Glutathione and Immunophenotypes of T and B Lymphocytes in HIV-Infected Individuals[a]

FRANK J. T. STAAL, MARIO ROEDERER, LEONORE A. HERZENBERG, AND LEONARD A. HERZENBERG

Department of Genetics
Stanford University School of Medicine
Stanford, California 94305

INTRODUCTION: GLUTATHIONE AND THE REGULATION OF SIGNAL TRANSDUCTION

Glutathione (GSH), the predominant intracellular thiol, is a cysteine-containing tripeptide (γ-glutamyl-cysteinyl-glycine) found at relatively high concentrations (1-10 mM) in all eukaryotic cells. It plays a number of roles that are crucial to cell survival and function. It serves as a cofactor for several enzymes; it is required for the synthesis of DNA precursors; it reduces protein disulfides, and thus regulates sulfhydryl-dependent enzymes; and it reduces intracellular oxidants (free radicals, reactive metabolic intermediates) and thereby protects the cell from damage by these agents.[1-3] Thus it is also the main regulator of the cellular redox potential and functions as a buffer of reducing agents. Furthermore, our recent studies show that GSH has the ability to inhibit the transduction of cytokine-generated signals that activate the NF-κB transcriptional activation factor. So, the expression of a variety of genes, including those controlled by the HIV long terminal repeat (LTR), is favored when intracellular GSH is depleted and is blocked when this depletion is prevented.[4,5]

The NF-κB transcription factor was first identified as a nuclear factor that binds to the enhancer of the kappa light chain[6] and was subsequently shown to greatly increase HIV transcription and replication.[7] We have shown that intracellular thiols are rapidly depleted in certain cell types by stimulation with tumor necrosis factor α (TNFα) and/or phorbol myristate acetate (PMA) under conditions that efficiently activate NF-κB. Diamide pretreatment depletes intracellular GSH and markedly facilitates the cytokine-stimulated NF-κB activation. By contrast, *N*-acetyl-L-cysteine (NAC), a nontoxic drug used clinically to replenish GSH and scavenge intracellular oxidants, replenishes intracellular thiols and inhibits or prevents NF-κB activation. Thus, the transduction of the NF-κB activation signal generated by stimulation with TNFα and certain other cytokines proceeds when thiol levels are low and is decreased or blocked when thiols are maintained at high levels.[4]

HIV replication would be expected to proceed more rapidly in immunosuppressed individuals who have infections that induce inflammatory responses. Indeed, serum levels of TNFα and other inflammatory cytokines are known to become progressively

[a]This work was supported by Genetics Department Funds (F. J. T. Staal) and NIH Grants CA42509 and HD01287.

elevated,[8-12] whereas plasma thiol- and leukocyte-GSH levels are lower in HIV-infected individuals. We hypothesize that inflammatory responses in HIV-infected individuals shift the thiol/oxidant balance so that NF-κB activation and HIV transcription are favored. The findings presented above prompted us to investigate the intracellular GSH levels in B and T lymphocytes of HIV-infected individuals. Previous studies, using bulk biochemical assays to measure GSH, have shown that HIV$^+$ individuals have lower cystine levels than uninfected controls in plasma,[13] GSH levels in lung epithelial lining fluid,[14] and GSH in extracts from peripheral blood mononuclear cells (PBMC).[13] Studies presented here, employing methods to study individual cells instead of bulk assays, confirm and substantially extend these findings. We use multiparameter fluorescence-activated cell sorter (FACS) analyses to measure intracellular GSH levels in PBMC subsets (T cells, B cells, and monocytes) from 123 HIV$^+$ individuals whose clinical status ranges from asymptomatic to AIDS.

GSH LEVELS IN T CELLS FROM HIV-INFECTED INDIVIDUALS ARE LOWERED

Rice *et al.*[15] introduced the use of monochlorobimane (MCB) as a FACS-based measurement for intracellular GSH levels. MCB, which is nonfluorescent and readily crosses the cell membrane, is coupled intracellularly to GSH by GSHS-transferase. The resulting adduct is fluorescent and trapped within cells. Under appropriate conditions, the intracellular fluorescence is proportional to the GSH content within the cells;[16] thus, the FACS can be easily used to quantitate intracellular GSH levels. We have extended this assay for use with immunophenotyping, in order to determine the precise levels of GSH within defined subsets of PBMC.

