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Disregulation of Leukocyte Glutathione in AIDS

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AIDS is a complex disease, encompassing a wide variety of symptoms: immune deficiency, consisting of both immunosuppression and immune activation; loss of CD4 T cells; metabolic disorders (wasting); increased opportunistic infections; inflammatory stress; neurological deficiencies; and increased occurrences of cancers. This wide variety of effects is paradoxical in view of the relatively small number of infected cells present in an infected individual.

It is this paradox that has led to the conclusion that AIDS is accompanied by a widespread disregulation of immune function. Dysfunctions have been noted in almost all compartments of the immune system: in particular, anergy and other functional abnormalities of T cells, B cells, and monocytes (reviewed in reference 1). It is now becoming evident that at least part of the reason for the anergy is a result of overstimulation. Overstimulation could occur through cytokine receptors (brought about by increased levels of cytokines), through CD4 (by gp120), or possibly through viral products such as TAT (which can activate uninfected cells). The chronic, low-level stimulation results in a refractory (anergic) state, causing a lack of further responsiveness and possible apoptosis in response to stimulation. Several groups have postulated that this process is responsible for the loss of CD4 T cells that is characteristic of AIDS (e.g., see reference 2).

Several years ago, Dröge and colleagues demonstrated a significant alteration in the serum levels of several amino acids in the sera from HIV-infected individuals.[3] Since then, several confirming observations have lent credence to the hypothesis that AIDS is accompanied by a metabolic disregulation. At the level of the individual, this is evidenced by the common "wasting" syndrome, during which infected individuals have severely depressed appetites, reduced absorption of nutrients, and weight loss.[4] Wasting is often evident during full-blown AIDS and is usually followed by death.

Perhaps most significant of these observations was the depression in cysteine and glutathione (GSH) levels in sera, peripheral blood mononuclear cells (PBMC), and lung-lining fluid from infected individuals.[3,5–8] GSH, a cysteine-containing tripeptide, is the major intracellular source of free thiol and thus the major antioxidant. GSH plays important roles in many cellular processes, including DNA synthesis, enzymatic reactions, and (most importantly) the protection against damage by oxidizing agents, free radicals, and reactive metabolic intermediates.[9,10]

We have adapted an assay for measuring intracellular GSH by FACS[11] in order to

determine the GSH levels in defined subpopulations of PBMC.[6] This simple assay relies on the intracellular reaction of monochlorobimane (MCB) with GSH (catalyzed by GSH-*S*-transferase) to form a highly fluorescent adduct that is trapped within the cells. The fluorescence is proportional to the total cell-associated GSH and can be measured by excitation with a UV laser (e.g., 361-nm line from an argon ion laser) and emission anywhere from 460 to 560 nm. It is suitable for use with simultaneous immunofluorescence measurements using monoclonal antibodies conjugated with fluorescein, phycoerythrin, or the tandem dye cyanine-5-phycoerythrin (all excited by the 488-nm line from an argon ion laser). Cells can be fixed with

