Increased Levels of Oxidized Glutathione in CD4$^+$ Lymphocytes Associated With Disturbed Intracellular Redox Balance in Human Immunodeficiency Virus Type 1 Infection

By Pål Aukrust, Asbjørn M. Svardal, Fredrik Müller, Bodil Lunden, Rolf K. Berge, Per M. Ueland, and Stig S. Frøland

We investigated the intracellular glutathione redox status in isolated lymphocyte subpopulations and monocytes in patients with human immunodeficiency virus type 1 (HIV-1) infection and in healthy controls. CD4$^+$ lymphocytes from HIV-1–infected patients were primarily characterized by a substantial increase in oxidized glutathione levels and a considerable decrease in the ratio of reduced to total glutathione, in most cases below 0.5 in patients with symptomatic HIV-1 infection, rather than decreased levels of reduced glutathione. The increase in oxidized glutathione was strongly correlated with low numbers of CD4$^+$ lymphocytes in peripheral blood and impaired stimulated interleukin-2 production and proliferation in peripheral blood mononuclear cells, which is compatible with an immunopathogenic role for these redox disturbances. The HIV-1–infected patients with the most advanced clinical and immunologic disease were also characterized by an increase in levels of reduced glutathione in monocytes, suggesting that the glutathione redox cycle may be differentially regulated in CD4$^+$ lymphocytes and monocytes. We could not confirm previous reports suggesting cysteine deficiency as a major cause of disturbed glutathione homeostasis during HIV-1 infection. The demonstrated glutathione abnormalities were correlated with raised serum levels of tumor necrosis factor α. These findings suggest that a therapeutical approach, which can restore the glutathione redox dysbalance in CD4$^+$ lymphocytes and decrease the inflammatory stress, may be worthwhile exploring in HIV-1 infection.

INCREASED OXIDATIVE STRESS has been suggested to play an important pathogenic role in many human diseases including infection with human immunodeficiency virus type 1 (HIV-1).[1,2] Highly reactive oxygen species (ROS) are formed during a variety of biochemical reactions, and this steady-state formation of pro-oxidants is normally balanced by a similar rate of their consumption by antioxidants.[2] Oxidative stress results from imbalance between formation and neutralization of the pro-oxidants. Glutathione, a cysteine-containing tripeptide, is present in eukaryotic cells as the dominant intracellular thiol and represents the major intracellular redox buffering compound,[3] is involved in recycling of other antioxidants (eg, ascorbic acid),[4] plays a role in a variety of reactions such as synthesis of proteins and DNA precursors, and acts as a cofactor for many glycolytic enzymes.[3]

Several immune functions with relevance to HIV-1 infection are dependent on adequate intracellular glutathione levels, eg, lymphocyte activation by mitogens,[5] natural killer cell activation[6] and T-lymphocyte–mediated cytotoxicity.[7] Moreover, tumor necrosis factor α (TNFα)–stimulated HIV-1 transcription in vitro is enhanced by depleting intracellular levels of reduced glutathione.[8] There is also evidence for abnormalities in glutathione homeostasis in vivo during HIV-1 infection. Glutathione deficiency in HIV-1–infected patients have been reported in plasma,[9] in lung epithelial-lining fluid,[9] in peripheral blood mononuclear cells (PBMCs)[10] and in CD4$^+$ and CD8$^+$ lymphocytes.[11,12] However, the methods used in measuring intracellular glutathione levels have recently been questioned.[13] It has been suggested that glutathione depletion in HIV-1 infection is caused partly by decreased availability of cysteine that is rate limiting in glutathione synthesis,[10,14,15] although in one study glutathione depletion in serum was accompanied by normal cysteine levels.[16]

In the present study we used different experimental approaches to further analyze glutathione abnormalities and plasma cysteine redox status in HIV-1–infected patients. First, as glutathione levels may be differentially regulated in different cell types[17,18] we analyzed glutathione levels in isolated lymphocyte subpopulations and monocytes. Second, because previous studies have measured only reduced glutathione levels in these cell subpopulations,[12,13] we attempted to assess redox status by measuring the ratio between reduced and total intracellular glutathione. Third, the determination of redox thiol status in plasma is particularly challenging because redox conditions change rapidly (within seconds) after blood sampling.[19] Therefore, we measured circulating thiol levels by a novel procedure based on trapping thiols by collecting blood directly into evacuated tubes containing thiol-reactive agents.[20] Finally, to further elucidate the possible significance of glutathione abnormalities in HIV-1 infection, the association between these abnormalities and important clinical and immunologic characteristics in HIV-1–infected patients was evaluated.

MATERIALS AND METHODS

Patients and controls. Blood samples were obtained from twenty two HIV-1–infected patients. All individuals had serum antibodies to HIV-1 in two separate serum samples as determined by enzyme-

From the Section of Clinical Immunology and Infectious Diseases, Medical Department A, and Research Institute for Internal Medicine, University of Oslo, The National Hospital, Rikshospitalet, Oslo, Norway; and the Department of Clinical Biology, Divisions of Pharmacology and Clinical Biochemistry, University of Bergen, Haukeland Hospital, Bergen, Norway.

Submitted November 2, 1994; accepted February 16, 1995.

Supported by Norwegian Research Council for Science and Humanities, Norwegian Cancer Society, Medinnova Foundation, Anders Jahre's Foundation, and Odd Kåre Rabben's Memorial Fund for AIDS Research.

Address reprint requests to Pål Aukrust, MD, Section of Clinical Immunology and Infectious Diseases, Medical Department A, Rikshospitalet, N-0027 Oslo, Norway.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/95/8601-0020$3.00/0

Table 1. Clinical and Immunologic Characteristics of the Study Group

	HIV-1–Infected Patients	Controls
Number	22	15
Age in years, median	34	35
ranges	30-54	28-53
25th-75th percentiles	32-44	31-43
Males/females	18 (82%)/4 (18%)	12 (80%)/3 (20%)
$CD4^+$ lymphocytes ($\times 10^6$/L)	120* (55-295)	550 (340-750)
$CD8^+$ lymphocytes ($\times 10^6$/L)	620* (440-810)	315 (260-350)
$CD19^+$ lymphocytes ($\times 10^6$/L)	185 (68-275)	150 (90-230)
Monocytes ($\times 10^6$/L)	365† (280-435)	280 (240-350)
Serum levels of TNFα (pg/mL)	29* (22-52)	8 (0-10)

Ten patients were classified as asymptomatic (CDC group A) and twelve as symptomatic (eight in CDC group B and four in CDC group C) HIV-1–infected patients. Data are given as medians and 25th-75th percentiles if not otherwise stated.

* $P < .001$.

† $P < .05$.

linked immunosorbent assay and confirmed by Western blot. Patients were clinically classified according to the revised criteria from Centers for Disease Control and Prevention (CDC).[21] Some clinical and immunologic characteristics are given in Table 1. Patients with ongoing acute infection at the time of blood collection (3 weeks before to 1 week after), intravenous drug users, patients abusing alcohol, patients in which antiretroviral therapy had been initiated during the last 6 months and patients with levels of alanine aminotransferase >50 U/L or serum creatinine levels >100 μmol/L were not included in the study. Controls were fifteen sex- and age-matched healthy, volunteer HIV-1–seronegative blood donors (Table 1). The percentage of smokers was equal in the two groups. Informed consent was obtained from all patients and controls.