Surprisingly, these measurements showed that there are distinct types of human CD4$^+$ and CD8$^+$ T cells based on intracellular GSH levels.[17] Unlike in the mouse, human T cells can be divided into high-GSH cells and low-GSH cells. Although the relative proportion of these two classes varies considerably from individual to individual, the absolute GSH levels within these cells are highly conserved among individuals (FIG. 1).

Analysis of GSH levels in T cells from 123 HIV-infected individuals reveals significant differences from normals. The high-GSH T cells are selectively lost (in both the CD4$^+$ and CD8$^+$ T-cell subpopulations), a process that is already apparent in the early stages of the disease (FIG. 2). Individuals in two uninfected control groups (25 at random, healthy controls and 7 uninfected homosexual males (at risk-control)) have similar GSH levels and always have high-GSH T cells (FIG. 3). Using the Mann-Whitney U test, HIV$^+$ patients had significantly lower relative intracellular GSH than the control groups in CD4$^+$ T cells (asymptomatic: 74% of normal, $p = 0.0023$; ARC: 67% of normal, $p = 0.003$; AIDS: 62% of normal, $p = 0.0001$) and CD8$^+$ T cells (asymptomatic: 74% of normal, $p = 0.001$; ARC: 69% of normal, $p = 0.003$, AIDS: 66% of normal, $p = 0.0001$).

The selective loss of high-GSH T cells in HIV-infected individuals is further illustrated in FIGURE 4, which shows the intracellular GSH level at the 10th, 50th (median), and 90th percentiles for CD4$^+$ and CD8$^+$ T cells in each individual, averaged for each subject category. As the FIGURE shows, the 90th percentile values are much lower in HIV-infected individuals, whereas the 10th percentile GSH-value does not change significantly. Because the 90th percentile value is located within the high-GSH class in uninfected individuals, the strong decline seen in this value for HIV-infected subjects points to a selective loss of high-GSH T cells. By contrast, the relative constancy of

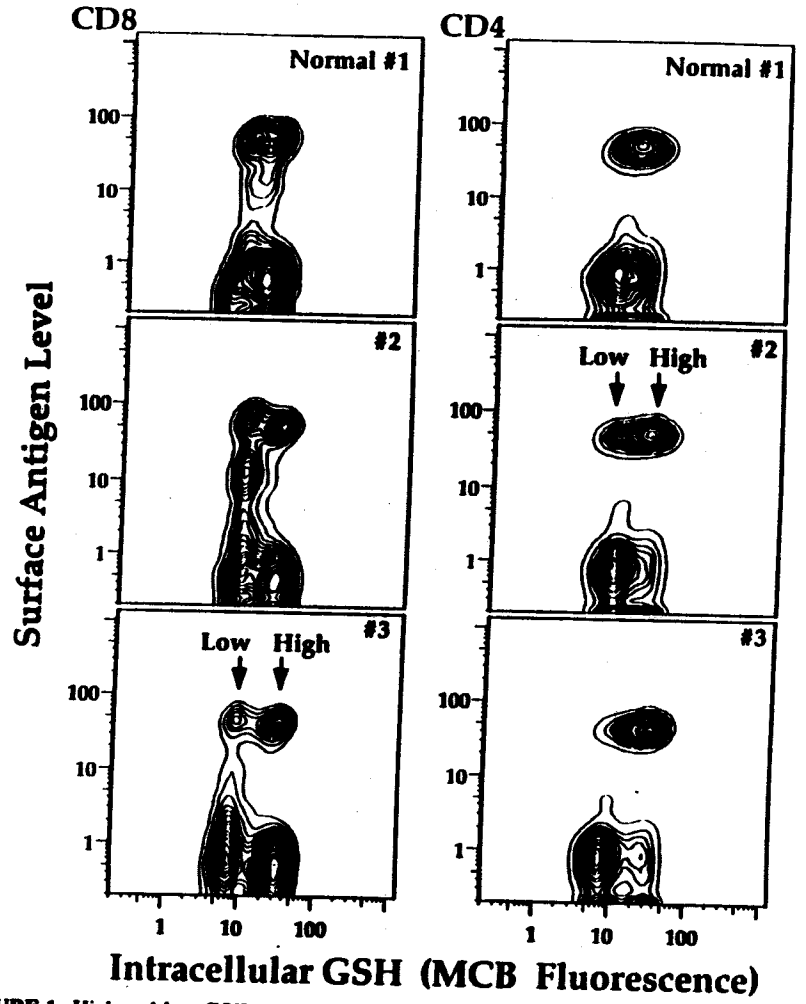

FIGURE 1. High and low GSH-containing T-cell subsets in three normal, healthy individuals. The intracellular GSH level (as determined by MCB fluorescence) is plotted versus CD4 or CD8 expression. There is variation in the frequency of the high GSH subset among individuals, but all healthy individuals have these cells.

the 10th percentile values indicates that cells in the low-GSH subset tend not to lose GSH.