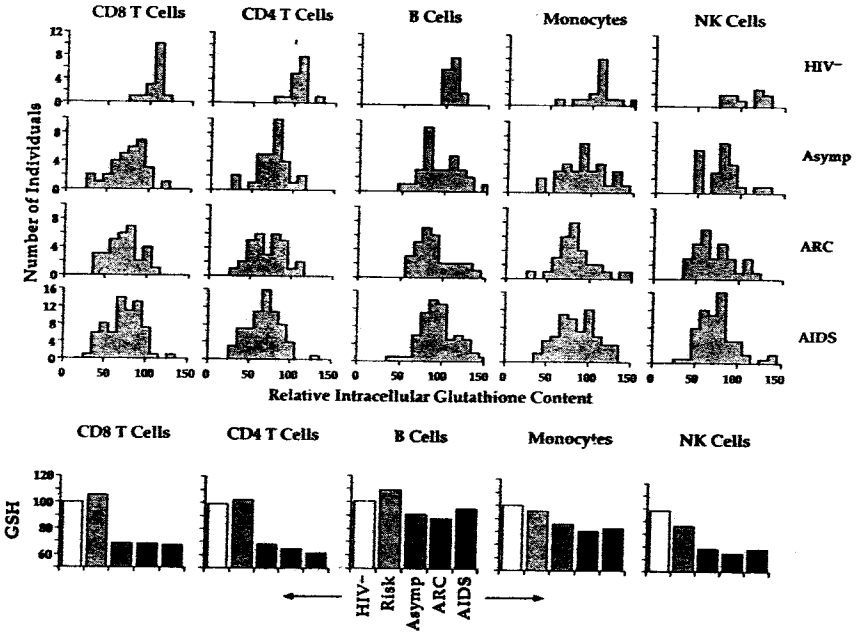

FIGURE 1. (Top) The distribution of the median GSH level in PBMC subsets for different categories of individuals is shown as a histogram. (Bottom) The mean GSH level in each PBMC subset is shown for each category. This cohort has been described previously.[7] Although there is a disregulation in the GSH levels for all subsets, there is a statistically significant decline in the levels for CD4 and CD8 T cells. The decline is not correlated with the stage of the infection.

paraformaldehyde to inactivate HIV with no effect on any of the measured parameters. (See APPENDIX for a complete description of the method.)

Using this assay, we have confirmed the observations by Dröge and Crystal in which the intracellular GSH levels from PBMC are decreased in HIV-infected individuals.[7,12,13] However, this decrease is confined primarily to the T cells;[7] B cells and monocytes show some loss, but it is not statistically significant (FIGURE 1). The loss of GSH was not correlated with the absolute CD4 number and was only slightly correlated with the stage of the disease. This suggests that the disregulation of intracellular GSH occurs very early after infection with HIV, as do many of the immune dysfunctions.

A most interesting aspect of the loss of GSH in the T cell compartment is that it primarily due to the loss of a specific class of T cells with unusually high intracellul GSH levels.[6,7] All normal healthy individuals have heterogeneous levels of GSH both CD4 and CD8 T cells; this heterogeneity is considerably larger than that foun in other leukocyte subpopulations. The high-GSH T cells are usually evidenced by shoulder in the distribution of GSH (FIGURE 2A). However, the distributions GSH in T cells from HIV-positive individuals are markedly different. In thes individuals, there is generally a homogeneous (log-normal) distribution of GS levels (FIGURE 2B), as is the case for other PBMC subpopulations in both infecte and uninfected individuals. Note that the loss of high-GSH T cells cannot be ascribe to the loss of CD4 T cells in general because it occurs in both the CD4 and CD lineages equally.

Because of the difficulty in quantitating the frequency of high- and low-GSH cells (due to the considerable overlap in their distributions), we devised an analytic method to represent the distributions of GSH in the T cells.[7] This method, in essence, measures the width of the distribution of GSH (as, for instance, the coefficient of variation does). Because the relative frequency of high- and low-GSH cells varies from 20% to 80% in normal individuals, we chose to determine the GSH content of the 10th, 50th, and 90th percentiles in the distribution for T cells in each individual (the *n*-th percentile is that amount of GSH for which *n*% of the cells have less GSH; thus, the 50th percentile is the median GSH content for the subset). If the assumption is made that the GSH content of the high- and low-GSH T cells does not vary from individual to individual (whereas the relative frequency of the two types does vary, from 20% to 80%), then the 10th and 90th percentiles will be relatively constant across individuals. This is because the 10th percentile will always be in the low-GSH population and the 90th percentile will always be in the high-GSH population. (Note that the median will fall in the more prevalent population.)

Finally, by calculating the ratio of the 90th to the 10th percentile, we can obtain an estimate of the width of the distribution of GSH in a population of cells. A specific loss of high-GSH T cells would be evidenced by a decrease in both the 90th percentile and the 90:10 ratio. A specific loss of low-GSH T cells would be evidenced by an increase in the 10th percentile and a decrease in the 90:10 ratio. Last, if GSH were lost from all cells without preference for the high- or low-GSH T cells, then both the 90th and the 10th percentiles would fall and the ratio could stay the same.

This analysis confirms the visual impression obtained from FIGURE 2B: namely there is a specific loss of the high-GSH T cells (FIGURE 3). Interestingly, there is no decline in the GSH from the low-GSH T cells, much like the case for B cells and monocytes.

