

# Elevation of plasma thioredoxin levels in HIV-infected individuals

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## Abstract

**Thioredoxin (Trx), a ubiquitous protein intimately involved in redox and protein disulfide reductions, has been shown to be released from cells and to have cytokine-like activities. In addition, Trx has been implicated in the redox regulation of immunological responses and shown to be deficient in tissues from AIDS patients. In studies presented here, plasma Trx levels were measured by ELISA in plasma samples from HIV-infected individuals ( $n = 136$ ) and HIV-negative controls ( $n = 47$ ). To account for the release of Trx into plasma due to hemolysis, the Trx measurements were corrected according to the level of hemoglobin in the plasma sample. Data presented show that, in contrast to tissue Trx levels, corrected plasma Trx levels are significantly higher in HIV-infected individuals than in controls ( $P < 0.0001$ ). Furthermore, ~25% of the HIV-infected individuals studied have plasma Trx levels greater than the highest level found in controls (37 ng/ml). Detailed multiparameter FACS analysis of peripheral blood mononuclear cells (PBMC) from the infected individuals demonstrates that those with higher plasma Trx levels (37 ng/ml or greater) tend to have lower overall CD4 counts. In addition, increases in plasma Trx levels correlate with decreases in monochlorobimane staining (indicative of lower intracellular glutathione levels in PBMC) and with changes in surface antigen expression (CD62L, CD38 and CD20) that occur in the later stages of HIV infection. These correlations suggest that elevation of plasma Trx levels may be an important component of advanced HIV disease, perhaps related to the oxidative stress that often occurs at this stage.**

## Introduction

Several reports implicate oxidative stress in the regulation of HIV expression and the progression of HIV infection to AIDS. In particular, glutathione (GSH), the major intracellular antioxidant, has been shown to be decreased in HIV-infected individuals (1,2). Inflammatory cytokines, which are produced in greater amounts in HIV-infected individuals, stimulate the transcription and replication of HIV (3). GSH-replenishing prodrugs, such as *N*-acetyl-L-cysteine, inhibit the HIV replication stimulated by these cytokines in *in vitro* studies (2,4,5). These redox-related findings motivated the studies reported here, which document increases in the plasma levels of thioredoxin

(Trx), a key redox mediator, in plasma samples from HIV-infected individuals.

Trx is a ubiquitous protein (mol wt 12,000) with a known three-dimensional structure containing two redox-active cysteine residues (-Cys-Gly-Pro-Cys-). It acts in combination with NADPH and Trx reductase to catalyze protein disulfide reductions (6-8). Human Trx was first detected in platelets (9), and subsequently measured in cultured fibroblasts by its ability to catalyze the reduction of insulin disulfides with NADPH and calf Trx reductase (10). Holmgren and colleagues developed a radioimmunoassay for Trx in calf serum and

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erythrocytes (11), and more recently, human Trx isolated from placenta was purified to homogeneity and used in a radioimmunoassay that demonstrated that there is relatively little Trx in human plasma whereas human erythrocytes (RBC) have high levels of Trx (A. Ehrnberg and A. Holmgren, in preparation).

Trx expression is induced by oxidative stress and has been shown to have anti-oxidant activity (12,13). Trx is released by lymphocytes and other types of cells (14,15) and is identical to a previously recognized molecule, adult T cell leukemia-derived factor (ADF), produced by human leukemia cells transformed with HTLV-I (16,17). (Trx and ADF are equivalent, but only the abbreviation Trx is used in the text.) Trx has several known cytokine-like functions (18,19) and plays an important role in the redox regulation of immunological responses (20,21). Previous studies have shown that Trx is selectively lost in dendritic cells of lymph nodes in AIDS patients and in cytopathic cells of acute HIV infection (22). Recently, the presence of Trx in human serum has been demonstrated by affinity purification (23). Studies presented here show that Trx is elevated in plasma from HIV-infected individuals.

Plasma samples for our Trx studies were drawn from HIV-infected individuals whose clinical laboratory parameters were measured and whose peripheral blood mononuclear cells (PBMC) were characterized for lymphocyte subset representation and surface marker expression by flow cytometry (FACS). Thus, FACS data available for each of our subjects allowed us to determine the relationship between Trx levels and various FACS measures of HIV infection. Data from these studies show that plasma increases in Trx levels correlate with a constellation of PBMC marker changes associated with increased severity of HIV disease.

## Methods

### *HIV-infected subjects*

HIV-infected subjects ( $n = 136$ ) were recruited from the San Francisco area as part of a larger group being screened for entry into a clinical drug trial. Virtually all of the subjects recruited for the study reported here have  $<500$  CD4 T cells/ $\mu$ l; none reported a current opportunistic infection and none were taking large amounts of antioxidants, vitamins or minerals. Forty-four subjects were receiving antiretroviral treatment at the time of analysis (either AZT, ddI or ddC alone, or combinations of these).