Calculating the ratio of GSH levels at the 90th and 10th percentiles for CD4$^+$ and CD8$^+$ T cells in individual subjects provides a measure of the representation of the high GSH subclass. This 90/10 ratio is independent of the relative GSH level, so that normalization to a control subject is not required. For both CD4$^+$ and CD8$^+$ T cells, the 90/10 ratio is significantly different at the 95% confidence level between normal controls and asymptomatic HIV$^+$ individuals, and between ARC and AIDS patients.

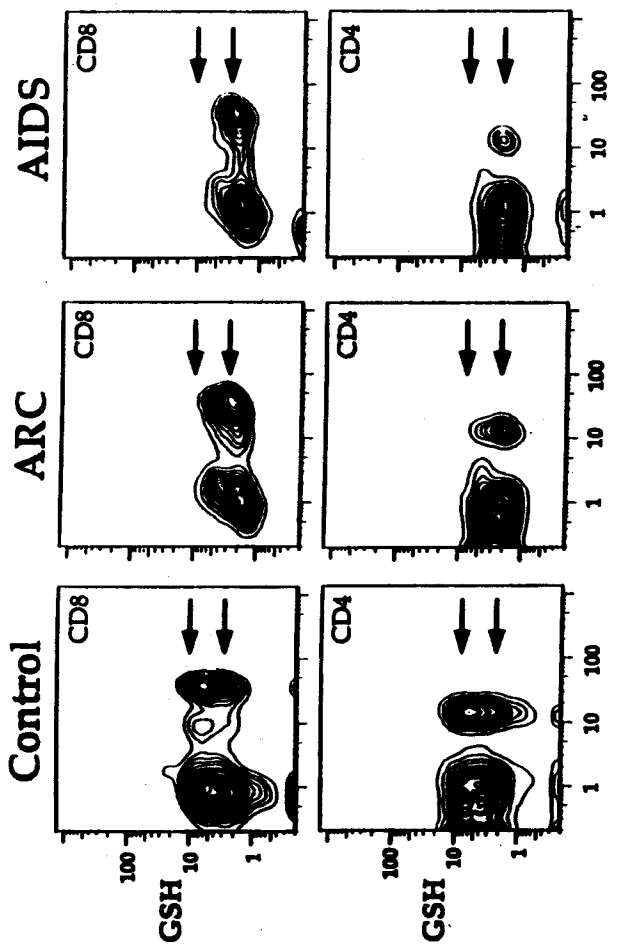

FIGURE 2. FACS analysis of intracellular GSH in $CD4^+$ and $CD8^+$ T cells. Dual parameter plot of GSH level (MCB fluorescence) versus CD4 or CD8 fluorescence. Left: uninfected control; middle: ARC patient; right: AIDS patient. The frequency of the high GSH-containing cells is decreased in HIV-infected persons.

