutathione.

A simultaneous determination of plasma α -tocopherol and β -carotene was performed by high-performance liquid chromatography (HPLC) [19]. For measurement of ascorbic acid, plasma was stabilized with an equal volume of 10% metaphosphoric acid. The precipitate was removed by centrifugation at 4000 *g* for 5 min at 4°C , and the supernatant was stored at -80°C , until analysis of ascorbic acid by HPLC with colorimetric detection [20].

Plasma levels of malondialdehyde (MDA), as measured on the

basis of its thiobarbituric complexes, were measured by HPLC [19]. To prevent peroxidation during the assay, the chain-breaking antioxidant butylated hydroxytoluene was added to the samples at the start of the assay.

CD3⁺ T cells (10^6 cells/mL) were incubated in 96-well trays (Costar), either in medium alone (RPMI 1640 with L-glutamine [2 mmol/L] HEPES buffer [Gibco] [25 mmol/L], and 10% heat-inactivated pooled human AB⁺ serum) or in medium with stimulant (anti-CD3 monoclonal antibodies, clone SpvT₃B [17]; final concentration, 1.2 ng/mL), with and without different concentrations of glutathione-monoethyl ester (Bachem). The cell-surface markers were cross-linked by use of monodispersed immunomagnetic beads coated with sheep anti-mouse IgG (Dynal), in a cell:bead ratio of 1:1. After 48 h, $1\ \mu\text{Ci}$ of [³H]-thymidine was added to cell cultures, and, 16 h later, cultures were harvested onto glass filter strips by use of an automated multisampler (Skatron) and subsequently were analyzed by β scintillation.

PBMCs (10^6 cells/mL) resuspended in medium (see above) were incubated in 96-well trays (Costar), either in medium alone or in medium with purified protein derivative from *Mycobacterium avium* (MAC-PPD; final concentration, 1:100) [21], with and without different concentrations of glutathione-monoethyl ester (Bachem). After 24 h, cell-free supernatants were harvested and were stored at -80°C .

TNF- α levels were determined by enzyme immunoassay (Biosource International) [22]. Cell viability was determined by propidium-iodide staining [23]. Plasma HIV-RNA levels were determined by quantitative reverse polymerase chain reaction (Amplicor HIV Monitor; Roche Diagnostic Systems) (detection limit, 200 copies/mL plasma). The numbers of CD4⁺ and CD8⁺ lymphocytes in peripheral blood were determined by immunomagnetic quantification [22].

For each parameter, levels after initiation of HAART were compared with baseline levels, by Wilcoxon matched-pairs test. Coefficients of correlation were calculated by Spearman's rank-correlation test. Differences between 2 groups were calculated by Mann-Whitney *U* test. Data are given as medians and interquartile ranges (IQRs), unless otherwise stated. Two-sided $P < .05$ was considered to be significant.

Results. The immunologic and virologic responses to HAART in this study population have been reported elsewhere [24], and they show a pronounced fall in virus load, with a maximal decrease in HIV-RNA copies per milliliter of plasma after 26 weeks (median [IQR], 2.55 [1.39–5.10] \log_{10} ; $P < .001$). There was a concomitant, prolonged increase in both CD8⁺ T cell count (maximum increase [IQR], 320 [60–530] $\times 10^6/\text{L}$; $P < .01$) and CD4⁺ T cell count (maximum increase [IQR], 190 [70–280] $\times 10^6/\text{L}$; $P < .001$), in peripheral blood.

At baseline, CD4⁺ T cells from HIV-infected patients were characterized by a decreased ratio of reduced glutathione to

total glutathione, increased oxidized glutathione, and decreased levels of reduced glutathione ($P < .01$, for all parameters), with no significant changes in total levels of this thiol, compared with those in control subjects (figure 1). During HAART, there was a significant increase in this ratio, reflecting a decrease in oxidized glutathione and an increase in reduced glutathione (figure 1). In contrast to the rapid changes in virus load and T cell count, these changes in glutathione metabolism were first seen after 26 weeks, and, although several changes were observed, normalization was not observed, compared with what was observed in control subjects (figure 1).