Blood sampling protocol. Blood samples were drawn between 8 and 10 AM after an overnight fast. For serum sampling, sterile vacuum blood collection tubes without any additives were immediately immersed in melting ice and were allowed to clot for less than 1 hour before centrifugation at 400*g* for 10 minutes. For determination of various thiol components in plasma, blood was routinely collected into three evacuated tubes containing heparin as anticoagulant and either monobromobimane (mBrB; Molecular Probes, Eugene, OR) or *N*-ethylmaleimide (NEM; Sigma Chemical Co, St Louis, MO) as thiol-derivatizing reagent or no addition and placed in melting ice.[20] The blood was centrifuged within 15 minutes at 10,000*g* and 4°C for 5 minutes to obtain platelet-free plasma. To NEM- and mBrB-treated plasma, a solution of sulfosalicylic acid (final concentration of 5%; Merck AG, Darmstadt, Germany) containing dithioerythritol (DTE; final concentration of 50 μmol/L; Sigma) was added before storage at −70°C.

Cell separation. PBMCs were obtained from heparinized blood by Isopaque-Ficoll (Lymphoprep; Nycomed Pharma AS, Oslo, Norway) gradient centrifugation within 1 hour after blood sampling in twenty HIV-1–infected patients and all controls. Mononuclear cells were washed twice in Hanks' balanced salt solution (GIBCO, Paisley, UK) and finally resuspended at a concentration of 15×10^6 PBMC/mL in phosphate-buffered saline (PBS) and 0.3% (final concentration) bovine serum albumin (BSA; Calbiochem, La Jolla, CA). Further positive selection of cell subsets by monodisperse immunomagnetic beads was done at 4°C as previously described.[22,23] Briefly, PBMCs were mixed with Dynabeads (Dynal, Oslo, Norway) coated with appropriate antibodies (anti-CD4, Dynabeads M-450 CD4; anti-CD8, Dynabeads M-450 CD8; anti-CD14, Dynabeads M-450 CD14; anti-CD19, Dynabeads M-450 Pan-B) in a cell-to-bead ratio of 1:10. The concentration of the actual cell subset was determined by immunomagnetic quantification.[24] The mixture was incubated in a test tube on a rocking platform for 30 minutes and rosetting cells were isolated by application of a samarium cobalt magnet (Dynal) to the side of the test tube. After five consecutive washes in cold PBS/0.3% BSA, the cell pellet was immediately stored in liquid nitrogen. The nonrosetting cells received a new round of treatment with appropriate Dynabeads as described above. Thus, from the same portion of PBMC, $CD14^+$, $CD19^+$, $CD4^+$, and $CD8^+$ cells were consecutively isolated.

The purity of the obtained cell populations was greater than 98% as assessed by staining of cytospin preparations of positively selected cells by the alkaline phosphatase–antialkaline phosphatase procedure[25] using a panel of monoclonal antibodies (MoAbs): anti-CD3 (clone SK 7; Becton Dickinson, San Jose, CA), anti-CD4 (clone SK 3; Becton Dickinson), anti-CD8 (clone SK 1; Becton Dickinson), anti-CD19 (clone HD 37; Dakopatts, Glostrup, Denmark), anti-CD56 (clone T 199; Dakopatts) and anti-CD14 (clone Mϕ-P9; Becton Dickinson).

Determination of cysteine and glutathione levels in plasma. As previously described,[20] the amounts of reduced and oxidized thiols were obtained from blood collected into solutions containing mBrB and NEM, respectively. The total amount of thiols (oxidized + reduced + protein-bound form) was assayed in nontreated plasma in which a solution of sulfosalicylic acid (final concentration of 5%) containing DTE (final concentration of 50 μmol/L) was added before thawing.[20]

Determination of intracellular glutathione levels in different lymphocyte subsets and monocytes. The isolated lymphocytes and monocytes were extracted with 0.3 mL ice-cold 5% sulfosalicylic acid containing 50 μmol/L DTE, and the precipitated protein and the immunomagnetic beads were immediately removed by centrifugation. Storing of cells with immunomagnetic beads did not influence the intracellular glutathione levels (data not shown). Total free glutathione (reduced glutathione + glutathione disulfide + soluble glutathione mixed disulfide; for simplicity referred to as total glutathione level in the text) and reduced glutathione were determined in the acid extract according to a modification[20] of a chromatographic procedure described previously.[19] The fraction of oxidized glutathione (glutathione disulfide + soluble glutathione mixed disulfide) was calculated by subtracting the amount of reduced from the total amount of glutathione.

Determination of glutamate and glutamine in serum. Glutamate (Glu) and glutamine (Gln) were determined in deproteinized serum by an assay based on derivatization with *o*-phthaldialdehyde and fluorescence detection.[26]

Interleukin-2 (IL-2) production and lymphocyte proliferation assays. PBMCs were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in triplicates with or without stimulants in flat-bottomed 96-well microtiter trays (Costar, Cambridge, UK) at a concentration of 10^6 cells/mL in RPMI 1640 with 2 mmol/L L-glutamine and 25 mmol/L HEPES buffer (GIBCO) supplemented with gentamicin (40 μg/mL) and 10% heat-inactivated pooled human AB^+ serum. Stimulants were phytohaemagglutinin (PHA; Murex, Dartford, UK; final concentration, 1:500), staphylococcal enterotoxin B (SEB; Sigma; final concentration, 100 ng/mL) and influenza virus A/Singapore/6/86 (INF; a kind gift from The National Institute of Public Health, Oslo, Norway; final concentration, 1:1000). In cultures for IL-2 production, IL-2 consumption was blocked by addition of a mouse-antihuman IL-2–receptor antibody (final concentration of 5 μg/mL, anti-CD25 clone 3G10; Boehringer Mannheim, Biochemica, Mannheim, Germany).[27] Supernatants were procured after 7 days of culture and stored at −70°C.

Lymphocyte proliferation was assessed by pulsing the cells with

1 μCi of [^{3}H]thymidine 48 hours after seeding. Cultures were harvested 16 hours later onto glass filter strips, using an automated multisample harvester (Skatron, Lier, Norway). [^{3}H]thymidine incorporation was determined by liquid scintillation counting.

Cytokine enzyme immunoassays (EIAs). IL-2 and TNFα concentrations were quantified by EIAs provided by Cayman Chemical (Ann Arbor, MI) (IL-2) and Medgenix (Fleurus, Belgium) (TNFα) according to the manufacturer's guidelines using microtiter wells coated with one (Cayman) or several (Medgenix) MoAbs against distinct epitopes of the actual cytokine.

Determination of lymphocyte subsets and monocytes in peripheral blood. The numbers of $CD4^+$, $CD8^+$, $CD19^+$ lymphocytes and $CD14^+$ monocytes were determined by immunomagnetic quantification, which has been shown to agree well with flow cytometry.[24]

Statistical analysis. For comparison of two groups of individuals, the Mann-Whitney U test was used. When more than two groups were compared, the Kruskal-Wallis test was used as previously described.[28] Coefficients of correlation (r) were calculated by the Spearman rank test. The calculations were performed using the Statistica (StatSoft, Tusla, OK) and SOLO (BMDP Statistical Software, Los Angeles, CA) software packages. Data are given as medians and 25th to 75th percentiles if not otherwise stated. P values are two sided and considered significant when less than .05.