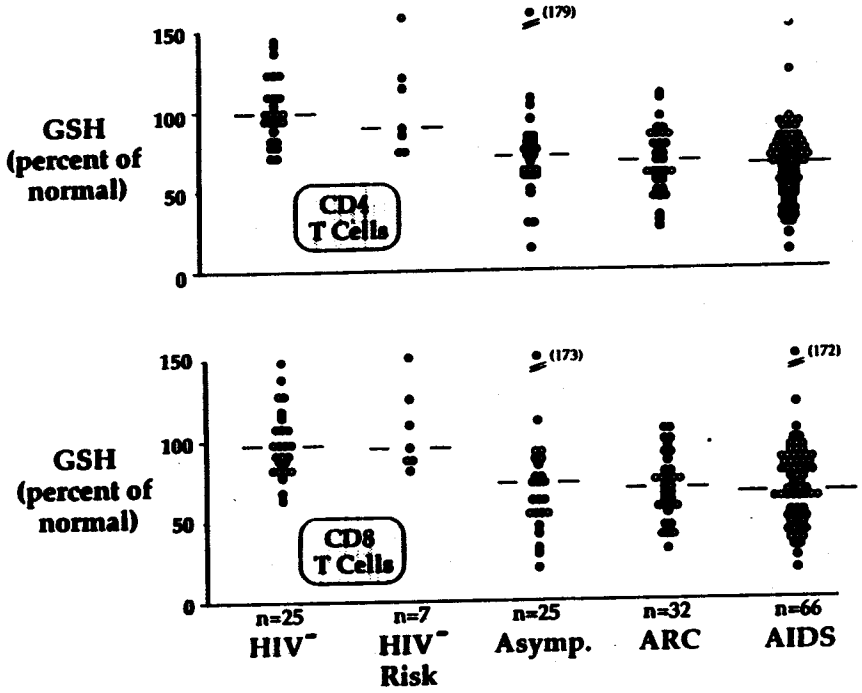

FIGURE 3. Intracellular GSH levels in $CD4^+$ and $CD8^+$ T cells are lower in HIV-infected individuals than in uninfected controls. Intracellular GSH is determined as the median MCB fluorescence, with the median of normal healthy controls (random control) set as 100 for each subset. Each individual is indicated with a small circle; the median GSH level for each category is shown with a bar.

In fact, using this method, we detect a significant difference between asymptomatic individuals and AIDS patients (Scheffe F-test, 95% confidence level).

The loss of the high-GSH T cells can be explained by a number of hypotheses, none of which has been ruled out: (1) high GSH cells are sensitive to other conditions of the HIV infections (*e.g.* high TNFα levels) and selectively die; (2) the high GSH levels are more difficult to maintain, especially under the oxidative stresses of opportunistic infections or high TNFα levels, and the cells are converted into low-GSH cells; (3) the *de novo* generation of these cells is altered by some aspect of the HIV infection.

The decline in GSH levels in the T cells of HIV-infected individuals does not appear to be directly related to the loss of CD4 (or CD8) cells, inasmuch as there is no correlation between the absolute number of cells remaining in either of these subsets with the intracellular level of GSH per cell. By contrast, there is a strong correlation ($r = 0.88$) between the levels of GSH in each person's CD4 and CD8 cells, both in HIV-infected individuals and in uninfected individuals.

GSH LEVELS AND CD20 EXPRESSION IN B LYMPHOCYTES DURING HIV INFECTION

The most striking B-cell abnormality in the HIV infection is the hypergammaglobulinemia present in over 90% of patients with AIDS.[18] This increase in serum Ig consists

