Erythrocytic Glutathione and Plasma Cysteine Status of Human Immunodeficient Patients

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Both deficient and normal blood levels of glutathione (GSH) and cysteine (Cys) have been reported in HIV patients, a discrepancy that has been attributed to different methodologies. The goal of this study was to apply our analytical method to this problem. Blood samples from HIV patients and healthy subjects were collected, immediately stabilized, and quantified using high performance liquid chromatography with dual electrochemical detection. The results showed that the erythrocytic GSH levels were the same in healthy subjects and in HIV patients regardless of their CD4 lymphocyte level. Only those with the lowest CD4 level plus opportunistic infections had subnormal GSH concentrations ($P < 0.001$). GSH plus glutathione disulfide (GSSG) levels also were normal in patients. However, the Cys contents were higher in patients than in controls ($P < 0.05$). These findings demonstrated that HIV patients have normal erythrocytic GSH concentrations and supranormal Cys levels. [Exp Biol Med Vol. 226(9):866–869, 2001]

Key words: HIV; blood glutathione; cysteine

For many years we have investigated biochemical phenomena associated with the aging process. These studies were based on a concept of anabolism to catabolism ratio changes during the life span (1). Thus, anabolism predominates during growth, anabolism and catabolism are essentially equal during maturity, and catabolism is greater during the aging stage. The decline in anabolism was verified by a sequence of aging decreases in $NADP^+$/NADPH enzyme activities and coenzyme levels (2–4).

In later studies, glutathione (GSH) concentrations in various tissues were determined during the life span. A noteworthy discovery was a sharp decline in tissue GSH levels in the senescent C57BL/6J mouse and the yellow fever mosquito, *Aedes aegypti* (2–4). Another key finding was that the blood GSH profile in the mature and aging mouse was paralleled in less accessible organs such as heart, liver, kidneys, lung, spleen, and brain (5, 6, 8, 9). Thus, blood GSH reflected the whole body status.

A similar GSH and aging relationship occurs in humans. Healthy males and females from ages 20 to 94 years old were recruited from Louisville, Kentucky and their blood GSH status was determined. Normal GSH concentration was determined in the 20- to 40-year-old subjects. Reduced glutathione (GSSH) deficiency occurred in subjects older than 40 year and the number increased with age, reaching as high as 50% of the 60- to 79-year-old age group (10).

GSH deficiency was also found in another group of elderly subjects in Michigan with an inverse relationship between age and GSH level. In that epidemeologic study, GSH together with age and measure of suppressed anger accounted for 39% of the variance of an index for morbidity (11).

The findings in healthy persons suggested an investigation of GSH in unhealthy subjects (12). Newly admitted hospital patients who had a variety of major chronic diseases were consecutively recruited along with healthy controls. Blood GSH levels were determined and correlated with their diagnoses. Over 36% of the 74 patients were GSH deficient ($P < 0.001$).

At this time we became aware of reports that GSH was deficient in plasma and its cellular components of HIV-seropositive subjects, regardless of CD4 lymphocyte level (13–17). Since Cys is the rate-limiting precursor of GSH, its decrease inferred that Cys deficiency was the cause of low GSH levels and implicated low levels of both GSH and Cys in the pathogenesis of HIV infection. The importance of GSH and Cys to HIV infection has been well documented in these articles.

Indeed, GSH deficiency was associated with impaired survival in HIV disease. *In vitro* studies showed that low GSH levels both promote HIV expression and impair T cell function. Thus, GSH depletion in CD4 cells was linked with HIV disease progression (18).

In contrast to these deficiency findings, normal levels have been reported by other investigators using different

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methodologies and whole blood to determine GSH status (19–22). They did not find a decrease in plasma GSH or its precursors like cysteine. Thus, one conclusion was that GSH is not compromised, but is redistributed (22).

At this time we found both blood GSH and Cys deficiencies in healthy, aging subjects and also in individuals with chronic disease (10–12). Also, in atherosclerotic patients, normal GSH levels were found, but significantly increased levels of free plasma Cys (23). For these reasons we were curious about the methods of others and their use of plasma GSH level as an indicator of disease.

Other HIV studies have focused on plasma or its components like lymphocytes, but to our knowledge, there have been no reports in patients of reduced and oxidized forms of blood GSH and plasma Cys. Therefore, our current aims were to quantify these forms in HIV patients, determine their relationship to CD4 levels, and compare them with healthy control subjects.

Materials and Methods

Reduced glutathione, glutathione disulfide (GSSG), cysteine, and cystine (CSSC) were purchased from the Sigma Chemical Company (St. Louis, MO). Monochloroacetic acid, HPLC-grade methanol, metaphosphoric acid (MPA), and acetonitrile were purchased from the Fisher Scientific (Pittsburgh, PA) and heptanesulfonic acid was from Alltech Associates (Deerfield, IL). All other chemicals were reagent grade, and deionized water was used.