The major advantage of the 90:10 ratio is that it does not require standardization of the MCB assay. It is also independent of the time of labeling with MCB and of the concentration of MCB in the assay (data not shown), making it a far more rigorous analysis than median MCB levels. Finally, the 90:10 ratio shows the most significant changes in intracellular GSH between HIV-infected and uninfected individuals. In fact, there is even a statistically significant decline from the asymptomatic to AIDS stage.[7]

We have begun to try to determine phenotypic differences between the high- and low-GSH T cells, in addition to the differences in GSH content. Analysis with a number of fluorescently conjugated antibodies against cell surface determinants

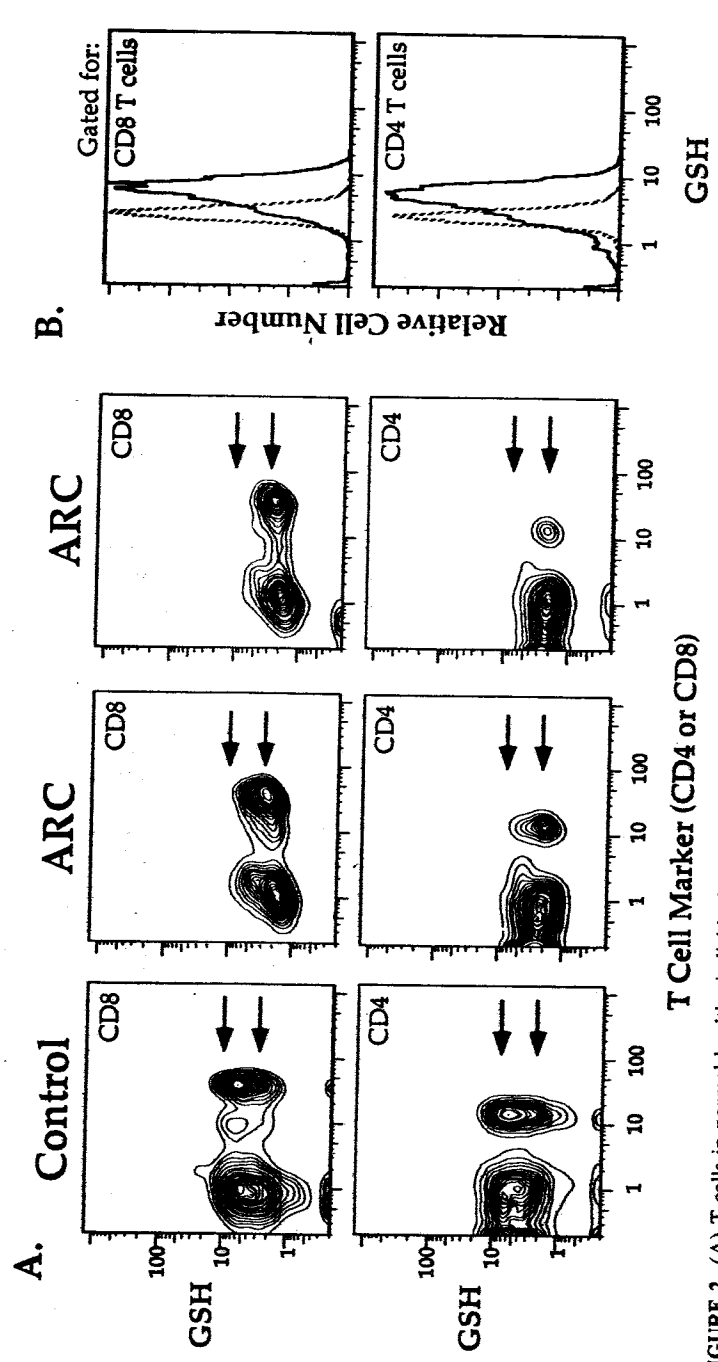

FIGURE 2. (A) T cells in normal healthy individuals have a heterogeneous GSH distribution, including low- and high-GSH T cells (arrows in the leftmost panels). The HIV-infected individuals selectively lose the high-GSH T cells. (B) Histograms of the GSH distribution in CD8 T cells (top) or CD4 T cells (bottom) for the uninfected individual (solid line) or for the first HIV-infected individual (hatched line). There is no size-dependence of the intracellular GSH for these cells. The low end of the GSH distribution does not shift; however, the high end decreases dramatically. See also FIGURE 3. (Reproduced from reference 7.)