Before initiation of HAART, the HIV-infected patients had significantly decreased plasma levels of vitamin C, compared with those in control subjects ($P < .001$), and, notably, HAART induced a marked increase in this antioxidant vitamin, with the highest levels occurring at the end of the study period (figure 2). A similar pattern was also seen for vitamin E, although the increase was more modest (figure 2). Although levels of both vitamin C and vitamin E increased during HAART, the concentrations were not normalized, compared with those in control subjects (figure 2). In contrast to the rise in levels of vitamins, levels of MDA, as a parameter of lipid peroxidation, significantly decreased during HAART, but, as observed for the antioxidant vitamins, normalization was not observed during the study period (figure 2). Similar to what was observed for the glutathione parameters, the effects of HAART on levels of vitamin C, vitamin E, and MDA were first seen after 26 weeks

of HAART (figure 2). For β -carotene, concentrations were within normal limits throughout the study (figure 2).

During HAART, the maximal change in virus load was negatively correlated with the maximal changes in both vitamin C ($r = -0.67$; $P < .005$) and the ratio of reduced glutathione to total glutathione in $CD4^+$ T cells ($r = -0.59$; $P < .01$). After 1 year, 7 patients were classified as patients with virologic-treatment failures (HIV-RNA level, >200 copies/mL). Although, with regard to prior treatment regimen, baseline virus load, and CDC classification, these patients were comparable to those without virologic-treatment failure; the patients with virologic-treatment failure had significantly decreased vitamin C levels, compared with those in other patients (median [IQR], 7.19 [3.71 – 8.32] $\mu\text{mol/L}$ vs. 10.64 [9.25 – 11.32] $\mu\text{mol/L}$; $P < .01$), after 1 year.

The changes in intracellular thiol levels were also correlated with the changes in $CD4^+$ T cell count during HAART. Thus, in these cells, the maximal increase in the number of $CD4^+$ T cells was positively correlated with changes in both the ratio of reduced glutathione to total glutathione ($r = 0.71$; $P < .001$) and reduced glutathione ($r = 0.62$; $P < .01$).

To further study the relationship between glutathione and T cells, in HIV infection, we examined, in a separate experiment, whether glutathione supplementation in vitro could improve T cell proliferation in HIV-infected patients during HAART. In this in vitro experiment, we included only patients without virologic treatment failure, to examine the potential role that glutathione

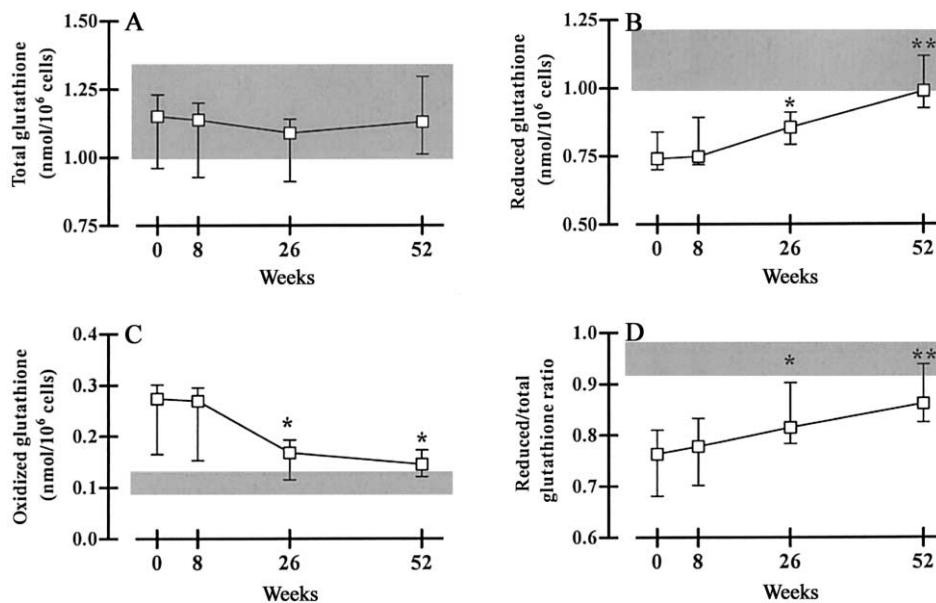


Figure 1. Intracellular glutathione-redox status in $CD4^+$ T cells from 20 human immunodeficiency virus–infected patients, before and 8, 26, and 52 weeks after initiation of highly active antiretroviral therapy. Total glutathione (A), reduced glutathione (B), oxidized glutathione (C), and reduced glutathione to total glutathione ratio (D) are shown. Data are medians and interquartile ranges (IQRs). * $P < .01$ and ** $P < .001$, versus baseline. The shaded areas indicate the IQRs in 10 control subjects.