Historical clinical data concerning HIV-related symptoms or conditions were obtained for 84 of the 136 individuals studied. Of these, 12 had a history of an AIDS defining illness or opportunistic infection as defined in Category C in the CDC case definition (24) and an additional 32 had a history of other HIV-related conditions (Category B). The remaining 40 subjects for whom we have data did not report any indication of a past history of HIV-related symptoms or conditions and were scored in Category A.

### *Controls*

Forty seven healthy HIV-uninfected individuals were studied as controls. These included laboratory personnel as well as

individuals who were recruited as controls for the clinical drug trial. The infected and control groups were approximately matched for age although the control group had a slightly higher number of younger people (no correlation was found between Trx level and age). The infected group was mostly male, and the control group included both male and female individuals.

### *Blood samples*

Blood was drawn from 136 HIV-infected individuals (all participants signed informed consent forms). Blood samples were drawn in heparin tubes and sent to Stanford University. Following some preprocedure interval time after drawing (0–8 h), plasma samples were separated by centrifugation at 3000 r.p.m. for 10 min at room temperature and stored at  $-70^{\circ}\text{C}$ . Plasma samples were freeze-thawed one to three times just before being tested.

Blood samples were simultaneously measured for a complete blood count, alkaline phosphatase (ALP), and aminotransferases [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] by an accredited commercial laboratory. HIV antibody sero-status was determined by ELISA and confirmed by Western blotting assays. In addition, PBMC samples for multiparameter FACS measurements were prepared and analyzed as indicated below.

Two consecutive plasma samples were drawn 1 week apart for most of the HIV-infected individuals in our study. We report average Trx values for these two samples whenever both were drawn, except for the few occasions when the Trx values proved to be widely discordant (difference  $>35$  ng/ml). In these cases, the lower Trx value was used, since this is the more conservative option. The FACS and clinical values referred to in this study are reported only for the first sample drawn (first visit with subject).

### *ELISA for Trx*

Two murine mAb to non-overlapping epitopes of human Trx (ADF-11 and ADF-21) were provided by Fuji Rebio (Tokyo, Japan), for use in a sandwich ELISA for Trx. To assay for Trx, 96-microwell plates (Nunc, Roskilde, Denmark) were pre-coated with 100  $\mu$ l of a 15  $\mu$ g/ml anti-Trx mouse mAb (ADF-21) in 0.1 M citrate coating buffer (pH 3.5). Then, 200  $\mu$ l of blocking buffer (50 mM phosphate buffer, 1% BSA, 0.05% Tween 20, pH 6.0) and 20  $\mu$ l of sample were added to the wells and incubated at room temperature for 2 h. After washing three times with 0.05% Tween 20 supplemented PBS (TPBS), 200  $\mu$ l of a 0.1  $\mu$ g/ml horseradish peroxidase-labeled, anti-Trx mAb (ADF-11) diluted in a second buffer (50 mM phosphate buffer, 1% BSA, 0.05% Tween 20, pH 8.0) was added and incubated at room temperature for 2 h.

After washing three times with TPBS, 100  $\mu$ l of substrate solution [0.5 mg/ml 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) dissolved in 0.1 M triethanolamine-succinate buffer containing 0.03% hydrogen peroxide, pH 4.2] was added and incubated at room temperature. One hour later, 100  $\mu$ l of stopping solution (1% oxalic acid, pH 1.9) was added and the absorption at 405 nm was measured by ELISA reader (Vmax kinetic microplate reader; Molecular Devices, Menlo Park, CA). Recombinant human Trx (rTrx), provided by

Ajinomoto (Kawasaki, Japan), was used as a standard, using 2-fold dilutions from 320 to 5 ng/ml.

A standard curve for Trx (5–320 ng/ml) was calculated by Softmax (version 2.01). Trx concentrations of plasma samples were calculated by fitting the standard curve values for rTrx to a four-parameter logit-log curve model shown as follows:  $Y = (A - D)/(1 + (X/C)^B) + D$ , where  $A$  is the maximal absorption,  $B$  is the reaction order,  $C$  is the association constant and  $D$  is the background absorption. Intra-assay coefficient of variation for rTrx was 5.1% ( $n = 70$ ) in 10 different experiments. There was no correlation between plasma Trx levels and preprocedure time (data not shown). Plasma Trx titers were not influenced by freezing and thawing (data not shown). Intra-assay coefficient of variation for the same plasma samples ( $n = 89$ ) was 5.2% in triplicates. Interassay coefficient of variation for the same plasma samples ( $n = 26$ ) was 18.7% in three experiments.