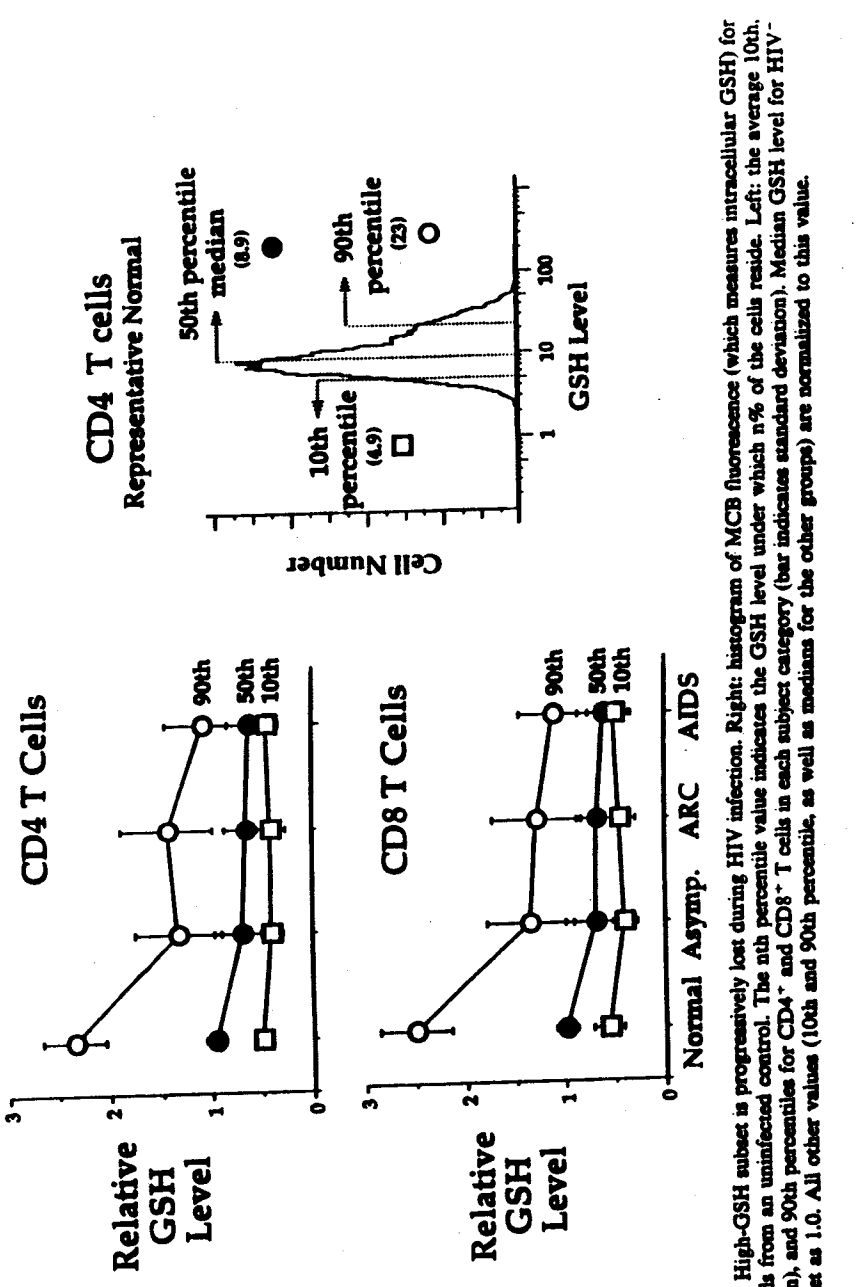

FIGURE 4. High-GSH subset is progressively lost during HIV infection. Right: histogram of MCB fluorescence (which measures intracellular GSH) for $CD4^+$ T cells from an uninfected control. The nth percentile value indicates the GSH level under which n% of the cells reside. Left: the average 10th, 50th (median), and 90th percentiles for $CD4^+$ and $CD8^+$ T cells in each subject category (bar indicates standard deviation). Median GSH level for HIV^- controls is set as 1.0. All other values (10th and 90th percentile, as well as medians for the other groups) are normalized to this value.

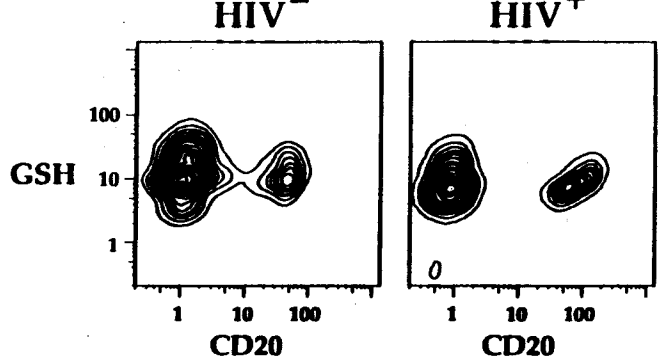

FIGURE 5. CD20 expression changes upon HIV infection and is correlated with intracellular GSH level. Dual parameter plot of MCB fluorescence versus CD20 fluorescence (CD20 expression). Left: uninfected control; right: HIV-infected individual (representative example).

almost primarily of IgG and IgA and is almost always polyclonal in nature, except when superseded by a monoclonal Ig as the manifestation of an evolving non-Hodgkin's lymphoma.[19] Concomitant with this hypergammaglobulinemia may be an increase in autoantibody production and an increased incidence of circulating immune complexes.

In vitro manifestations of B-cell abnormalities are well-documented[20,21] and can be summarized as follows: (1) increased number of circulating B cells spontaneously secreting Ig; (2) decreased proliferative response of B cells to T cell-dependent (PWM) and T cell-independent (SAC) mitogens (it is thought that the inability of B cells in AIDS to respond to mitogens is because they are maximally stimulated rather than being due to some irreversible intrinsic effect); and (3) inability of B cells in AIDS to mount a humoral response to new specific antigens. (This has been demonstrated by the inability of these cells to respond to keyhole limpet hemocyanin (KLH) and to sheep red blood cells (SRBC).)