demonstrates that there are indeed some subtle differences between the classes. Perhaps the most significant difference is that virtually all of the high-GSH T cells are CD45RA$^+$ and CD45RO$^-$ ("virgin" T cells), whereas the low-GSH T cells can be found in both types of T cells (virgin T cells and CD45RA$^-$, CD45RO$^+$ "memory" T cells; FIGURE 4). By itself, this proves a functional difference between the high- and low-GSH T cells. There are many functional differences between the CD45RA$^+$ and the CD45RA$^-$ T cells already known (e.g., see references 14 and 15). However, it

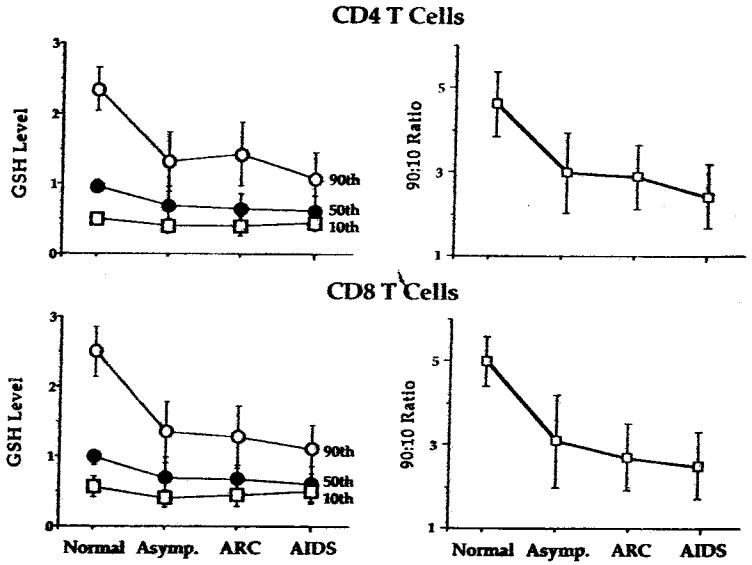

FIGURE 3. Analysis of the GSH distribution with T cells reveals that high-GSH cells are specifically lost. (Left) The values for the 10th, 50th (median), and 90th percentile GSH levels in CD4 and CD8 T cells were averaged for each subject category (error bars are ±1 SD). There is a dramatic decrease in the 90th percentile (which represents the high-GSH T cells) and virtually no change in the 10th percentile (which represents the low-GSH T cells). These values are normalized to uninfected controls; the 50th percentile (median) for uninfected controls is set to 1.0. (Reproduced from reference 7.) (Right) The ratio of the 90th to the 10th percentile, a value that is independent of normalization, was calculated for each individual and was then averaged by subject category. Using this parameter, the difference between the asymptomatic and AIDS categories is significant ($p < 0.05$). This suggests that there is a progressive deterioration in the GSH regulation with advancement of the disease.

remains to be determined whether there are functional differences between the CD45RA$^+$ high-GSH and the CD45RA$^+$ low-GSH cells.

Other cell surface phenotypic differences can also be ascribed to the high- and low-GSH T cells (TABLE 1). However, at this time, there is no combination of antibody staining that will resolve the two classes of T cells. Based on the phenotypes listed in TABLE 1, it is tempting to postulate that the high-GSH T cells are those that have never been stimulated by antigen (i.e., newly arising) because they have none of the activation phenotypes. This hypothesis is strengthened by our observation that, in

thymic tissue, mature single-positive thymocytes have high GSH levels, whereas the immature double-positive thymocytes have low GSH levels (unpublished observations).