#### *Hemoglobin (Hb) in plasma*

Hb concentration in plasma samples was measured with a diagnostic kit for plasma Hb according to the procedure described by the supplier (Sigma, St Louis, MO). Hemolyzed blood samples were prepared from heparinized whole blood samples put in a lysis buffer (17 mM Tris-HCl, 140 mM  $\text{NH}_4\text{Cl}$ ) to lyse RBC. Trx and Hb levels in RBC-lysed blood samples were examined by the same methods for plasma samples.

#### *Flow cytometry measurements: lymphocyte subset frequencies, surface antigen densities and intracellular monochlorobimane (MCB) staining*

These methods have been described in detail elsewhere (25). Briefly, PBMC were isolated from heparinized whole blood by Ficoll-Paque density centrifugation (Pharmacia, Uppsala, Sweden). The isolated PBMC were washed and stained with MCB in the presence of probenecid (2.5 mM) to inhibit fluorescent product export (26). Next, the PBMC were washed in HEPES RPMI media without carbonate added (Gibco, Grand Island, NY), supplemented with 4% FCS and stained at 4°C with sets of fluorochrome-coupled (FITC, phycoerythrin or Cy-chrome) mAb reagents detecting the following surface markers: CD14, CD16 and CD45; CD3, CD8 and CD4; CD20, HLA-DR and CD5; CD7, CD16 and CD5; CD3, CD38 and CD4; HLA-DR, CD8 and CD4; CD62L, CD45RA and CD4; CD62L, CD45RA and CD8; CD11a, CD45RA and CD4; CD11a, CD45RA and CD8. Levels of surface markers were measured with a FACStar Plus (Becton Dickinson, San Jose, CA) flow cytometer and computed with FACS/Desk software written in our laboratory (27).

The MCB measurements, which provide an index of intracellular GSH (28,29), were normalized to the MCB values obtained for a simultaneously stained aliquot of a frozen PBMC standard sample maintained in liquid nitrogen until use. MCB levels shown here reflect this normalized value. The precise quantitative relationship between MCB fluorescence and intracellular GSH levels in PBMC subsets is unknown. In GSH depletion studies of both PBMC and the Jurkat cell line, the MCB levels are correlated monotonically with intracellular GSH levels (26,30). Comparisons of median MCB levels in individual subsets should thus provide a stable relative measure of intracellular GSH. However, the

dependence of MCB staining on intracellular enzymes that couple the MCB to GSH (e.g., GSH-S-transferase) and the possibility that different levels of such enzymes are expressed in individual subsets suggest that comparisons of MCB levels may be more reliable when made within a given subset than when made between different subsets. Indeed, preliminary studies comparing intracellular GSH levels measured by MCB staining with GSH levels measured by bulk biochemical assays indicate that this may be the case for certain T cell subsets.

#### *Statistical analysis*

Unless otherwise indicated in the text, statistical differences between each group were evaluated by the non-parametric Wilcoxon rank sums test and correlations were evaluated by Kendall's non-parametric analysis. All statistical evaluations were performed with JMP Version 3 (SAS, Cary, NC) software and an Apple Macintosh computer (Apple Computer, Cupertino, CA).

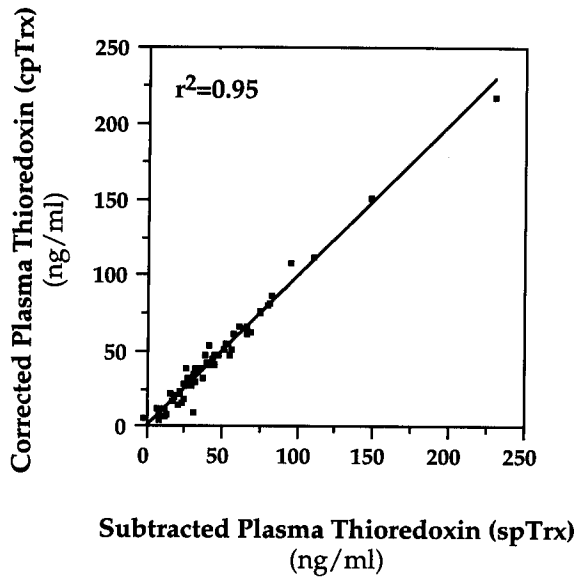
## **Results**

#### *Correction of plasma Trx levels for erythrocyte-derived Trx*

The demonstration that erythrocytes have high Trx levels relative to the amount of Trx found in plasma (11) means that measurements of plasma Trx levels (by ELISA) must be corrected for the amount of Trx introduced by the low level of hemolysis that typically occurs during the drawing, transport and separation of plasma samples. In the absence of this correction, there is a significant correlation ( $P < 0.0001$ ) between the plasma Hb and Trx levels; once the correction is made, this correlation is no longer detectable and the increase in Trx levels in HIV-infected people becomes accurately measurable.