RESULTS

Intracellular glutathione levels in $CD4^+$ lymphocytes. Several differences were shown when intracellular glutathione levels in $CD4^+$ lymphocytes in healthy controls and in different clinical stages of HIV-1 infection were compared (Fig 1). First, there was a considerable increase in intracellular levels of oxidized glutathione among HIV-1 seropositive patients with the highest levels in patients with symptomatic HIV-1 infection (CDC group B and C had an ≈700% increase compared with controls). Second, the marked increase in oxidized glutathione levels accounted for an increase in total glutathione in $CD4^+$ lymphocytes from HIV-1 seropositive patients, and a significant decrease in the ratio of reduced to total glutathione, particularly among patients with symptomatic HIV-1 infection. All but two of eight patients in CDC group B and C had a ratio of reduced to total glutathione below 0.55 compared with none among controls. Third, patients with symptomatic HIV-1 infection had a decrease in intracellular levels of reduced glutathione (≈30% decrease compared with controls), although the difference did not reach statistical significance.

Intracellular glutathione levels in other lymphocyte subsets and monocytes. In contrast with $CD4^+$ lymphocytes, no significant differences were found in intracellular levels of either oxidized or total glutathione in $CD8^+$ and $CD19^+$ lymphocytes from HIV-1–infected patients and controls (Table 2). However, HIV-1 seropositive patients exhibited a slight, but statistically significant increase in the ratio of reduced to total glutathione in these lymphocyte subsets reflecting increased levels of reduced glutathione among HIV-1–infected patients (Table 2). There were no significant differences in these glutathione parameters between asymptomatic and symptomatic HIV-1 seropositive patients (data not shown).

Monocytes from patients with symptomatic HIV-1 infection had significantly increased levels of both reduced and total glutathione as compared with both healthy controls and asymptomatic HIV-1 seropositive patients (Fig 2). Furthermore, in HIV-1–infected patients there was a significant inverse correlation between reduced glutathione levels in $CD4^+$ lymphocytes and both total and reduced glutathione levels in monocytes ($r = -.72$, $P < .001$; $r = -.68$, $P < .001$; respectively).

Plasma levels of glutathione and cysteine. As illustrated in Table 3, plasma levels of total as well as oxidized glutathione were decreased in HIV-1 seropositive patients compared with healthy controls (≈20% and 30% decrease, respectively), although only the difference in oxidized glutathione levels reached statistical significance.

Except for a slight but statistically significant decrease (≈10%) in oxidized cysteine levels, we could not show any significant decrease in cysteine species in plasma among HIV-1 seropositive patients compared with healthy controls (Table 3). Furthermore, in these patients, no significant correlations were found between any of the circulating cysteine parameters and the intracellular glutathione species (data not shown).

There were no significant differences in plasma levels of the glutathione and cysteine species between asymptomatic and symptomatic HIV-1–infected patients (data not shown).

Serum levels of glutamate. Hyperglutamataemia has been suggested to be related to the glutathione redox disturbances during HIV-1 infection,[29] and, therefore, we measured serum levels of Glu in all patients and controls. Because interconversion of Gln to Glu may occur ex vivo, the data for serum levels of Gln and Glu + Gln are also given.[30] There were no significant differences in these parameters either between HIV-1 seropositive patients and healthy controls (Table 3) or between asymptomatic and symptomatic HIV-1–infected patients (data not shown).

Glutathione parameters in HIV-1–infected patients in relation to lymphocyte subsets and monocyte counts in peripheral blood. In the present study, a low number of $CD4^+$ lymphocytes in peripheral blood was significantly correlated with an increased level of oxidized glutathione in this lymphocyte subpopulation, and as can be seen in Fig 3, this correlation was particularly striking among symptomatic HIV-1 seropositive patients (Fig 3). Also, a decrease in the ratio of reduced to total glutathione ($r = .73$, $P < .001$) and an increase in total glutathione levels ($r = -.60$, $P < .01$) in $CD4^+$ lymphocytes were significantly correlated with low numbers of $CD4^+$ lymphocytes. In contrast, depletion of reduced glutathione levels in $CD4^+$ lymphocytes found among symptomatic HIV-1–infected patients (Fig 1) was not significantly correlated with decreased $CD4^+$ lymphocyte counts (data not shown). In fact, in monocytes, elevated levels of both reduced (Fig 3) and total glutathione ($r = -.49$, $P < .05$) was correlated with low numbers of $CD4^+$ lymphocytes (Fig 3). No significant correlation was found between oxidized glutathione levels in monocytes and $CD4^+$ lymphocyte counts (data not shown).

Plasma levels of total glutathione, but not plasma levels of oxidized glutathione nor any of the measured cysteine species (data not shown), were significantly correlated with numbers of $CD4^+$ lymphocytes ($r = .58$, $P < .01$). Although neither the HIV-1 seropositive group as a whole, nor any