Here we report additional abnormalities in B lymphocytes during HIV infection. We have focused on the expression of the classical pan B-cell marker CD20 and on the intracellular GSH levels in B cells. The CD20 antigen has been cloned[22] and has been shown to function as a Ca channel.[23] CD20 regulates B-cell activation and is differently phosphorylated in resting and activated cells;[24] anti-CD20 antibodies trigger a resting B cell to enter the cell cycle,[25] and CD20 expression is increased up to fivefold on *in vitro*-activated B cells (T. Tedder, personal communication).

B lymphocytes from HIV-negative individuals that a homogeneous CD20 expression and GSH level, as revealed by FACS-staining of PBMC isolated by Ficoll density centrifugation. By contrast, all HIV-infected individuals whom we have examined show a shift in their B-cell staining pattern. In most individuals, GSH levels remain in the normal range, whereas CD20 expression is increased; however, the increase in CD20 is correlated in the GSH level (FIG. 5). This results in a distinctive diagonal when plotting GSH levels versus the expression of CD20. One of the individuals we investigated appeared to have been infected 2-3 weeks before analysis, and his B cells showed this shift, too. This indicates that the alteration in staining pattern occurs very early after infection with HIV.

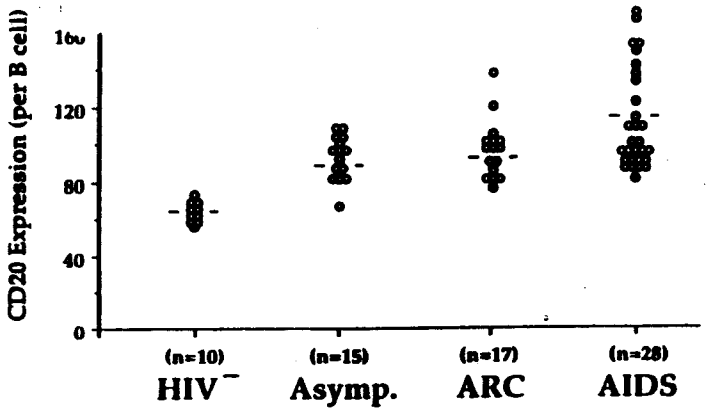

FIGURE 6. Increased CD20 expression on B cells of HIV-infected individuals. CD20 expression was determined as median fluorescence of FITC-labeled anti-CD20 antibody. Each individual is indicated by a small circle; mean CD20 expression for each category is shown with the bar.

As demonstrated in FIGURE 6, essentially all HIV-infected individuals have a higher CD20 expression on their peripheral B lymphocytes. The increased CD20 expression is strongest in the AIDS patients (on average almost twofold); the CD20 expression is significantly different for all HIV-infected individuals as compared to normal (at the 99% confidence level), as well as for the AIDS patients compared to asymptomatic and ARC (Scheffe F-test). In our data set, there is a strong, negative correlation between the number of $CD4^+$ T cells and the CD20 expression on the B cells in a given individual (nonparametric Spearman rank correlation test, $p < 0.0001$). Thus, AIDS patients have increased expression of CD20 and decreased numbers of $CD4^+$ T cells.

We have also measured the frequency of $CD5^+$ B cells on all individuals included in our study. Thus far we have not observed any significant differences in the frequency of $CD5^+$ B cells in infected versus noninfected individuals (data not shown). We have observed that $CD5^+$ B cells have a slightly higher GSH content than conventional B cells, but this is the case in both infected and uninfected individuals. Another marker included in our multicolor FACS analysis is the activation antigen CD25, which corresponds to the IL2 receptor. The expression of IL2-R was not different (that is, essentially absent) in HIV-infected patients as compared to our uninfected control group.

The median GSH level in the B cells of HIV-infected individuals varies considerably. Some patients have decreased intracellular GSH levels (as low as 45% of normal), and some have increased levels (up to 180% of normal). By contrast, uninfected individuals have a very narrow and highly conserved range of GSH level in their B cells. This heterogeneity in GSH levels and the shift in GSH-CD20 staining pattern indicate that the regulation of GSH is altered in B lymphocytes from HIV-infected individuals.