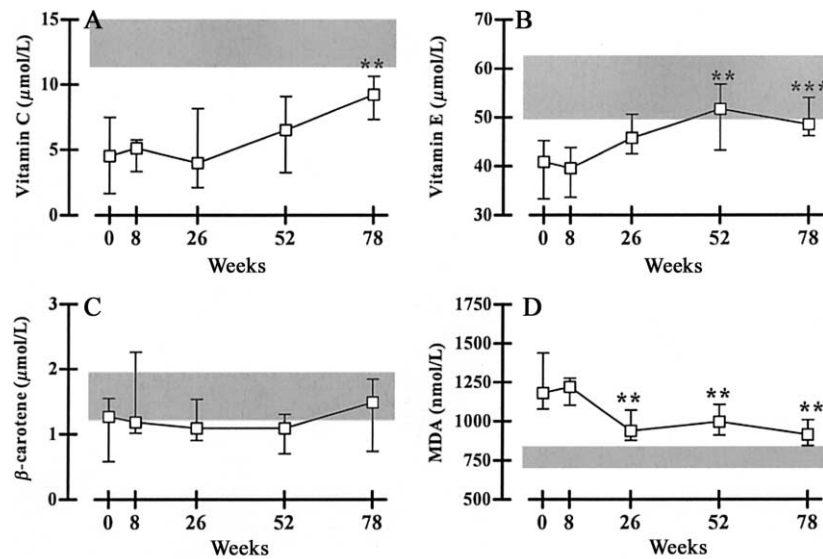


Figure 2. Plasma levels of vitamin C (A), vitamin E (B), β-carotene (C), and malondialdehyde (MDA) (D), before and 8, 26, 52, and 78 weeks after initiation of highly active antiretroviral therapy, in 20 human immunodeficiency virus–infected patients. Data are medians and interquartile ranges (IQRs). * $P < .01$ and ** $P < .001$, versus baseline. The shaded areas indicate the IQRs in 10 control subjects.

supplementation plays in addition to apparently successful HAART, since there is an increasing awareness that other therapeutic modalities may be of interest as supplementations to HAART [15]. In 6 patients (4 in CDC group A and 2 in CDC group B, before HAART) who were not included in the longitudinal HAART study (figures 1 and 2), whose median duration of HAART was 1 year (range, 8–16 months), and who had no signs of virologic treatment failure (as defined above), we found still-decreased T cell proliferation after anti-CD3 stimulation, compared with that in control subjects (figure 3A). More important, when glutathione-monoethyl ester, acting as glutathione precursor, was added to cell culture before stimulation, we found a dose-dependent increase in anti-CD3–stimulated T cell proliferation, which, in all but 2 patients, reached levels within the normal range (figure 3A). Glutathione-monoethyl ester had no effect on spontaneous T cell proliferation.

We have reported elsewhere a HAART-induced decrease in plasma levels of TNF-α for this study population [24]. Of note, this decrease in TNF-α was negatively correlated with the maximal changes in reduced glutathione in CD4⁺ T cells ($r = -0.52$; $P < .05$). Moreover, when examining PBMCs in the 6 patients who were included in the T cell–proliferation experiment (see above), we found that glutathione-monoethyl ester decreased the spontaneous release of TNF-α in a dose-dependent manner (figure 3B). No such effect was seen with regard to levels of MAC-PPD–stimulated TNF-α. Finally, glutathione-monoethyl ester supplementation in vitro, in the actual concentrations used in the present study, was not toxic to either T cells or PBMCs, as assessed by propidium-iodine staining.