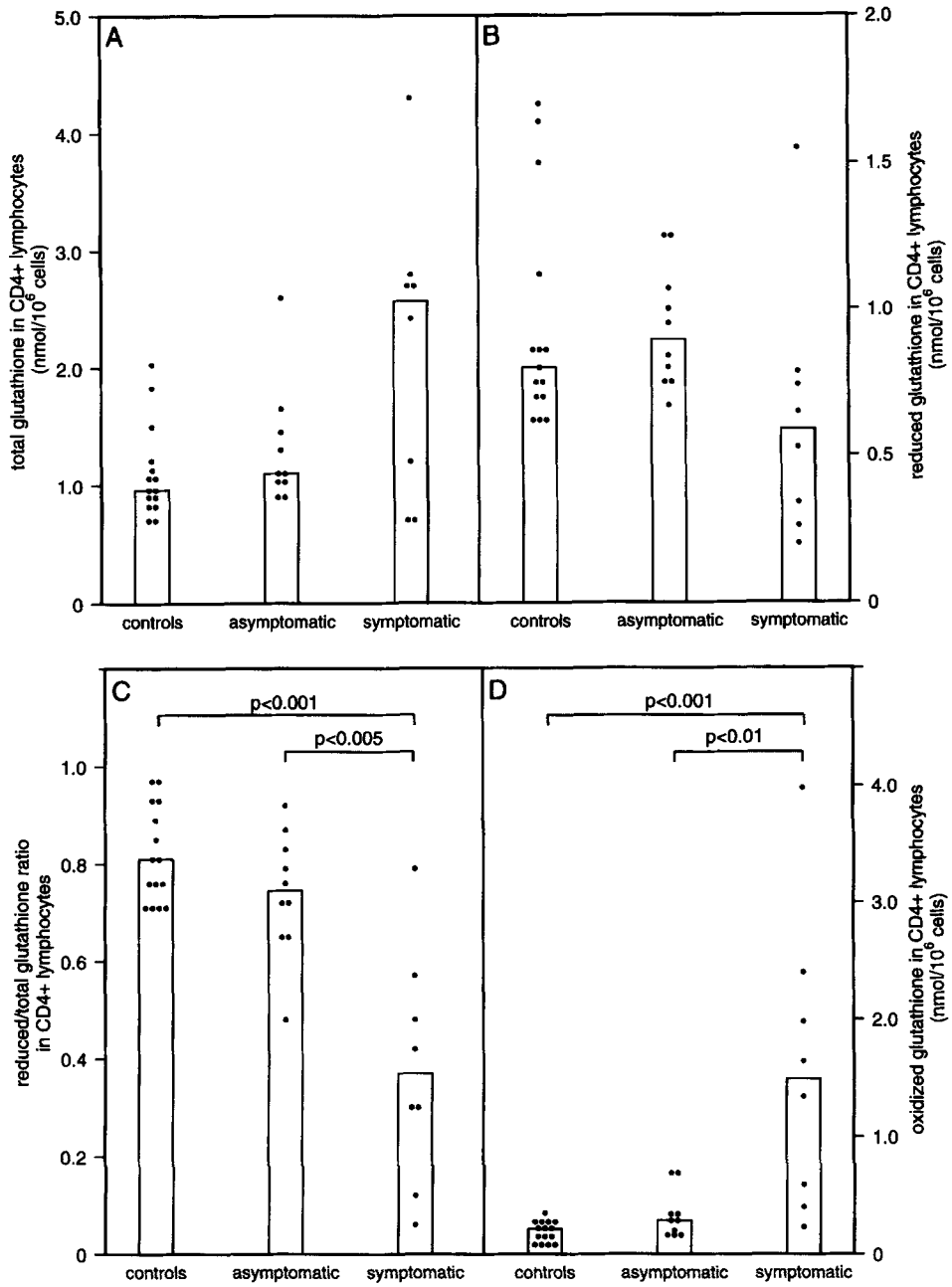

Fig 1. Intracellular glutathione levels in isolated CD4⁺ lymphocytes in 10 patients with asymptomatic HIV-1 infection (CDC group A), 8 with symptomatic HIV-1 infection (CDC groups B and C) and 15 healthy controls. (A) Total glutathione levels (P = .08, Kruskal-Wallis test). (B) Reduced glutathione levels (P = .06, Kruskal-Wallis test). (C) Ratio of reduced to total glutathione. (D) Oxidized glutathione levels.

of the two clinical subgroups of patients had significantly increased serum levels of glutamate compared with controls, there was a significant inverse correlation between glutamate levels and CD4⁺ lymphocyte counts in the patient group (r = −.44, P < .05).

Thus, HIV-1 seropositive patients with the most pronounced immunodeficiency were primarily characterized by a marked increase in oxidized glutathione levels in CD4⁺ lymphocytes, severely decreased ratio of reduced to total glutathione in these cells, and in addition, elevated levels of reduced and total glutathione in monocytes and decreased total glutathione levels in plasma.

Also for CD8⁺ lymphocytes, but not for CD19⁺ lymphocytes and monocytes, we found a significant inverse correlation between intracellular oxidized glutathione levels and circulating numbers of the same cellular subset in peripheral blood (R = −.57, P < .01).

Cellular glutathione levels in HIV-1–infected patients in relation to serum levels of TNFα. HIV-1 infection is associated with a chronic immune activation,[28] which, in the present study, was reflected by raised serum levels of TNFα among HIV-1 seropositive patients (Table 1). The raised TNFα levels were significantly correlated with elevated levels of oxidized glutathione in CD4⁺ lymphocytes, and as can be seen in Fig 4, this correlation was particularly striking in the asymptomatic HIV-1 seropositive patients. Raised serum

Table 2. Intracellular Glutathione Parameters in $CD8^+$ and $CD19^+$ Lymphocytes Among HIV-1–Infected Patients and Controls

	HIV-1–Infected Patients (n = 20)	Controls (n = 15)
$CD8^+$ lymphocytes		
Reduced glutathione (nmol/10^6 cells)	0.91 (0.71-1.07)	0.76 (0.61-0.94)
Total glutathione (nmol/10^6 cells)	1.06 (0.92-1.45)	0.97 (0.85-1.31)
Oxidized glutathione (nmol/10^6 cells)	0.15 (0.08-0.32)	0.21 (0.19-0.32)
Ratio of reduced to total glutathione	0.87* (0.76-0.92)	0.76 (0.70-0.79)
$CD19^+$ lymphocytes		
Reduced glutathione (nmol/10^6 cells)	1.05* (0.75-1.38)	0.76 (0.58-0.92)
Total glutathione (nmol/10^6 cells)	1.13 (0.77-1.45)	0.87 (0.71-0.97)
Oxidized glutathione (nmol/10^6 cells)	0.08 (0.00-0.12)	0.11 (0.06-0.19)
Ratio of reduced to total glutathione	0.98* (0.91-1.00)	0.90 (0.80-0.94)

Data are given as medians and 25th-75th percentiles.
* $P < .02$ compared with controls.

Table 3. Plasma Levels of Glutathione and Cysteine and Serum Levels of Glutamate and Glutamine in HIV-1–Infected Patients and Controls

	HIV-1–Infected Patients (n = 22)	Controls (n = 15)
Glutathione (μmol/L)		
Total	4.69 (3.7-6.3)	6.0 (4.6-7.8)
Oxidized	2.1* (1.8-2.8)	3.1 (2.5-3.3)
Cysteine (μmol/l)		
Total	292.2 (266.5-319.3)	318.1 (289.2-337.2)
Oxidized	113.1† (96.9-128.8)	124.9 (115.6-149.5)
Reduced	11.0 (8.3-13.3)	10.9 (8.3-11.6)
Protein-bound	168.1 (142.2-208.4)	182.3 (138.0-197.8)
Glutamate (μmol/L)	55.7 (38.0-84.5)	67.7 (46.6-86.7)
Glutamine (μmol/L)	453.0 (425.7-533.2)	479.0 (445.7-542.7)
Glutamate + glutamine	517.7 (474.9-581.0)	544.0 (532.9-609.1)

The protein-bound fraction of cysteine was calculated by subtracting the quantities of the reduced and oxidized cysteine form from the total amount. Data are given as medians and 25th-75th percentiles.
* $P < .001$ compared with controls.
† $P < .05$ compared with controls.

levels of TNFα were also significantly correlated with decreased ratio of reduced to total glutathione in $CD4^+$ lymphocytes ($r = -.60$, $P < .01$). Moreover, in patients with symptomatic HIV-1 infection, elevated TNFα levels were correlated with decreased levels of reduced glutathione in $CD4^+$ lymphocytes, although this correlation did not reach statistical significance ($r = -.69$, $P = .05$).

Cellular glutathione levels among HIV-1–infected patients in relation to stimulated IL-2 production and lymphocyte proliferation. To elucidate the relationships between cellular redox status and IL-2 production and proliferative response in PBMC, cells from the same samples as used in glutathione analyses were activated by PHA, SEB, and INF in 16 patients and 11 controls to simultaneously determine IL-2 production in cell supernatants and DNA incorporation of [^{3}H]thymidine. Although the differences obtained with PHA did not reach statistical significance, stimulated IL-2 production and lymphocyte proliferation were decreased in HIV-1 seropositive patients compared with healthy controls with all stimuli (data not shown). Both INF- and SEB-stimulated, but not PHA-stimulated IL-2 production, were significantly inversely correlated with oxidized glutathione levels