In summary, we have identified the following abnormalities in B lymphocytes from HIV-infected individuals: (1) HIV-infected individuals have increased expression of the classical pan B-cell marker CD20, (2) the expression of CD20 is stronger in the later stages of HIV infection, (3) the range of CD20 expression is wider in HIV-infected individuals than in uninfected controls, (4) the GSH levels of HIV-infected individuals are very heterogeneous: both decreases and increases are observed, and (5) intracellular GSH levels and CD20 expression become correlated.

CONCLUDING REMARKS

We have found significant alterations in the GSH status of B lymphocytes and T lymphocytes of HIV-infected individuals. The critical need for adequate levels of GSH in lymphocyte function is well-established: GSH is important for mixed lymphocyte reactions,[26] T-cell proliferation,[26] T- and B-cell differentiation,[27] cytotoxic T-cell activity,[27] NK activity,[28] and cell protection against oxidants.[29] In view of the requirement of adequate GSH levels for proper lymphocyte function, it is possible that a decrease in the levels of this metabolite may contribute the immunodeficiency seen in the later stages of HIV infection.

A correlate of the findings presented is that measurement of intracellular GSH levels in T cells and CD20 expression on B cells (or the combination of CD20 expression and GSH levels) could provide new surrogate markers for HIV infection. The use of these markers can overcome the need to use mortality or the development of severe opportunistic infections as end points in clinical trials. Thus, these kinds of measurements may provide meaningful methods for clinical staging and prognosis for HIV infection.

ACKNOWLEDGMENTS

We thank Drs. Jeff Bubp, Lary Mole, Dennis Israelski, and Stanley Deresinski for providing patient samples and clinical data. We thank Paul Raju, Michael Anderson, and Jeff Bubp for assistance with the experiments. The generous gift of monoclonal antibodies by Becton Dickinson and the support from "Project Inform," Zambon Inc., and Digital Equipment Inc. are highly appreciated.

REFERENCES

1. KOSOWER, E. M. & N. S. KOSOWER. 1969. Lest I forget thee, glutathione. Nature 224 (215): 117-120.
2. DOLPHIN, D., O. AVRAMOVIC & R. POULSON. 1989. Glutathione: Chemical, Biochemical, and Medical Aspects. Coenzymes and Cofactors. Vols. 3A: 476-517 and 3B: 45-85. John Wiley. New York.
3. TANIGUCHI, N., T. HIGASHI, Y. SAKAMOTO & A. MEISTER. 1989. Glutathione Centennial: Molecular Properties and Clinical Applications. Academic Press. New York.
4. STAAL, F. J. T., M. ROEDERER, L. A. HERZENBERG & L. A. HERZENBERG. 1990. Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA 87: 9943-9947.
5. ROEDERER, M., F. J. T. STAAL, P. A. RAJU, S. W. ELA, L. A. HERZENBERG & L. A. HERZENBERG. 1990. Cytokine-stimulated HIV replication is inhibited by *N*-acetyl-L-cysteine. Proc. Natl. Acad. Sci. USA 87: 4884-4888.
6. SEN, R. & D. BALTIMORE. 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46: 705-716.
7. NABEL, G. & D. BALTIMORE. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 326: 711-713.
8. LAHDEVIRTA, J., C. P. J. MAURY, A.-M. TEPPO & H. REPO. 1988. Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. Am. J. Med. 85: 289-291.