The HIV patients were recruited from the infectious disease program of our Department of Medicine. Their antiretroviral therapy was based on the recommendations of the Department of Health and Human Services. At the time of this study, April 1993 through March 1994, patients with CD4 counts below 500 were treated with combination therapy of two nucleoside reverse transcriptase inhibitors. The most common antibiotic used was trimethropim/sulfametoxazole for prophylaxis of *Pneumocystis carinii* pneumonia in all patients with CD4 below 200. Although patients had opportunistic infections such as *Pneumocystic carnii* pneumonia and *Cryptococcal meningitis*, all patients were enrolled in the study during follow-up at the outpatient clinic when they were clinically stable from any type of co-infection. HIV viral load testing was not available at the time of this study.

The 103 HIV patients had a mean age ± SEM of 35.5 ± 0.87 years in a range of 16 to 53 years. They were grouped according to their CD4 lymphocyte levels (cells/μl) as follows: CD4>500; 500>CD4>200, CD4<200, and CD4<200 plus other infections. Twenty-six healthy adult controls (45.8 ± 3.0 years in a range of 30 to 80 years) were from the University of Louisville Medical School and the Louisville community. Informed consents approved by our Institutional Review Board were signed by the patients and control subjects.

Blood samples were collected by venipuncture with EDTA Vacutainers (Becton Dickinson, Rutherford, NJ), chilled immediately in crushed ice, and processed within 1 hr. The stability of GSH, GSSG, Cys, and CSSC in blood stored at 0°C had previously been determined, and only slight changes occurred up to 6 hr. A portion of each sample was analyzed for number of red cells, hemoglobin concentration, and hematocrit by a commercial laboratory. Protein was precipitated by the addition of 800 μl of 5% (w/v) MPA to 200 μl of blood. Plasma was obtained by centrifugation of blood at 1000*g* for 20 min in a refrigerated centrifuge, and red cell and buffy layers were removed and discarded. Plasma was deproteinized by addition of an equal volume of 5% MPA. After centrifugation, the acid-soluble supernatants of blood and plasma were analyzed simultaneously for GSH, GSSG, Cys, and CSSC with our high performance liquid chromatography method with dual electrochemical detection (25). The detection limits were 25 pmol for Cys and GSH, and 50 pmol for CSSC and GSSG. Fresh standards were prepared for each analysis.

GSH and GSSG values were determined in blood, and Cys, and CSSC values, in plasma. Validation of the method included recovery experiments with additions of authentic GSH and Cys to blood with recoveries of 93% to 112%.

Data are presented as mean ± SEM. Statistical analyses were performed with SPSS for Windows (Version 9.0). Comparisons between groups were made by one-way analysis of variance (ANOVA). When significance was indicated, a Tukey-Kramer mean pairwise *post hoc* analysis was used.

Results

Erythrocytic GSH, GSSG, and total GSH levels in HIV-infected patients were the same as for control subjects (Table I) with one exception. Only the lowest CD4<200 plus infection group had a higher GSH value than the con-

Table I. Erythrocytic Glutathione in HIV Patients

	Control (n = 24)	CD4>500 (n = 19)	500>CD4>200 (n = 44)	CD4<200 (n = 25)	CD4<200+ inf (n = 15)
RBC (10^6/ml)	4.72 ± 0.099	4.63 ± 0.20	4.44 ± 0.11	4.34 ± 0.012[a]	3.72 ± 0.16[b]
GSH (μmol/10^{10} RBC)	1.98 ± 0.086	2.04 ± 0.82	2.06 ± 0.59	2.18 ± 0.79	2.33 ± 0.11[a]
GSSG (μeq/10^{10} RBC)	0.172 ± 0.018	0.098 ± 0.020[a]	0.101 ± 0.014[b]	0.125 ± 0.017	0.116 ± 0.027
GSH + GSSG (μeq/10^{10} RBC)	2.15 ± 0.089	2.13 ± 0.080	2.16 ± 0.063	2.30 ± 0.079	2.44 ± 0.11

Note. All values are mean ± SEM.
[a] $P < 0.02$ compared with control group. [b] $P < 0.001$ compared with control group.

trol ($P < 0.017$) and the other HIV groups. This may be due to the significantly lower red blood cell counts for that group ($P < 0.001$).

The GSSG levels varied depending on the CD4 levels. The CD4>500 and 500>CD4>200 groups were significantly lower than the controls, but the CD4<200 groups were like the controls. In contrast, all GSH plus GSSG groups regardless of CD4 concentration were like the controls.

The Cys, CSSC, and total Cys levels in plasma are shown in Table II. The concentrations of Cys were higher in the HIV patients than the controls ($P < 0.01$–0.03) except for the CD4<200 plus infection group, which was statistically the same as normal. However, total Cys in all patients did not differ from control subjects.