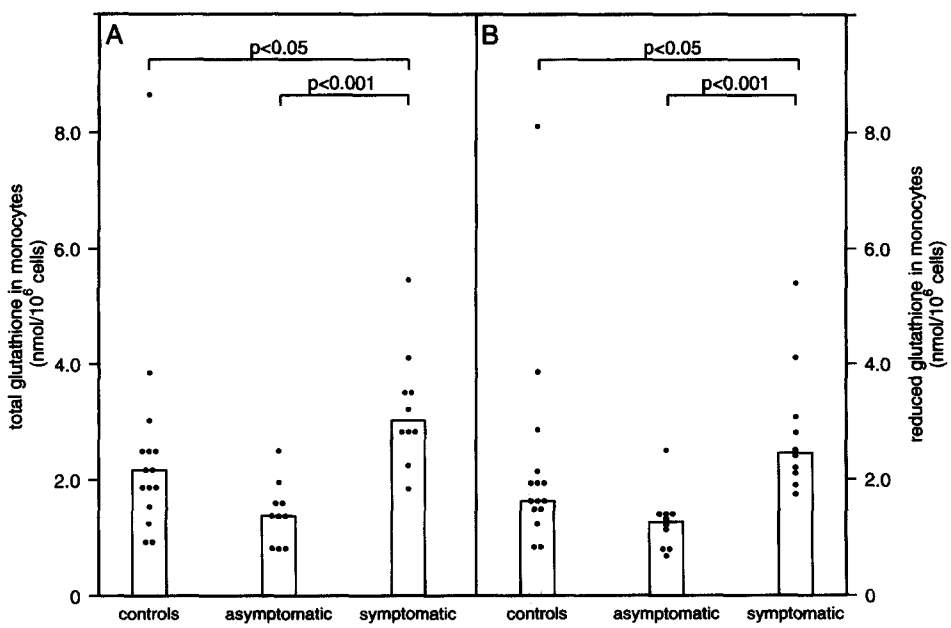

Fig 2. Intracellular glutathione levels in isolated monocytes in 10 patients with asymptomatic HIV-1 infection (CDC group A), 10 with symptomatic HIV-1 infection (CDC group B and C) and 15 healthy controls. (A) Total glutathione levels. (B) Reduced glutathione levels.

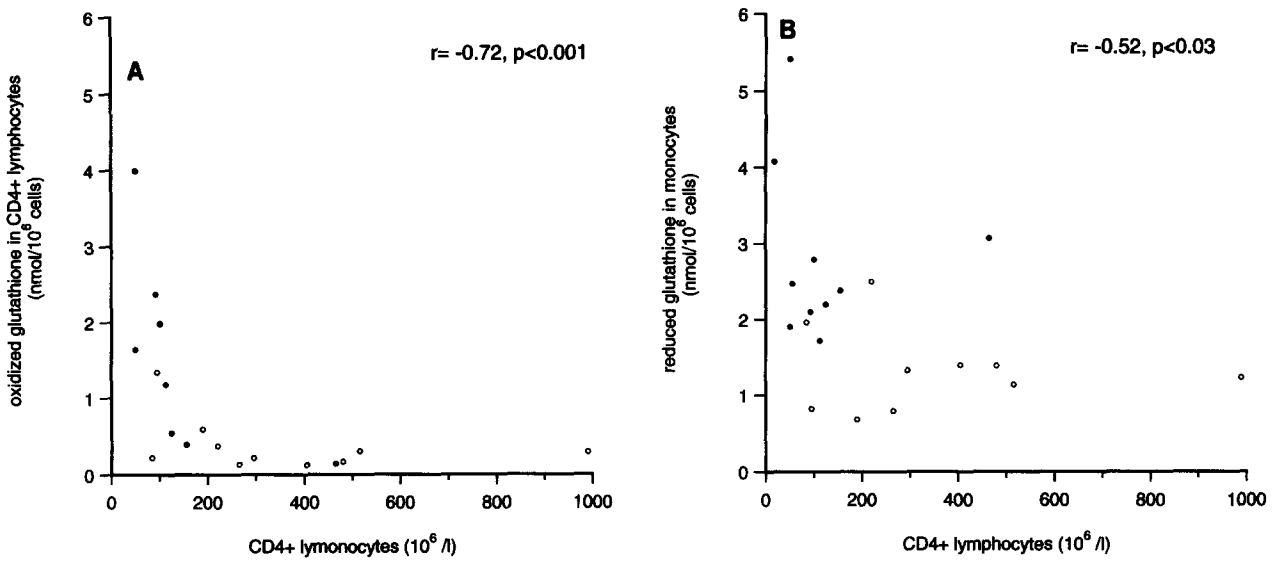

Fig 3. Correlations in HIV-1 seropositive patients (asymptomatic are represented with open symbols and symptomatic patients with closed symbols) between numbers of CD4⁺ lymphocytes in peripheral blood and (A) intracellular levels of oxidized glutathione in CD4⁺ lymphocytes (n = 18), (B) intracellular levels of reduced glutathione in monocytes (n = 20). Oxidized glutathione levels in CD4⁺ lymphocytes were significantly inversely correlated with numbers of CD4⁺ lymphocytes in symptomatic ($r = -.90$, $P < .001$), but not asymptomatic patients ($r = -.30$, $P > .05$). The inverse correlation between reduced glutathione levels in monocytes and CD4⁺ lymphocyte counts did not reach statistical significance within these two separate subgroups of patients (data not shown).

in CD4⁺ lymphocytes ($r = -.60$, $P < .03$ and $r = -.70$, $P < .005$; SEB and INF, respectively) and significantly positively correlated with ratio of reduced to total glutathione in these cells ($r = .50$, $P < .05$ and $r = .65$, $P < .01$; SEB and INF, respectively). Furthermore, we found that HIV-1–infected patients with the most impaired lymphocyte proliferation response had significantly higher oxidized glutathione levels and significantly lower ratio of reduced to total glutathione in CD4⁺ lymphocytes than other HIV-1 seropositive patients (Fig 5).

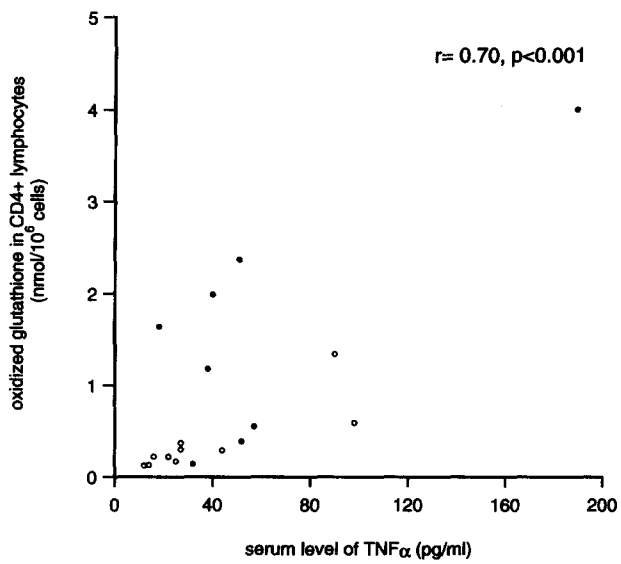

Fig 4. Correlation in 18 HIV-1 seropositive patients (asymptomatic are represented with open symbols and symptomatic patients with closed symbols) between intracellular levels of oxidized glutathione in CD4⁺ lymphocytes and serum levels of TNFα. Correlations within the two clinical subgroups of patients were $r = .92$, $P < .001$ and $r = .40$, $P > .05$ for asymptomatic and symptomatic patients, respectively.

DISCUSSION

Decreased levels of reduced glutathione in PBMC, CD4⁺, and CD8⁺ lymphocytes have previously been reported during HIV-1 infection and has been interpreted as reflecting a disturbed intracellular redox status.[11,12] In a recent review article it was hypothesized without basis in published data, that the decrease in reduced glutathione in HIV-1–infected patients is accompanied by a decrease in intracellular levels of oxidized glutathione.[31] However, the present study represents the first report determining glutathione redox balance in lymphocyte subpopulations and monocytes in HIV-1–infected patients by measuring intracellular levels of both reduced and total glutathione. We show that CD4⁺ lymphocytes from these patients appear to be characterized by a substantial increase in oxidized glutathione and a considerable decrease in the ratio of reduced to total glutathione rather than decreased levels of reduced glutathione. This increase in oxidized glutathione was strongly correlated with low numbers of CD4⁺ lymphocytes in peripheral blood and a number of other important immunologic features of HIV-1 infection suggesting a possible important immunopathogenic role for these redox disturbances.