The correction for hemolysis is readily made by using the Hb level in the plasma sample as an index of hemolysis and subtracting the corresponding amount of (erythrocyte-derived) Trx from the overall Trx level measured in the plasma sample. Best results are obtained by discarding highly hemolyzed plasma samples (<15 in this study) in which pHb > 65 mg/dl. To establish the correction values, we first determined the correspondence between the amount of Trx and the amount of Hb released by hemolyzed erythrocytes in fully hemolyzed blood samples from a series of 77 HIV-infected and 18 control individuals. To do this, we directly measured Trx and Hb in each hemolyzed sample (diluted 1/400 in lysis buffer) and determined the ratio of these values (hTrx/hHb) for each individual.

Since there was no significant difference in the ratios obtained for HIV-infected and control individuals ( $P = 0.09$ ), we combined data from all individuals in these groups ( $n = 95$ ) to calculate an average hemolyzed Trx/Hb ratio in RBC [0.937 (ng/ml)/(mg/dl)]. Corrections based on this average hemolyzed Trx/Hb ratio are equivalent to corrections based on direct measurements for each individual. As Fig. 1 shows, the corrected plasma Trx values obtained with the average ratio correlate linearly with Trx values obtained by correcting each sample individually according to its directly measured



**Fig. 1.** The correlation between corrected plasma Trx (cpTrx) and subtracted plasma Trx (spTrx).  $n = 88$  (73 HIV-infected individuals and 15 controls). As noted in the text, spTrx are the plasma Trx values corrected using the ratio of hemolyzed Trx to hemolyzed Hb (hTrx/hHb) determined on a subject by subject basis [spTrx = pTrx - (hTrx/hHb) × pHb, where pTrx is the measured plasma Trx and pHb is the plasma Hb]. cpTrx are the plasma Trx values determined using an average value for the hemolyzed Trx to hemolyzed Hb (cpTrx = pTrx - 0.937 × pHb).

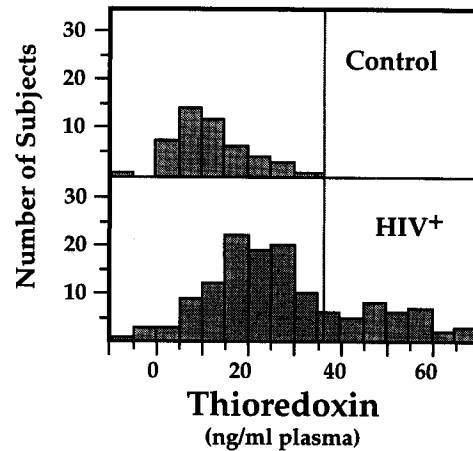
ratio ( $r^2 = 0.95$ , based on analysis of 73 HIV-infected individuals and 15 controls).

We used the average value (0.937), in combination with the plasma Hb level measured for each sample, to compute the Trx contribution due to hemolysis and subtracted that value from the overall Trx level measured for the sample. Thus, the corrected plasma Trx values reported here reflect the difference between the overall Trx level in plasma and the amount of Trx introduced by erythrocyte lysis, i.e. corrected plasma Trx = Trx measured in plasma - [(hTrx/hHb) (pHb)], where hTrx/hHb is the average ratio (0.937) of the Trx to Hb determined for 95 fully hemolyzed blood samples and pHb is the Hb measured directly for each plasma sample in the study.

The average correction (0.937 × pHb) for the group studied (HIV-infected and control) is  $17.4 \pm 8.6$  ng/ml, which corresponds to subtracting  $47 \pm 23\%$  of the measured plasma Trx levels. To the extent that the half-life of Hb (31) is equivalent to the half-life of Trx in blood (A. Mitsui, Basic Research Institute, Ajinomoto, Kawasaki, Japan, unpublished observation), this correction also corrects for plasma Trx due to *in vivo* hemolysis. The relatively large magnitude of the correction as determined above indicates the importance of accounting for the portion of the measured plasma Trx that is due to erythrocyte lysis. The clear difference obtained for corrected Trx levels in the HIV-infected and control groups (see below) demonstrates the validity of this methodology.