In terms of the HIV-infected individuals, in whom the high-GSH T cells are lost, there are several reports of disruption of the memory/virgin distribution of T cells.[16–19] However, there is some disagreement as to what the exact defect may

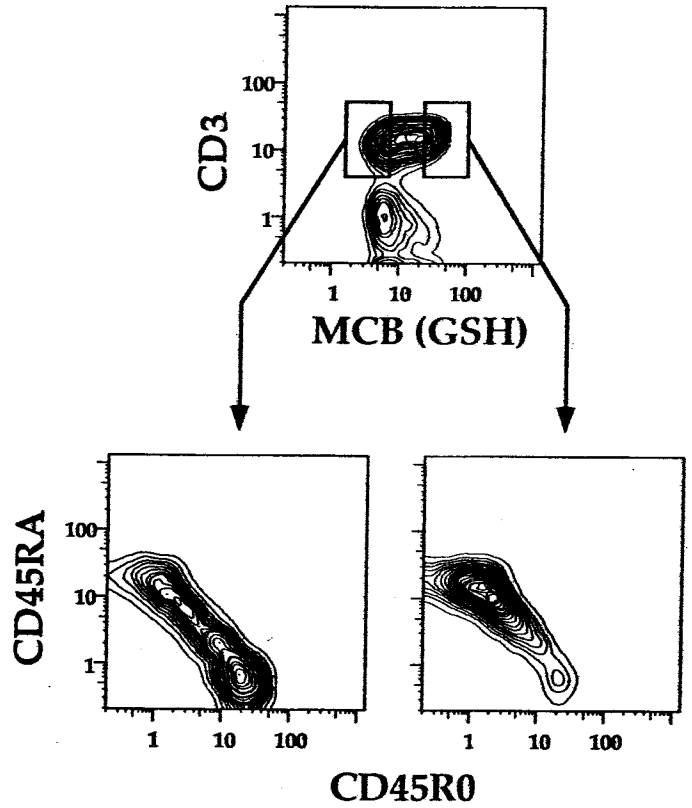

FIGURE 4. High-GSH T cells are predominantly CD45RA$^+$. PBMC from a normal individual were simultaneously stained for GSH, CD3, CD45RA, and CD45RO. The high- and low-GSH T cells were selected by software gating (top panel); the CD45RA and CD45RO distributions for these cells are shown (bottom panels). The high-GSH T cells are almost exclusively CD45RA$^+$; the low-GSH T cells are of both CD45RA$^+$ and CD45RO$^+$ phenotypes.

be.[20,21] In any case, our observations that the high-GSH T cells are lost after HIV infection do not necessarily imply a loss of virgin T cells: it could be that these cells have simply lost the high level of intracellular GSH, but are still resident in the periphery. In fact, we cannot distinguish between the following hypotheses that can account for the loss of high-GSH T cells: (1) specific killing of these cells; (2) specific removal from the periphery of these cells; (3) a disruption in the production of novel

TABLE 1. Phenotype of the GSH-defined T Cell Classes

Marker	Low-GSH T Cells	High-GSH T Cells
CD45RA	+ or −	+
CD45RO	+ or −	−
CD7	+ or −	+
CD56	+ or −	−
CD2	+ or ++	+
Leu8	+ or −	+
CD11a	+ or ++	+
CD18	+ or ++	+
CD44	+ or ++	+

high-GSH T cells; and, as mentioned, (4) the loss of GSH to become low-GSH T cells.

Because of the GSH (and other low molecular weight thiol) deficiency in HIV-infected individuals, we and others have suggested that GSH replacement