Discussion. There are several studies of disturbed glutathione metabolism in HIV-infected patients [3–7], and a few studies also have reported decreased plasma levels of vitamin C [15]. In the present study, we have shown that, during HAART, the decrease in virus load and the increase in CD4⁺ T cell count are accompanied by both an improvement in the abnormal glutathione-redox status and an increase in the subnormal levels of antioxidant vitamins; however, HAART did not induce full normalization of these parameters, and, furthermore, in HIV-infected patients receiving HAART, glutathione supplementation in vitro increased anti-CD3–stimulated T cell proliferation and suppressed the spontaneous release of TNF-α from PBMCs. These findings lend further support to the idea that enhanced oxidative stress contributes to the pathogenesis of HIV infection, and our in vitro findings suggest that therapeutic intervention aimed at normalization of these oxidative disturbances could be of interest, even in the “HAART era.” However, it remains to be proven that such effects of glutathione supplementation also are operative in vivo in HIV-infected patients receiving HAART.

In the present study, we have shown that HIV-infected patients are characterized by a decrease in both reduced glutathione and vitamin C, the 2 most important hydrophilic antioxidants; and such a pattern is of particular interest. Thus, reduced glutathione is one of the most important reducing agents in the regeneration of ascorbic acid from its oxidized form, and, in turn, vitamin C deficiency may lead to enhanced utilization of reduced glutathione, an event that would further promote depletion of both antioxidants [26]. Accordingly, the

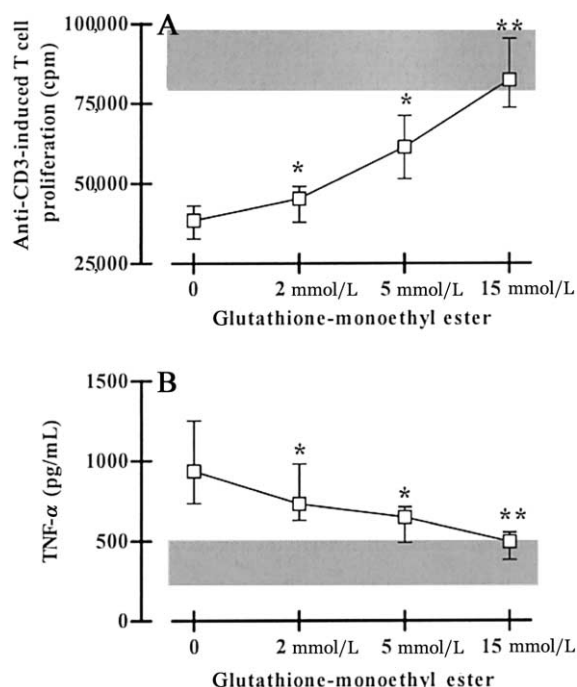


Figure 3. Effect of different concentrations of glutathione-monoethyl ester on anti-CD3-stimulated T cell proliferation (A) and on the release of tumor necrosis factor- α in unstimulated peripheral blood mononuclear cells (B), in 6 human immunodeficiency virus-infected patients who had received highly active antiretroviral therapy (HAART) for a median duration of 1 year (range, 8–16 months) and who had no signs of virologic treatment failure (see Patients, materials, and methods and Results). The patients in these in vitro experiments were not included in the longitudinal HAART study as outlined in figures 1 and figure 2. Data are medians and interquartile ranges (IQRs). * $P < .05$ and ** $P < .01$, versus no addition of glutathione-monoethyl ester. The shaded areas indicate the IQRs in 6 control subjects. cpm, counts per minute; TNF, tumor necrosis factor.

combined decrease of these 2 antioxidants, as seen in HIV-infected patients receiving or not receiving HAART, may represent a vicious circle, by contributing to enhanced oxidative stress. From a therapeutic point of view, synergistic effects between these 2 functionally similar antioxidants have been reported in some animal models [27], and a similar approach has also been suggested for HIV infection [28].