#### Elevation of plasma Trx levels in HIV-infected individuals

The distribution of plasma Trx values for HIV-infected individuals overlaps the distribution for controls (Fig. 2); however,



**Fig. 2.** The distribution of plasma Trx levels among HIV-infected individuals ( $n = 136$ ) and controls ( $n = 47$ ). These levels are significantly different between control and HIV-infected individuals ( $P < 0.0001$ ). Levels of plasma Trx were calculated as described in the text. A reference line has been drawn at a plasma Trx of 36.1 ng/ml, the maximum value found in the control group.

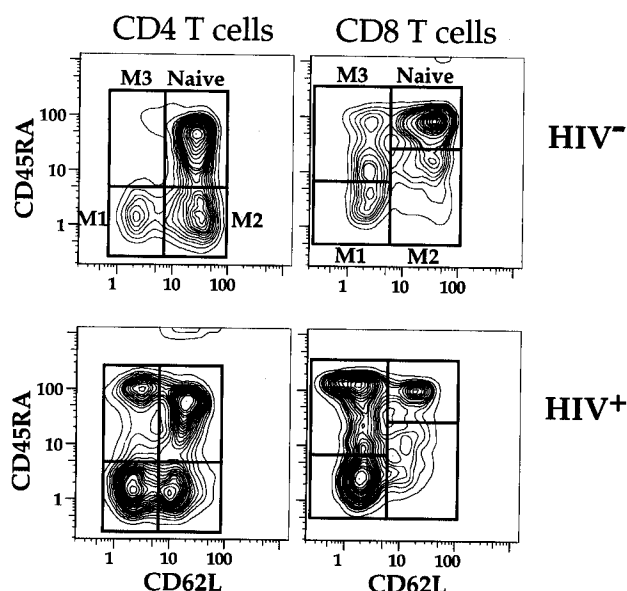
plasma Trx levels in 25% (34/136) of the infected individuals were above the highest value obtained for the control population ( $>37$  ng/ml). Furthermore, the entire plasma Trx distribution for HIV-infected individuals is shifted upwards with respect to the control distribution, i.e. the median, 75th and 90th percentile values for the infected group are well above those for the controls. Median plasma Trx levels were 24.5 ng/ml for 136 HIV-infected individuals and 11.7 ng/ml for 47 controls; 90th percentile levels were 54.5 versus 26.6 ng/ml respectively. Trx levels in 84% of infected individuals were higher than the control median.

Non-parametric comparison of the plasma Trx distributions shows that the difference between the HIV-infected and control individuals is highly significant ( $P < 0.0001$ ). Plasma Trx values for the control group were normally distributed. Parametric comparison of the mean Trx levels in the groups also shows that the difference between the groups is highly significant:  $27.6 \pm 16.7$  ng/ml for the infected and  $12.6 \pm 8.7$  ng/ml for the control group (ANOVA/*t*-test;  $P < 0.0001$ ).

#### Correlations of plasma Trx levels with FACS-measured parameters

**CD4 T cell counts.** Plasma Trx levels in HIV-infected individuals tend to be higher when absolute CD4 counts are low ( $P = 0.01$ ), but show no correlation with the overall absolute CD8 T cell count ( $P = 0.4$ ). Trx increases are also correlated with decreases in the absolute numbers of cells in the naive subsets of CD4 and CD8 T cells ( $P = 0.03$  for both subsets). We have shown elsewhere that the numbers of cells in these naive subsets decrease together as HIV infection progresses, apparently in advance of the overall decrease in CD4 T cells (32,33).

**Surface antigen expression on lymphocytes.** Multiparameter FACS analysis subdivides CD4 and CD8 T cells into naive and memory subsets based on the expression of the cell



**Fig. 3.** CD4 and CD8 T cell subsets in PBMC from a healthy HIV-uninfected control and an HIV-infected adult as determined by three-color flow cytometry. To generate these 5% probability FACS plots, lymphocytes were first selected from the PBMC using forward and side scatter gates. Within lymphocytes, the CD4 and CD8 T cells were discriminated based on the expression of CD4 and CD8. The plots on the left have been gated on CD4<sup>+</sup> T cells and the plots on the right have been gated on CD8<sup>+</sup> T cells. CD4 and CD8 T cells can be further subdivided into naive and memory subsets as determined by the expression of CD62L (L-selectin) and CD45RA. For both CD4 and CD8 T cells, the naive subset is defined as CD62L<sup>+</sup> and CD45RA<sup>+</sup>. At least three memory subsets can be identified in CD4 and CD8 T cells: cells expressing neither CD62L or CD45RA, or cells expressing only CD62L or only CD45RA. Shown for comparison are representative examples from an HIV-uninfected control (upper plots) and an HIV-infected adult (lower plots). In healthy controls there are very few CD4 cells with the CD62L<sup>-</sup>CD45RA<sup>+</sup> phenotype (labeled as M3); however, in HIV-infected individuals this population becomes more prominent.

surface antigens, CD62L (L-selectin) and CD45RA (Fig. 3). The naive CD4 and CD8 T cell subsets normally are characterized by the expression of high levels of CD62L and high levels of CD45RA. However, although CD62L is also clearly expressed on naive and memory T cells in HIV-infected individuals, the CD62L levels tend to decrease as HIV disease progresses (34). This decrease correlates with increased plasma Trx levels (Table 1), particularly in the naive and M2 subsets.