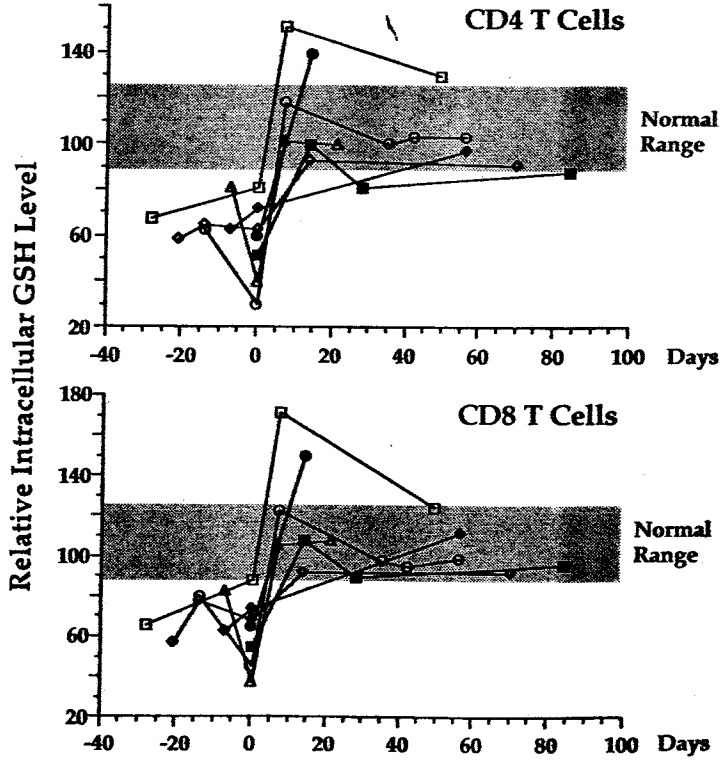

FIGURE 5. Oral NAC can restore GSH levels in T cells in HIV-infected individuals. See TABLE 2 for information about these individuals. The median GSH level in CD4 (top) or CD8 (bottom) T cells is shown as a function of the time at which NAC administration began. The shaded region is the range of GSH levels in 30 of 31 uninfected individuals.

TABLE 2. Profile of Patients Taking NAC[a]

Patient ID	Sex	Age	Race[b]	Risk Group	CDC Class[c]	CD4 Count[d]	Symptoms[e]	Medication[f]	NAC (mg/day)[g]	Symbol in FIGURE 5
SM020	M	43	C	homosexual	AIDS	125	Kaposi's sarcoma past PCP cachexia	AZT pentamidine	1000[h]	□
SM023	M	31	C	homosexual	ASX	17		AZT multivitamin	2000	○
SM027	M	38	H	homosexual	AIDS	23	cachexia	AZT septra	2000	●
SM037	M	43	C	homosexual	ARC	55	VZV	AZT, DDC, vitamin C	1500	■
SM039	M	50	C	homosexual	ASX	158		DDC vitamin C	1500	◇
SM043	M	31	C	heterosexual, multiple partners	AIDS	26	PCP	AZT	1000	◆
BC	M	47	C	homosexual	AIDS	40	weight loss	AZT	1200	△

[a]Source of NAC: Buyer's Club.
[b]C = Caucasian; H = Hispanic.
[c]CDC classification (ASX = asymptomatic; ARC = AIDS-related complex).
[d]Values indicate the absolute CD4 count just prior to beginning NAC administration (i.e., on day 0).
[e]PCP = *Pneumocystis cartinii* pneumonia; VZV = varicella zoster virus infection.
[f]AZT = zidovudine; DDC = dideoxycytosine.
[g]NAC dosage: 500 mg 2×, 3×, or 4× daily to reach indicated dose (except for BC, who took 600 mg 2× daily).
[h]Patient took L-cysteine, 500 mg, 4× daily for the first week.

therapy accompany other therapies for treatment of AIDS.[22,23] An excellent drug this purpose is *N*-acetylcysteine (NAC): it is exceedingly nontoxic, it is commo used for treatment of bronchitis in Europe and of acetaminophen overdose wo wide, it is cheap, it has been shown to have a variety of anti-inflammatory activi both *in vitro* and *in vivo*, and it can restore depleted GSH levels *in vivo*.

We have studied seven HIV-infected individuals who decided to self-admin NAC. Baseline measurements for up to three weeks were obtained; measureme were then made after these individuals started taking NAC. As shown in FIGURE the intracellular GSH levels returned to within the normal range immediately af NAC was taken. Some of the information about these individuals is summarized TABLE 2. Of course, this study is nonblinded; however, it is highly suggestive t NAC will be a useful adjunct in AIDS therapy, especially for restoring GSH level

Measurements of intracellular GSH in subsets of PBMC reveal a rich heteroge ity. Because of the known dependence of lymphocyte function on intracellular G levels,[22] it is important to understand this heterogeneity. For instance, what is difference in functionality between the high- and low-GSH T cells? We predict t the susceptibility to activation may be quite different for these cells: the high-GS cells may be more easily activated by antigen (because high GSH levels prom antigen-specific or lectin-mediated activation) and the low-GSH T cells may be n easily stimulated by inflammatory mediators such as TNF (because depletion GSH levels promotes inflammatory stimulations). These two classes may have different roles in immune response.