Although HAART markedly improves immunologic parameters, some immunodeficiency seems to persist to a variable degree. In the present study, we have shown that impaired T cell proliferation in HIV-infected patients receiving HAART and, notably, glutathione supplementation in vitro seem to correct this T cell defect. In previous studies, decreased levels of reduced glutathione in T cells have been shown to impair interleukin (IL)-2 production, IL-2 responses, and cytotoxic T cell activity and to favor T helper (Th) 2 responses over Th1 responses [9, 29–32]. The importance of glutathione metabolism for T cell function is underscored by the fact that an ~30% decrease in reduced glutathione in T cells almost completely

blocks T cell proliferation, through T cell-receptor/CD3-activation [33]. The relevance of these findings to HIV infection has been shown by Cayota et al., who demonstrated that restoration of the glutathione-redox balance, by antioxidant supplementation in vitro, is able to restore the impaired CD4⁺ T cell proliferation in HIV-infected patients [34]. In the present study, we have shown that a similar phenomenon also may be seen in patients with a satisfactory response to HAART; and some recent studies have suggested that glutathione supplementation in vivo also may improve immunologic functions in patients receiving HAART [35].

Several mechanisms—such as low intake of antioxidants or their precursors, malabsorption, and, in peripheral tissue, enhanced cysteine metabolism with a consequent loss of sulfur—may account for glutathione and antioxidant deficiency during HIV infection [3, 9, 36]. Moreover, HIV may also, directly through a Tat-related mechanism, promote disturbed glutathione metabolism [37, 38], and it could be hypothesized that the improvement in glutathione-redox status during HAART could reflect decreased levels of HIV-Tat protein. However, disturbed glutathione metabolism and decreased antioxidant levels may also reflect enhanced consumption that is secondary to persistently enhanced inflammation in these patients. Thus, TNF- α could impair glutathione-redox status by various mechanisms—such as consumption of reduced glutathione, secondary to enhanced production of reactive oxygen species, and impairment of the glutathione-reductase system—thereby leading to decreased regeneration of reduced glutathione from oxidized glutathione [3, 8, 39, 40]. Moreover, enhanced oxidative stress may increase TNF- α production in various cells, and depletion of reduced glutathione may increase the inflammatory response to this cytokine [3, 8]. Thus, glutathione-redox disturbances and enhanced TNF- α activation may represent a pathogenic loop, leading to enhanced inflammation and oxidative stress, and our findings suggest that this mechanism may also be operating, at least to some degree, during HAART. This inflammatory interaction not only may contribute to impaired T cell function (see above) but also may promote both HIV replication [41–44] and T cell apoptosis [8, 45], thus contributing to CD4⁺ T cell depletion. The findings of the present study, demonstrate suppression of the spontaneous release of TNF- α in PBMCs from HIV-infected patients during HAART, suggest that glutathione supplementation could represent a therapeutic approach to block the vicious circle involving oxidative stress and TNF- α . This concept is also supported by some in vivo studies of HIV-infected patients, as well as by some murine models of AIDS [46, 47].

On the basis of recent findings implicating oxidative stress as playing a pathogenic role in atherogenesis [48, 49], our findings could also be of importance for an understanding of certain longtime side effects of HAART (e.g., hyperlipidemia poten-

tially predisposing to atherosclerotic disorders). In fact, persistent TNF- α activation appears to be involved in the pathogenesis of HAART-associated lipodystrophy syndrome [50]. Moreover, drug-associated dysfunction of mitochondria is believed to play a central role in the etiology of several of the various adverse symptoms that occur in HIV-infected patients treated with nucleoside reverse-transcriptase inhibitors [51], and, again, disturbed glutathione metabolism may enhance such toxic effects [52, 53]. Thus, although there is some improvement, disturbed glutathione-redox status and decreased antioxidant levels seem to persist even during HAART, and these effects not only may contribute to persistent immunodeficiency in these patients but also may potentially enhance the toxicity of such therapy.

The present study suggests that enhanced oxidative stress and disturbed glutathione metabolism exist, at least to some degree, during apparently successful HAART. These effects seem to be related to persistent TNF- α activation in HIV-infected patients. As a supplement to HAART, combination therapy with glutathione-replenishing agents, antioxidants, and therapeutic modalities that down-regulate TNF- α activity could be an interesting therapeutic approach in HIV-infected patients. Such therapy not only may contribute to both restoration of immune responses and down-regulation of HIV replication but also may attenuate the toxic effects of HAART.

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