The present study indicates that important issues may be overlooked when intracellular glutathione levels are analyzed in mixed cell populations (eg, PBMCs). Although symptomatic HIV-1–infected patients tended to have de-

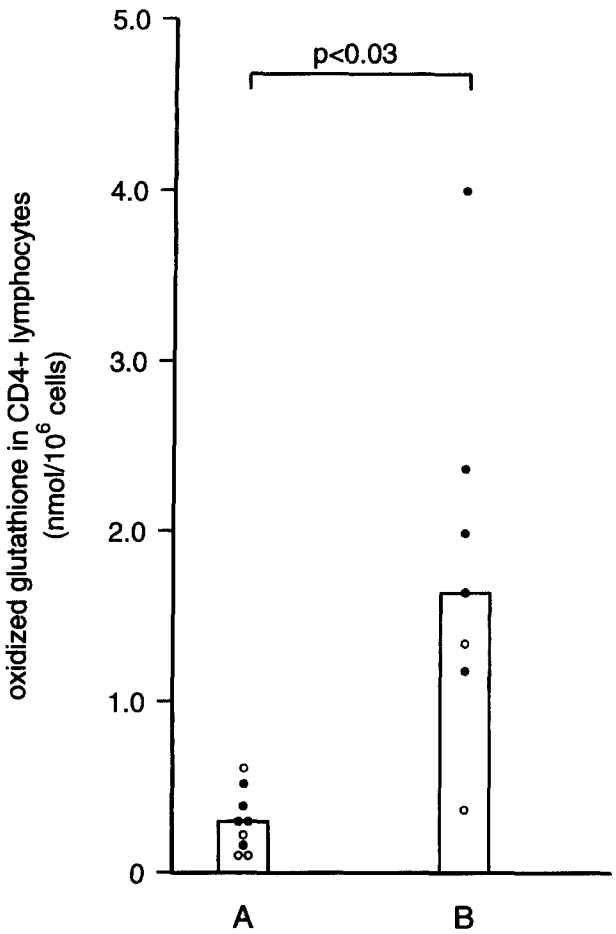

Fig 5. Intracellular levels of oxidized glutathione in CD4⁺ lymphocytes in two defined groups of HIV-1–infected patients. Group A (n = 9): Impaired lymphocyte proliferation response (<50% decrease compared with controls) only when stimulated with INF. Group B (n = 7): Impaired lymphocyte proliferation response when stimulated with INF and at least one of the other two stimuli used (SEB and PHA). Asymptomatic are represented with open and symptomatic patients with closed symbols. Patients in group B also had significantly decreased ratio of reduced to total glutathione in CD4⁺ lymphocytes compared with patients in group A (0.32 [0.28 – 0.48] compared with 0.74 [0.61 – 0.79], $P < .03$).

creased intracellular levels of reduced glutathione in $CD4^+$ lymphocytes, there was an increase in this glutathione species in monocytes from these patients. Such an increase have previously been reported in some HIV-1–infected individuals.[11] We found that HIV-1 seropositive patients with the most pronounced immunodeficiency and most advanced clinical disease had increased levels of oxidized glutathione in $CD4^+$ lymphocytes, but they also exhibited the highest levels of total and reduced glutathione in monocytes suggesting an immunopathogenic role for the increased glutathione levels in these cells. Furthermore, these findings suggest that glutathione redox cycle may be differentially regulated in $CD4^+$ lymphocytes and monocytes.

Staal et al[11,12] have previously reported significantly decreased levels of reduced glutathione in both $CD4^+$ and $CD8^+$ lymphocytes from asymptomatic as well as symptomatic HIV-1–infected patients. This may seem to be in some conflict with our results. These discrepancies may be related to methodological differences; Staal et al measured cellular levels of reduced glutathione using monochlorobimane labeling and flow cytometry, and technical flaws when using this method for quantitive determination of reduced glutathione in PBMC have recently been described.[13]

The most striking finding in the present study was the markedly increased levels of oxidized glutathione in $CD4^+$ lymphocytes in patients with HIV-1 infection. In mammalian cells, only a small fraction of total glutathione exists in the oxidized form.[3] The marked increase in oxidized glutathione levels in $CD4^+$ lymphocytes during HIV-1 infection indicates increased oxidative stress in these cells. Although intracellular depletion of reduced glutathione levels is the predominant response to acute increase in ROS generation, chronic inflammatory stress including enhanced ROS production may result in elevation of total glutathione and a persistent and considerable elevation of oxidized glutathione levels as the major characteristics. In both cases there is a decrease in the ratio of reduced to total glutathione.[17,32,33] Thus, increased intracellular levels of oxidized glutathione or a decrease in the ratio of reduced to total glutathione seem to be better indicators of chronically enhanced oxidative stress than depletion of reduced intracellular glutathione.[17,32] In the present study the majority of patients with symptomatic HIV-1 infection had a ratio of reduced to total glutathione in $CD4^+$ lymphocytes below 0.5, and this has to our knowledge not been reported in lymphocyte subpopulations in any human disease.

The considerable intracellular accumulation of oxidized glutathione in $CD4^+$ lymphocytes during HIV-1 infection most probably reflects increased generation of this glutathione species. The significant positive correlation shown between serum levels of TNFα and concentrations of oxidized glutathione in $CD4^+$ lymphocytes suggests that increased inflammatory stress, which, in turn, may result in increased ROS generation,[2] is a major pathogenic factor in the induction of disturbed glutathione redox cycle in $CD4^+$ lymphocytes during HIV-1 infection. Indeed, TNFα stimulation both in vitro and in vivo has been shown to increase oxidized glutathione levels.[34,35]

In addition to increased generation, impaired reduction of oxidized glutathione or impairment of the plasma membrane Ca^{2+}-dependent adenosine triphosphatase, which actively secretes oxidized glutathione from cells,[36] may also be involved in the mechanisms causing accumulation of oxidized glutathione. A recent study in a chronically HIV-1–infected T-cell line,[37] could not show any impairment in the glutathione reductase activity, which is responsible for the regeneration of reduced glutathione.[3] However, the glutathione reductase reaction is dependent on reduced nicotinamide adenine dinucleotide phosphate (NADPH), and TNFα or other proinflammatory mediators that enhance ROS formation, may consume NADPH and thereby indirectly interfere with glutathione reductase function.[2] In addition, glutathione transhydrogenases participating in regeneration of reduced from oxidized glutathione,[38,39] may not function properly, as suggested by the observed downregulation of thioredoxin levels during HIV-1 infection.[40]

Cysteine availability is rate limiting for glutathione synthesis, and lymphocytes depend on extracellular cysteine concentrations for glutathione synthesis.[3,15] It has been suggested that decreased plasma levels of cysteine may play an important role in the immunopathogenesis of HIV infection.[10,15] However, we could not confirm published data indicating a marked decrease in circulating levels of reduced and oxidized cysteine levels during HIV-1 infection.[10,14,29,41,42] Furthermore, in contrast with a previous study,[29] we could not find any significant correlation between plasma levels of oxidized cysteine and $CD4^+$ lymphocyte counts in peripheral blood or any of the other investigated immunologic parameters among HIV-1–infected patients. These discrepancies may well be related to different procedures for blood collection and processing. A period of 2.5 minutes is sufficient for oxidation of a substantial fraction of glutathione,[19] and cysteine may be oxidized even more rapidly.[43] In the two studies showing decreased plasma levels of reduced cysteine during HIV infection, the time between blood collection and thiol derivatization or addition of acid to blood samples was greater than 2 minutes[14] and greater than 90 min,[10] respectively. In the present study, plasma cysteine was immediately derivatized during blood collection and essentially no oxidation could take place.[20] Furthermore, we found that protein-bound cysteine is the predominant form of this thiol in plasma with only ≈40% being in the free form. Thus, the results from previous studies only reporting plasma levels of free cysteine species,[10,14,41,42] will not reflect the total cysteine status in plasma. In addition, by forming mixed disulfides with proteins,[20] this free cysteine fraction will further decrease ex vivo if the thiol compounds are not derivatized during blood collection.[19] Finally, it is quite conceivable that the degree and rate of thiol oxidation ex vivo and total free-to-protein-bound-thiol ratio may be altered by disease activity during HIV-1 infection, further complicating the interpretation of data in previous studies comparing cysteine levels in HIV-infected patients with levels in healthy controls.