Changes in CD20, a B cell surface marker, are also correlated with changes in plasma Trx. We have shown previously that expression of CD20 increases in HIV-infected individuals, particularly at the later stages of HIV disease (35). Consistent with this finding, plasma Trx levels increase with CD20 expression (Table 1). Plasma Trx levels also correlate with the expression of another cell surface antigen, CD38. Increased expression of CD38 on CD8 T cells has been documented in HIV-infected individuals and is associated with HIV disease progression (36,37). In our study, we have found that plasma Trx levels increase as the level of expression of CD38 increases on CD4 and CD8 T cells (Table 1).

**Table 1.** Correlation of plasma Trx with antigen expression

Lymphocyte subset	Antigen	Significance of correlation (P value) <sup>a</sup>
Overall CD4 T cells	CD38	0.014 (pos)
CD4 naive T cells	CD62L	0.001 (neg)
CD4 CD62L <sup>+</sup> memory T cells (M2)	CD62L	0.001 (neg)
Overall CD8 T cells	CD38	0.019 (pos)
CD8 naive T cells	CD62L	0.001 (neg)
CD8 CD62L <sup>+</sup> memory T cells (M2)	CD62L	0.001 (neg)
B cells	CD20	0.019 (pos)

<sup>a</sup>P values are based on Kendall's non-parametric correlation analysis between the plasma Trx levels and the median level of expression of CD62L (L-selectin) and CD20 or the 90th percentile level of expression of CD38 in the designated PBMC subset in HIV-infected individuals. (The 90th percentile level of expression is more informative than the median for markers such as CD38 where there is a low-frequency population of positive cells (activated cells) which is of most interest.) Number of individuals analyzed = 100–134, depending on the analysis; (pos) = positive correlation; (neg) = negative correlation.

*MCB staining, a measure of intracellular GSH.* The median and 90th percentile MCB levels measured by flow cytometry for the individual PBMC subsets provide an index of the GSH content/status of the cells in the subset. Previous studies from our laboratory have shown that MCB levels tend to be lower in HIV-infected individuals, particularly in certain T cell subsets (38). Similarly, several laboratories have shown that GSH levels, as measured biochemically, are lower in HIV-infected individuals (1,39).

Comparison of Trx and FACS MCB staining data for individual subjects shows that plasma Trx levels tend to be higher when individuals have lower MCB staining levels, particularly in monocytes, granulocytes, NK and T cells ( $P = 0.001-0.03$ ). MCB levels in B cells do not show any correlation with Trx values ( $P = 0.3$ ); however, the negative correlation with MCB levels is clear in the overall CD4 and CD8 T cell populations, and the naive and memory subsets of each of these overall populations (Table 2).

#### Trx versus clinical parameters

The clinical stage of HIV disease was determined for 84 of the 136 HIV-infected individuals in this study. Twelve individuals reported a history of an AIDS defining illness [Category C in the CDC case definition (24)], 32 reported other HIV-related symptoms or conditions (Category B) and 40 were asymptomatic (Category A). Plasma Trx levels were not significantly different in these three groups of individuals ( $P = 0.2$ ). Nevertheless, other clinical indications associated with advanced HIV infection tend to be present when plasma Trx levels are higher.

For example, plasma Trx levels increase significantly (although not dramatically) as markers of macrocytic anemia become more pronounced in HIV-infected individuals (i.e. RBC number and Hb decrease and mean corpuscular volume increases) (see Table 3). Macrocytic anemia can be the result of antiretroviral treatment; however, we find no evidence that

**Table 2.** Correlation of plasma Trx with intracellular MCB staining

PBMC subset	Significance of correlation (P value) <sup>a</sup>
Monocytes	0.001
Granulocytes	0.002
NK cells	0.001
Lymphocytes	0.002
CD8 T cells	0.001
CD8 naive T cells	0.015
CD8 memory T cells	0.003
CD4 T cells	0.009
CD4 naive T cells	0.002
CD4 memory T cells <sup>b</sup>	0.030
B cells	NS <sup>c</sup>

<sup>a</sup>P values are based on Kendall's non-parametric correlation analysis between the plasma Trx level and the median level of intracellular MCB staining in the designated PBMC subset in HIV-infected individuals. Number of individuals analyzed = 101–135, depending on the analysis. The correlation is negative for all the subsets listed, except for B cells.