Discussion

The results show that HIV-infected patients have normal levels of erythrocytic GSH and significantly higher levels of plasma Cys, and they also confirm the findings of others who studied whole blood (19–22). The exception is that the group with CD4<200 plus opportunistic infections had significantly lower GSH and normal Cys levels. This may be expected of any patients with infection.

Our findings of HIV patients were consistent with the normal findings of the Nijmegen, Manchester, and Liverpool investigators (19–22). Similar methods were used by all of us, for the blood samples were quickly processed or chilled after collection to obviate autoxidation, and erythrocytic GSH rather than plasma GSH was determined as a criterion of sufficiency and deficiency.

Plasma GSH has rarely been detected in our experience with over 480 normal and HIV subjects. This is consistent with our finding that more than 99.5% of GSH in human blood was localized in erythrocytes and 97% of Cys was in plasma (24). Moreover, the plasma GSH determined by others is notoriously unstable, for their reported values varied widely with coefficients of variation from 20% to 90% (26). For these reasons, we regard erythrocytic GSH level rather than plasma GSH as the index of senescence in animal models and humans, and also as the index of deficiency in chronic disease.

The two opposing views of GSH status in HIV are due to their different definitions of GSH deficiency. GSH normality is based on erythrocytic GSH and on the 99.5% localization of GSH in erythrocytes (24). Also of relevant interest in the C57BL/6J mouse, the erythrocytic GSH level parallels the GSH levels in many organs during the maturity and senescence stages of the life span. Thus, erythrocytic GSH serves as an index of whole body GSH status.

Application of these findings to human populations has demonstrated GSH deficiencies in aging and also in chronic diseases. In contrast, GSH status was normal in this study of HIV.

Plasma GSH, the opposing view, is somewhat inaccurate, for it is most likely an experimental artifact due to the slight hemolysis that occurs in blood collection as suggested by Schofield *et al.* (19). They showed that despite using efforts to minimize hemolysis, lysed erythrocytes contributed an average of 25% of the plasma GSH. A review by Dröge and his group (29) now refers to intracellular GSH, although his original papers first described a plasma deficiency in HIV.

The dynamic state of plasma GSH has been observed by several groups (26–28). Most recently, a detailed study of thiols in human plasma was reported by Kleinman and Richie (26) who also observed a rapid, marked decrease in GSH and Cys 15 to 30 sec after adding authentic GSH and CYS to a fresh untreated plasma sample. This dramatic loss was prevented by chilling the sample quickly in ice and deproteinizing with MPA. Although the instability of blood GSH was recognized by some of the investigators reporting GSH deficiency, many waited as long as 2 to 10 min before processing the samples, thereby permitting autoxidation.

This procedure to stabilize the sample may also be applicable for other redox systems that are fragile to room temperature and to neutral-alkaline pH. To prevent sample autoxidation, it has been suggested that the blood could be collected directly into 5,5′-dithiobis(2-nitro benzoic acid) or DTNB.

In contrast, GSH deficiencies in plasma cells such as lymphocytes have been reported. Is this what is meant by "plasma" deficiency? If so, the term is misleading, for the impression given is that plasma, and not its cells, is GSH deficient. Also, a low GSH concentration in lymphocytes could have occurred by autoxidation, since the samples were not protected.

Although several studies have used monochlorobimane as a fluorescent label for measuring glutathione, other results have shown that the label is not specific for GSH and should not be used for this purpose (20).

The importance of stabilizing the blood sample was emphasized in our previous work on GSH in which 36% of

Table II. Plasma Cyst(e)ine in HIV Patients

	Control ($n = 26$)	CD4>500 ($n = 19$)	500>CD4>200 ($n = 44$)	CD4<200 ($n = 25$)	CD4<200+ inf ($n = 15$)
Cys (nmol/ml plasma)	7.91 ± 1.10	14.3 ± 2.19[a]	12.4 ± 1.25[a]	14.6 ± 2.68[a]	10.1 ± 1.46
CSSC (neq/ml plasma)	116 ± 6.61	109 ± 7.65	121 ± 6.56	112 ± 6.54	124 ± 12.8
CYS + CSSC (neq/ml plasma)	124 ± 6.09	123 ± 8.49	133 ± 6.83	127 ± 8.02	134 ± 13.1

Note. All values are mean ± SEM.
[a] $P < 0.03$ compared with control group.

newly admitted hospital patients with chronic diseases were GSH deficient (12). Perhaps viral diseases are distinctly different from metabolic diseases in regard to GSH status. Thus, there are further investigations necessary to elucidate these differences.

Other possible differences for the discrepancy in results may be different strains of HIV, the heterogeneity of HIV patients, and their prior treatment. Regardless, the GSH status of HIV patients should be carefully determined in future experiments of this subject.

In conclusion, the results of this study demonstrate that HIV-infected patients have normal levels of erythrocytic GSH and higher than normal Cys. How this occurs and yet plasma lymphocytes are GSH deficient is a problem for future study.

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