In accordance with a recent study reporting normal Glu + Gln levels in HIV-1–infected patients,[41] we could not confirm previous reports on markedly elevated serum Glu levels during HIV-1 infection, although in these studies there was no information on Gln or Gln + Glu levels.[29,42] Nevertheless, we found a significant inverse correlation between serum Glu levels and $CD4^+$ lymphocyte counts among HIV-1–infected patients, and a possible role for hyperglutamataemia during HIV-1 infection cannot be excluded.

Increased levels of oxidized glutathione may result in immunologic dysfunction either as a direct effect of increased oxidized glutathione levels[31,44] or indirectly through redox disturbances.[45] Altered intracellular redox status may impair functions of several important intracellular enzymes[39] and cofactors,[45] influence other intracellular redox systems,[39] and alter protein folding and microtubule assembly.[46] With respect to HIV-1 infection, several of these disturbances may be of particular importance. Apoptosis has been suggested to play an important immunopathogenic role in HIV-1 infection,[2,47] and notably, the intracellular increase in oxidized glutathione may activate redox sensitive transcriptional factors that may mediate such an activation-induced cell death.[48] The TNFα activation shown in these patients may further enhance the apoptotic process.[2] Although initially reported only in $CD4^+$ lymphocytes, abnormal apoptosis during HIV-1 infection seems also to involve $CD8^+$ lymphocytes.[47] It may be relevant that we found the circulating number of $CD8^+$ lymphocytes, but not of monocytes and $CD19^+$ lymphocytes, to be inversely correlated to the intracellular content of oxidized glutathione of the corresponding cellular subset. Furthermore, while physiologic concentrations of H_2O_2 may activate transcriptional factors in lymphocytes inducing enhanced IL-2 production and proliferation,[49] unphysiologically increased intracellular ROS levels and high levels of oxidized glutathione, may impair these functions both by inhibiting NFκB[31] and NFAT[50] DNA binding and by inhibiting intracellular processes upstream of activation of transcriptional factors, eg, regulation of protein-tyrosine kinase/protein-tyrosine phosphatase activity.[31,50,51] Finally, whereas increased oxidative stress may impair IL-2 production and lymphocyte proliferation, oxidant induced glutathione redox disturbances seem to strongly increase the cellular response to TNFα,[51] possibly leading to enhanced HIV-1 replication in HIV-1–infected patients.[1,8]

In conclusion, our study shows a considerable increase in oxidized glutathione in $CD4^+$ lymphocytes and a markedly disturbed intracellular redox balance in these cells, as the most significant disturbance in glutathione homeostasis during HIV-1 infection. However, we could not confirm previous reports of cysteine deficiency in HIV-1–infected patients. The markedly impaired glutathione redox status in $CD4^+$ lymphocytes was correlated with several parameters reflecting key immunologic dysfunctions. Therefore, we believe that restoration of glutathione redox balance in $CD4^+$ lymphocytes may be a therapeutic goal in HIV-1–infected patients using glutathione replenishing agents such as *N*-acetylcysteine. The results of the present study also suggest that therapeutic modalities that decrease the inflammatory stress, particularly the activation of the TNF system, could be important in modulation of intracellular glutathione redox balance during HIV-1 infection.

ACKNOWLEDGMENT

We thank Audun Høylandskjaer and Bjørn Netteland for excellent technical assistance.

REFERENCES

1. Roederer M, Ela SW, Staal FTJ, Herzenberg LA, Herzenberg LA: N-acetylcysteine: A new approach to anti-HIV therapy. AIDS Res Hum Retroviruses 8:209, 1992

2. Buttke TM, Sandstrom PA: Oxidative stress as a mediator of apoptosis. Immunol Today 15:7, 1994

3. Meister A, Anderson ME: Glutathione. Ann Rev Biochem 52:711, 1983

4. Meister A: Glutathione-ascorbic acid antioxidant system in animals. J Biol Chem 269:9397, 1994

5. Hamilos DL, Wedner HJ: The role of glutathione in lymphocyte activation. I. Comparison of inhibitory effects of buthionine sulfoximine and 2-cyclohexene-1-one by nuclear size transformation. J Immunol 135:2740, 1985

6. Yamauchi A, Bloom ET: Requirement of thiol compounds as reducing agents for IL-2 mediated induction of LAK activity and proliferation of human NK cells. J Immunol 151:5535, 1993

7. Dröge W, Pottmeyer-Greber C, Schmidt H, Nick S: Glutathione augments the activation of cytotoxic T lymphocyte in vivo. Immunobiology 171:151, 1986

8. Staal FTJ, Roederer M, Herzenberg LA, Herzenberg LA: Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. Proc Natl Acad Sci USA 87:9943, 1990

9. Buhl R, Jaffe HA, Holroyd KJ, Wells FB, Mastrangeli A, Saltini C, Cantin AM, Crystal RG: Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. Lancet 2:1294, 1989

10. Eck H-P, Gmünder H, Hartmann M, Petzoldt D, Daniel V, Dröge W: Low concentrations of acid-soluble thiol (cysteine) in the plasma of HIV-1 infected patients. Biol Chem Hoppe Seyler 370:101, 1989

11. Staal FJT, Roederer M, Israelski DM, Bubp J, Mole LA, McShane D, Deresinski SC, Ross W, Sussman H, Raju PA, Anderson MT, Moore W, Ela SW, Herzenberg LA, Herzenberg LA: Intracellular glutathione levels in T cell subsets decrease in HIV-infected individuals. AIDS Res Hum Retroviruses 8:305, 1992

12. Roederer M, Staal FJT, Osada H, Herzenberg LA, Herzenberg LA: CD4 and CD8 T cells with high intracellular glutathione levels are selectively lost as HIV infection progresses. Int Immunol 3:933, 1991

13. van der Ven AJAM, Mier P, Peters WHM, Dolstra H, van Erp PEJ, Koopmans PP, van der Meer JWM: Monochlorobimane does not selectively label glutathione in peripheral blood mononuclear cells. Anal Biochem 217:41, 1994

14. de Quay B, Malinverni R, Lauterburg BH: Glutathione depletion in HIV-infected patients: Role of cysteine deficiency and effect of oral N-acetylcysteine. AIDS 6:815, 1992

15. Dröge W, Eck H-P, Mihm S: HIV-induced cysteine deficiency and T-cell dysfunction—A rationale for treatment with N-acetylcysteine. Immunol Today 13:211, 1992