9. MINTZ, M., R. RA IT, J. M. OLESKE, E . CONNOR, M. R. KOENIGSBERGER, T. DENNY & L. G. . TEIN. 1989. Elevated serum levels of tumor necrosis factor are associated with progressive encephalopathy in children with acquired immunodeficiency syndrome. Am. J. Dis. Child. 143: 771-774.
0. FUCHS, D., A. HAUSEN, G. REIBNEGGER, E. R. WERNER, G. WERNER-FELMAYER, M. P. DIERICH & H. WACHTER. 1989. Interferon-gamma concentrations are increased in sera from individuals infected with human immunodeficiency virus type 1. J. AIDS 2: 158-162.
1. FUCHS, D., G. M. SHEARER, R. N. BOSWELL, M. CLERICI, G. REIBNEGGER, E. R. WERNER, R. A. ZAJAC & H. WACHTER. 1990. Increased serum neopterin in patients with HIV-1 infection is correlated with reduced *in vitro* interleukin-2 production. Clin. Exp. Immunol. 80: 44-48.
2. BREEN, E. C., A. R. REZAI, K. NAKAJIMA, G. N. BEALL, R. T. MITSUYASU, T. HIRANO, T. KISHIOTO & O. MARTINEZ-MAZA. 1990. Infection with HIV is associated with elevated IL-6 levels and production. J. Immunol. 144: 480-484.
3. ECK, H.-P., H. GMUNDER, M. HARTMANN, D. PETZOLDT, V. DANIEL & W. DROGE. 1989. Low concentrations of acid-soluble thiol (cysteine) in the blood plasma of HIV-1-infected patients. Biol. Chem. Hoppe-Seyler 370: 101-108.
4. BUHL, R., K. J. HOLROYD, A. MASTRANGELI, A. M. CANTIN, H. A. JAFFE, F. B. WELLS, C. SALTINI & R. G. CRYSTAL. 1989. Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. Lancet ii: 1294-1298.
5. RICE, G. C., E. A. BUMP, D. C. SHRIEVE, W. LEE & M. KOVACS. 1986. Quantitative analysis of cellular glutathione by flow cytometry utilizing monochlorobimane: some applications to radiation and drug resistance *in vitro* and *in vivo*. Cancer Res. 46: 6105-6110.
6. ROEDERER, M., L. A. HERZENBERG & L. A. HERZENBERG. 1992. Glutathione levels define leukocyte subsets. In preparation.
7. ROEDERER, M., F. J. T. STAAL, H. OSADA, L. A. HERZENBERG & L. A. HERZENBERG. 1991. CD4 and CD8 T cells with high intracellular glutathione levels are selectively lost as the HIV infection progresses. Int. Immunol. 3: 933-937.
8. LANE, H. C., H. MASUR, L. C. EDGAR & A. S. FAUCI. 1983. Abnormalities of B cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N. Engl. J. Med. 309: 453-458.
9. KATZ, I. R., S. E. KROWN & B. SAFAI. 1986. Antigen specific and polyclonal B cell responses in patients with AIDS. Clin. Immunol. Immunopathol. 39: 359-367.
0. LANE, H. C., J. M. DEPPER, W. C. GREENE & A. S. FAUCI. 1985. Qualitative analysis of immune function in patients with AIDS. Evidence for a selective defect in soluble antigen recognition. N. Engl. J. Med. 313: 79-83.
1. SCHNITTMAN, S. M., H. C. LANE & S. E. HIGGINS. 1986. Direct polyclonal activation of human B lymphocytes by the acquired immune deficiency virus. Science 233: 1084-1086.
2. CLARK, E. A. & J. A. LEDBETTER. 1989. Structure, function and genetics of human B cell-associated surface molecules. Adv. Cancer Res. 52: 81-149.
3. TEDDER, T. F., L. J. ZHOU, P. D. BELL, R. A. FRIZELL & J. K. BUBIEN. 1990. The CD20 surface molecule of B lymphocytes functions as a calcium channel. J. Cell Biochem. 14D(Suppl.): 195.
4. VALENTINE, M. A., K. E. KEIER, S. ROSSIE & E. A. CLARK. 1989. Phosphorylation of the CD20 phosphoprotein in resting B lymphocytes: regulation by PKC. J. Biol. Chem. 264: 11282-11287.
5. CLARK, E. A., G. L. SHU, B. LUSCHER, K. E. DRAVES, J. A. LEDBETTER & M. A. VALENTINE. 1989. Activation of human B cells. Comparison of the signal transduced by Il-4 to four different competence signals. J. Immunol. 140: 4071-4078.
6. MEISTER, A. 1988. Glutathione metabolism and its selective modification. J. Biol. Chem. 263: 17205-17208.
7. TSAMILOS, D. L. & H. J. WEDNER. 1985. The role of glutathione in lymphocyte activation. I. Comparison of inhibitory effects of buthionine sulfoximine and 2-cyclohexene-1-one on nuclear size transformation. J. Immunol. 135: 2740-2747.
8. DROGE, W., C. POTTMEYER-GERBER, H. SCHMIDT & S. NICK. 1986. Glutathione augments the activation of cytotoxic T lymphocytes *in vivo*. Immunobiology 172: 151-156.
9. GRIFFITH, O. W. & A. MEISTER. 1979. Glutathione; interorgan translocation, turnover, and metabolism. Proc. Natl. Acad. Sci. USA 76: 5606-5610.