The observation that the high-GSH T cells are virtually absent in HIV-infec individuals is consistent with the large body of evidence demonstrating an anerg the T cell compartment for antigen-specific responses. It may well be that restor of GSH levels could restore (at least partially) a normal response by these cells.

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APPENDIX

FACS Measurement of Intracellular GSH

Introduction

Glutathione (GSH) is a tripeptide found in relatively high concentrations (mM) in virally all cells. It maintains a reducing environment with cells by virtue of a free sulfhydryl on the cysteine residue. Measurement of its level within cells revolv around the reactive nature of this sulfhydryl. The basis for measurement is the use the fluorogenic reactant, monochlorobimane (MCB). MCB is a nonfluoresce molecule that reacts with free sulfhydryls to form a covalently linked product. Tl bimane-substituted sulfhydryl is highly fluorescent. Because of the relatively slo reacting nature of MCB, it reacts almost exclusively with GSH in cells; reaction wit free sulfhydryls in the form of proteins or cysteine is minimal. Additional specificity conferred by the requirement of GSH-*S*-transferase activity for the conjugation. Th final fluorescence is linearly related to the GSH concentration[11,24] (unpublishe observation). Note that high concentrations of MCB can inhibit some transferas and can result in lower fluorescences than standard conditions (in some cell types).

The reaction conditions noted herein result in a reaction of only a portion of th GSH with MCB. The reaction proceeds linearly with time; thus, it is very importar to carry out the reaction with special care to maintain equivalent reaction times.

Staining Materials

MCB can be obtained from Molecular Probes (Eugene, Oregon). Also require are deficient RPMI, fetal calf serum, sodium azide, HEPES, paraformaldehyd phosphate salts for PBS, and fluorescently conjugated antibodies. Deficient RPMI i free of biotin and riboflavin, making it optimal for immunofluorescence staining PBS can be substituted for it.

The following stock solutions should be made before experiments are begun:

(i) MCB (10 mM in 100% ethanol): Dissolve monochlorobimane in absolut ethanol to a final concentration of 10 mM. The resulting solution should b aliquoted and stored at −70 °C (keep the most recent aliquot at −20 °C and the remainder at −70 °C). Note that the MCB can precipitate a −70 °C; when taking out a stock, let it warm to room temperature an vortex it thoroughly prior to use (make sure the MCB is back in solution) Prior to staining, make a working stock solution by diluting the MCB stock 1:10 in staining medium (SM). Thus, the working solution is 1 mM MCB in 10% ethanol/SM.

(ii) Staining Medium (SM): Make SM fresh, that is, do not make large stock ahead of time. The fetal calf serum (FCS) should be kept frozen at −20 °C To deficient RPMI, add sodium azide to a final concentration of 0.1% [a stock solution of 10% NaN_3 (100×) in water can be kept at room tempera ture indefinitely for this purpose]; add HEPES to 10 mM [make a stock solution of 1 M HEPES (100×) with a pH such that a 1:100 dilution in RPMI results in a pH of 7.4; keep at 4 °C]; and add filtered FCS to a final concentration of 4%. For convenience, keep 235 mL of RPMI in bottles at 4 °C. To these, add 2.5 mL of 100× azide, 2.5 mL of 100× HEPES, and 10 mL of filtered FCS. Deficient RPMI has no riboflavin and no biotin, optimal for antibody staining. PBS can be used in place of SM for this assay.

(iii) Fixing Solution (FS): This solution will be 2% paraformaldehyde (w/v) in PBS. Heat to 65 °C until the paraformaldehyde completely dissolves; keep at 4 °C thereafter. This is a 4× stock solution.

(iv) Phosphate-buffered Saline (PBS): Any standard preparation of PBS will do.
(v) Fluorescently Conjugated Antibodies.