16. Jacobsen DW, Green R, Herbert V, Longworth DL, Rehm S: Decreased serum glutathione with normal cysteine and homocysteine levels in patients with AIDS. Clin Res 38:556, 1990 (abstr)

17. Jenkinson SG, Lawrence RA, Zamora CA, Deneke SM: Reduction of intracellular glutathione in alveolar type II pneumocytes following BCNU exposure. Am J Physiol 266:L125, 1994

18. Halliwell B, Cross CE: Reactive oxygen species, antioxidants, and acquired immunodeficiency syndrome, sense or speculation? Arch Intern Med 151:29, 1991

19. Svardal AM, Mansoor MA, Ueland PM: Determination of reduced, oxidized, and protein-bound glutathione in human plasma with precolumn derivatization with monobromobimane and liquid chromatography. Anal Biochem 184:338, 1990

20. Mansoor MA, Svardal AM, Ueland PM: Determination of in vivo redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in plasma. Anal Biochem 200:218, 1992

21. Center for Disease Control and Prevention: 1993 revised classification for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR 41:1, 1992

22. Funderud S, Nustad K, Lea T, Vartdal F, Gaudernack G, Stenstad P, Ugelstad J: Fractionation of lymphocytes by immunomagnetic beads, in Klaus GGB (eds): Lymphocytes: A Practical Approach. Oxford, UK, Oxford IRL, 1987, p 55

23. Funderud S, Erikstein B, Åsheim HC, Nustad K, Stokke T, Blomhoff HK, Holte H, Smeland EB: Functional properties of $CD19^+$ B lymphocytes positively selected from buffy coats by immunomagnetic separation. Eur J Immunol 20:201, 1990

24. Brinchmann JE, Vartdal F, Gaudernack G, Markussen G, Funderud S, Ugelstad J, Thorsby E: Direct immunomagnetic quantification of lymphocyte subsets in blood. Clin Exp Immunol 71:182, 1988

25. Mason DY: Immunohistochemical labeling of monoclonal antibodies by APAAP immunoalkaline phosphatase technique, in Bullock GR, Petruszvol P (eds): Immunohistochemistry, vol 3. London, UK, Academic, p 25

26. Krishnamurti CR, Heindze AM, Galzy G: Application of reversed-phase high-performance liquid chromatography using precolumn derivatization with o-phthaldialdehyde for the quantitative analyses of amino acids in adults and fetal sheep plasma. J Chromatogr 315:321, 1984

27. Uchiyama T, Broder S, Waldmann TA: A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac(+) cells. J Immunol 126:1393, 1981

28. Aukrust P, Liabakk N-B, Müller F, Lien E, Espevik T, Frøland SS: Serum levels of tumor necrosis factor α (TNFα) and soluble TNF receptors in HIV-1 infection—Correlations to clinical immunologic and virologic parameters. J Infect Dis 169:420, 1994

29. Eck H-P, Mertens T, Rosokat H, Fätkenheuer G, Pohl C, Schrappe M, Daniel V, Näher H, Petzoldt D, Drings P, Dröge W: $T4^+$ cell numbers are correlated with plasma glutamate and cystine levels: Association of hyperglutamataemia with immunodeficiency in diseases with different aetiologies. Int Immunol 4:7, 1992

30. Parvy P, Bardet J, Rabier D, Gasquet M, Kamoon P. Intra- and interlaboratory quality control for assay of amino acids in biological fluids: 14 years of French experience. Clin Chem 39:1831, 1993

31. Dröge W, Schulze-Osthoff K, Mihm S, Galter D, Schenk H, Eck H-P, Roth S, Gmünder H. Functions of glutathione and glutathione disulfide in immunology and immunopathology. FASEB J 8:1131, 1994

32. Deneke SM, Fanburg BL: Regulation of cellular glutathione. Am J Physiol 257:L163, 1989

33. Benard O, Balasubramanian KA: Effect of oxidant exposure on thiol status in the intestinal mucosa. Biochem Pharmacol 45:201, 1993

34. Ishii Y, Partidge CA, Vecchio PJD, Malik AB: Tumor necrosis factor-α–mediated decrease in glutathione increases the sensitivity of pulmonary vascular endothelial cells to H_2O_2. J Clin Invest 89:794, 1992

35. Chang SW, Ohara N, Kuo G, Voelkel NF: Tumor necrosis factor-induced lung injury is not mediated by platelet-activating factor. Am J Physiol 257:L232, 1989

36. Nicotera P, Moore M, Bellamo G, Mirabelli F, Orrenius S: Demonstration and partial characterization of glutathione disulphide stimulated ATP-ase activity in plasma membrane fraction from rat hepatocytes. J Biol Chem 260:1999, 1985

37. Sandstrom PA, Tebby PW, Cleave SV, Buttke TM: Lipid hydroperoxides induce apoptosis in T cells displaying a HIV-associated glutathione peroxidase deficiency. J Biol Chem 269:798, 1994

38. Meister A: Glutathione metabolism and its selective modification. J Biol Chem 263:17205, 1989

39. Holmgren A: Thioredoxin and glutaredoxin systems. J Biol Chem 264:13963, 1989

40. Masutani H, Naito M, Takahashi K, Hattori T, Koito A, Takatsuki K, Go T, Nakamura H, Fujii S, Yoshida Y, Okuma M, Yodoi J: Dysregulation of adult T-cell leukemia-derived factor (ADF)/thioredoxin in HIV-1 infection: Loss of ADF high-producer cells in lymphoid tissues of AIDS patients. AIDS Res Hum Retroviruses 8:1707, 1992

41. Hortin GL, Landt M, Powserly WG: Changes in plasma amino acid concentrations in response to HIV-1 infection. Clin Chem 40:785, 1994

42. Dröge W, Eck H-P, Näher H, Pekar U, Daniel V: Abnormal amino-acid concentrations in the blood of patients with acquired immunodeficiency syndrome (AIDS) may contribute to the immunological defect. Biol Chem Hoppe Seyler 369:143, 1988

43. Munday R: Toxicity of thiols and disulphides: Involvement of free radical species. Free Rad Biol Med 7:659, 1989

44. Hilly M, Pietri-Rouxel F, Coquil J-F, Mauger J-P: Thiol reagents increase the affinity of the inositol 1,4,5-triphosphate receptor. J Biol Chem 268:16488, 1993

45. Ziegler DM: Role of reversible oxidation-reduction of enzyme thiols-disulphides in metabolic regulation. Ann Rev Biochem 54:305, 1985

46. Ross D: Glutathione, free radicals and chemotherapeutic agents. Pharmacol Ther 37:231, 1988

47. Ameisen JC: Programmed cell death (apoptosis) and cell survival regulation: Relevance to AIDS and cancer. AIDS 8:1197, 1994

48. Sarafian TA, Bredesen DE: Is apoptosis mediated by reactive oxygen species? Free Rad Res 21:1, 1994

49. Schreck R, Rieber P, Baeuerle PA: Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 10:2247, 1991

50. Flescher E, Ledbetter JA, Schieven GL, Vela-Roch N, Fossum D, Dang H, Ogawa N, Tala N: Longitudinal exposure of human T lymphocytes to weak oxidative stress suppresses transmembrane and nuclear signal transduction. J Immunol 153:4880, 1994

51. Staal FTJ, Anderson MT, Staal GEJ, Herzenberg LA, Gitler C, Herzenberg LA. Redox regulation of signal transduction: Tyrosine phosphorylation and calcium influx. Proc Natl Acad Sci USA 91:3619, 1994