<sup>b</sup>An average of the MCB staining in the three memory T cell subsets (M1, M2 and M3) weighted on the basis of the fractional representation of each of these subsets is used in this correlation analysis with plasma Trx level.

<sup>c</sup>Not significant.

**Table 3.** Correlation of plasma Trx with clinical parameters

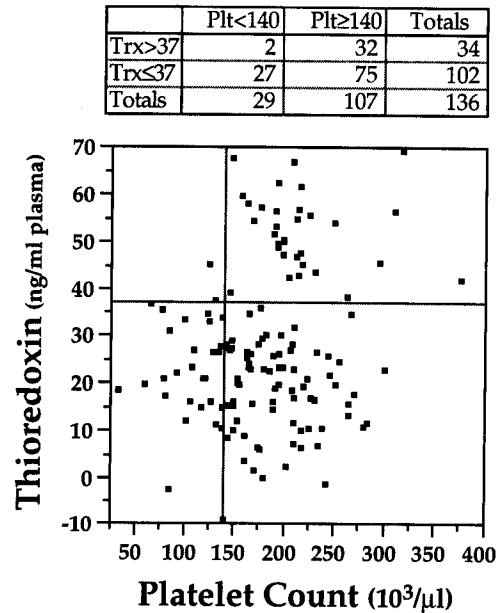
Parameter	Significance of correlation (P value) <sup>a</sup>
RBC count	0.001 (neg)
Hb	0.014 (neg)
Hematocrit	0.022 (neg)
Mean corpuscular volume	0.002 (pos)
Basophils	0.001 (pos)
Eosinophils	0.026 (pos)
AST	0.002 (pos)
ALT	0.036 (pos)
ALP	NS <sup>b</sup>

<sup>a</sup>P values are based on Kendall's non-parametric correlation analysis between the plasma Trx level and the indicated clinical parameter in HIV-infected individuals. Number of individuals analyzed (*n*) = 136 for all analyses except AST, ALT and ALP, for which *n* = 68; (pos) = positive correlation; (neg) = negative correlation.

<sup>b</sup>Not significant.

the correlation of the increased plasma Trx with markers of anemia is due to this treatment. Plasma Trx levels are not significantly elevated in the 45 HIV-infected individuals who were receiving antiretroviral treatment when compared with the 91 individuals who were not receiving this treatment (*P* = 1.0). For the 24 individuals whose antiretroviral therapy included AZT, there also is not a significant elevation in plasma Trx levels when compared with the remainder of the infected group (*P* = 0.5).

Trx levels also rise as CBC indices for basophils (*P* = 0.001) and eosinophils (*P* = 0.02) increase. Curiously, although decreases in platelet numbers are associated with advanced HIV infection in some individuals, HIV-infected individuals with



**Fig. 4.** The scatterplot of platelet count versus plasma Trx levels for HIV-infected individuals (*n* = 136). Reference lines have been drawn at a platelet count of  $1.4 \times 10^5/\mu\text{l}$  (the lower limit of the normal range) and at a plasma Trx level of 37 ng/ml (the upper limit of the normal range). The table above this plot lists the number of individuals in each quadrant. The distribution shown differs significantly from the distribution predicted by the marginal totals (*P* = 0.01, Fisher's exact test), e.g. there are fewer individuals than expected in the quadrant with high Trx and low platelet count (Trx > 37, PLT < 140).

low platelet counts ( $<140 \times 10^3/\mu\text{l}$ ) do not have elevated Trx levels ( $>37$  ng/ml) (Fig. 4).

Liver enzyme levels in blood also tend to increase with plasma Trx levels in HIV-infected individuals (Table 3). This correlation is most dramatic for AST (*P* = 0.002). Increases in plasma Trx correlate weakly with increases in ALT levels (*P* = 0.04), but there is no correlation with increased ALP (*P* = 0.2).

## Discussion

In this study, we have shown that plasma levels of Trx are markedly elevated in a subset of HIV-infected individuals. These individuals tend to have lower absolute CD4 T cell counts and to display other indications of advanced HIV disease. Elevated Trx levels do not, however, necessarily indicate advanced HIV infection since Trx levels are elevated in some apparently healthy HIV-infected people with CD4 counts  $>200$  cells/ $\mu\text{l}$ . By definition, none of the uninfected (control) individuals studied had elevated Trx values, since the highest plasma Trx level found in these control individuals was used as the boundary between elevated and normal levels (Fig. 2).