Staining Protocol

This protocol assumes that the cells have been isolated already: that is, Ficol-Paque density gradient separation of heparinized whole blood; isolation of the buffy coat; leukocytes centrifuged. All centrifugations are 10 min at 500*g* (normal viable cell centrifugations). The protocol is as follows:

(1) Resuspend the leukocyte preparation in 1 mL of SM in a 15-mL conical tube. Allow the sample to equilibrate to room temperature (at least 5 min). The cell count should be known (count them at this point if not).
(2) Add 40 μL of MCB working solution (1 mM, 10% EtOH). This reaction will proceed for exactly 20 min; use a stopwatch to keep time.
(3) Add 9 mL of cold SM to slow the reaction; put the tube on ice.
(4) Immediately underlay with 1 mL of serum: draw up 1 mL of serum into a Pasteur pipette; hold a finger over the top of the pipette and drop the pipette into the tube; allow the serum to flow into the tube; remove the pipette and discard—the serum will form a layer underneath the remaining fluid. For HIV-infected samples, use a 2-mL plastic pipette for the underlay process.
(5) Immediately centrifuge the cells.
(6) Aspirate the entire supernatant. All subsequent steps are to be carried out at 0 °C; all SM should be at 0 °C.
(7) Resuspend the cells at 40×10^6/mL in SM and aliquot 25 μL to the wells of a 96-well tray containing fluorescently conjugated antibodies (or use any immunofluorescence staining protocol that maintains 0 °C temperatures). Incubate for 15 min.
(8) Add 150 μL of SM to each well; centrifuge the cells. Aspirate with a drawn Pasteur pipette. Resuspend the cells in 200 μL of SM; centrifuge. Repeat this wash once more.
(9) If necessary, add the second-step stains (fluorescently conjugated avidin, usually); incubate for 15 min. Repeat step 8.
(10) Resuspend the cells in 150 μL of PBS; add 50 μL of FS. Allow to sit for at least 5 min. These samples are now stable for several hours (but they should be analyzed as soon as possible).

Notes on Staining Protocol

(1) It may be advantageous to use a circulating water bath set at 25 °C to equilibrate the samples more quickly.
(2–4) Make up enough working solution for all samples to be stained that day. Because the timing of the reaction is critical, the following protocol is suggested for multiple samples: Arrange the tubes in a rack; add the MCB working solution at 15-s intervals. At the end of the 20-min incubation, add 9 mL of SM at 15-s intervals in precisely the same order (then place on
With more than two or three samples, this procedure must be done by
people—one quenching the reaction and the second underlaying with se
Place all samples in the centrifuge at once after the last one is underlaid.
(5) Centrifugation should be at 0 °C, as for all following centrifugations. S
dard cell recovery centrifugation should be used (e.g., 400*g*, 10 min).
(6) By aspirating the entire top layer before the serum, the maximum amou
MCB can be removed. Keep the tube vertical until the SM has b
aspirated; then tilt it to remove as much of the serum as possible with
removing the pellet.
(7) This concentration results in the optimal 1×10^6 cells per stain.
(8–9) Standard antibody staining protocol should be used. These steps (8 an
summarize the protocol; notes about this protocol can be obtained f
Becton Dickinson (San Jose, California).
(10) Addition of 0.5% paraformaldehyde completely removes HIV activity,
sides every other viral contaminant. It does not affect the measuremen
bimane or fluorescent antibodies.

FACS Instrument Configuration

We use a FACStarPlus (Becton Dickinson) equipped with two argon ion lase
The primary laser is tuned to 488 nm (130 mW) and is used for forward a
orthogonal scatter measurements and for excitation of fluorescein (collected with
515–545 nm band-pass filter), phycoerythrin (575–595 nm), and Cychrome® (Ph
Mingen, San Diego, California) (678–696 nm). The secondary laser is tuned
351/361 nm (50 mW) and is used to measure bimane fluorescence (515–545 nm).
forward scatter threshold is used to exclude debris and residual erythrocyte
Typically, 30,000 events are collected for each sample. Data analysis (includi
gating, display, and statistical analyses such as percentiles) was performed usi
FACS-DESK software.