Erythrocytes typically contain high levels of Trx. Since some erythrocyte lysis during venipuncture is unavoidable, plasma Trx measurements will also reflect that Trx which is released by lysed cells. We account for this contribution by estimating lysis based on plasma Hb content, and then correcting total

Trx by subtracting the Trx due to the lysed erythrocytes. We find that almost 50% of measured plasma Trx can be attributed to erythrocyte lysis during venipuncture. Therefore, accurate measurements of plasma Trx must include such a correction factor. Finally, we found that the coefficient used in this correction factor was not different for HIV-infected versus HIV-uninfected individuals.

The source of the additional Trx in the plasma from HIV-infected individuals is unclear. It could derive from activated lymphocytes since several reports have demonstrated Trx release by lymphocytes activated *in vitro*. For example, lymphocytes infected by HTLV-I and by Epstein-Barr virus produce Trx (17,18) as do lymphocytes stimulated by mitogen or inflammatory cytokines (14,15,17). Alternatively, or in addition, the plasma Trx could derive from dying lymphocytes, since apoptosis of lymphocytes has been shown to be increased in HIV infection (40,41).

The correlation with anemia shown here suggests that erythrocyte lysis *in vivo* may contribute to the elevated Trx levels in some individuals since erythrocytes contain high levels of Trx (11). Erythrocyte lysis, however, is unlikely to be the main source of the elevated Trx because, as indicated above, studies with rTrx indicate that the Trx half-life in serum (in mice) is ~1 h (A. Mitsui, Basic Research Institute, Ajinomoto, Kawasaki, Japan, unpublished observation). Since this is the same order of magnitude as the half-life of plasma Hb (31), the correction we make to remove Trx originating from erythrocytes that lyse during sample preparation should largely correct for Trx originating from erythrocyte lysis *in vivo*.

Liver damage could account for plasma Trx since liver cells have high Trx levels and the liver contains the largest total amount of Trx in the human body (11,42,43). Consistent with this, plasma Trx levels have been shown to be elevated in some cases of acute viral hepatitis (23). However, although HIV infection is sometimes associated with liver dysfunction (44,45), we found no correlation in this study between Trx and a general elevation of the enzymes that usually report liver dysfunction, i.e. we found a relatively strong correlation with one of these enzymes (AST), but found relatively little or no correlation with the two others (ALT and ALP). Thus, elevated Trx levels are not simply correlated with liver dysfunction in HIV infection.

The recent cloning of the Trx promoter region, and the demonstration that the expression of Trx is inducible by inflammatory cytokines such as IL-6 and interferons (46), suggests that the well-known elevation of these cytokines during HIV infection is responsible for increasing the expression and release of Trx into plasma. The tendency for plasma Trx levels to increase as MCB (GSH) levels decrease is consistent with this hypothesis since lower GSH levels and oxidative stress in general have been shown to up-regulate inflammatory cytokine activity. The association of increased Trx levels and changes in the expression of surface markers that report activation of lymphocytes (CD62L, CD38 and CD20) also argues for the involvement of T cell activation and inflammatory cytokine activity in the increase of Trx in plasma during HIV infection. Thus, plasma Trx levels may reflect the lymphocyte activation that occurs in HIV infection and could prove useful as a measure of that activation.

Alternatively, or perhaps in addition, plasma Trx could be

derived from several other cell or tissue sources that are known to contain or release high levels of Trx. These include platelets (9), monocytes/macrophages (47,48) and endothelial cells (13). Interestingly, dysfunction of platelets and Trx reductase has been reported in Hermansky-Pudlak syndrome (49), and the association of platelet-activating factor and Trx has been reported to play an important role in the 'early pregnancy factor' phenomenon (50). Platelets constitute an intriguing candidate for the source of the elevated plasma Trx since, as we have shown, individuals with elevated plasma Trx levels do not show low platelet counts. In fact, platelet counts in individuals with high Trx levels virtually all fall into normal range (none are low) and, within this range, may even be somewhat higher than counts in individuals with low Trx levels. However, platelet counts in many individuals with normal Trx levels are equivalent to platelet counts in individuals with high Trx levels. Thus, if platelets do constitute a significant source of the elevated plasma Trx, additional factors must come into play, e.g. oxidative stress known to occur in HIV disease (1,2) may be responsible for inducing Trx release from platelets.

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#### Abbreviations

ADF	adult T cell leukemia-derived factor
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
GSH	glutathione
Hb	hemoglobin
MCB	monochlorobimane
PBMC	peripheral blood mononuclear cells
RBC	red blood cell
TPBS	0.05% Tween 20 supplemented PBS
Trx	thioredoxin

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