

# Chapter 54 Glutathione Measurement on the Flow Cytometer

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Glutathione (GSH) is an important regulator of cell function and its levels are decreased in some disease states. The measurement of GSH in clinical samples and disease models may provide important clues to the basis of the altered cell function occurring with disease as well as to the sequence of events occurring during the progression of disease. Intracellular GSH levels can be measured on the fluorescence-activated cell sorter (FACS) as the fluorescence produced from the GSH-s-transferase (GST) conjugation of monochlorobimane (MCB) with GSH. We have modified the original FACS MCB assay to increase both its reproducibility and to make it more quantitative. Screening of peripheral lymphocytes of HIV infected individuals with a modified MCB FACS assay identified a population of T cells that are progressively lost during the course of HIV infection. These studies also show that the mononuclear cell populations are positively correlated with each other in their median MCB fluorescence levels. Furthermore, the median MCB fluorescence levels of the CD4 T cells correlates loosely with their number in peripheral blood and with their whole blood GSH levels measured by HPLC.

prognostic indicator of cell function and insights into the sequence of events occurring during disease progression.

### Why do we use FACS GSH assay?

Over the last decade, significant progress has been made in the development of suitable FACS assays for intracellular GSH. These assays measure GSH levels within each cell analyzed and therefore record the heterogeneity of GSH levels within a population. When coupled to multiparameter immunofluorescence subsetting, the GSH levels of a particular cell type present within a mixture of cell types can be determined. In addition, with these assays, cells can be viably sorted by their GSH levels and then functionally analyzed. Questions still remain concerning the precise proportionality of the cellular fluorescence levels measured with these assays to the intracellular GSH levels of the cells; however, these questions do not obscure the power of this approach.

### GSH review

Glutathione,  $\gamma$  glutamyl-cysteinyl-glycine, is the most abundant intracellular thiol and is typically present within cells at millimolar concentrations. This peptide performs many distinct functions within the cell [1]. GSH removes peroxides and other reactive metabolic intermediates, either by directly inactivating them or by acting as a substrate for the enzymes that catalyze their removal. GSH is also an essential cofactor for enzymes involved in DNA synthesis.

Several different fluorescent reagents have been reported to measure GSH levels on the FACS. These include: monochlorobimane (MCB), monobromobimane, chloromethyl fluorescein diacetate, o-phthalaldehyde and, mercury orange (reviewed in [17]). All of these reagents detect GST by conjugation to its sulfhydryl group. However, they differ significantly in their GSH dependence for fluorescence and therefore also in their suitability as a FACS assay of GSH.

Intracellular GSH levels decrease with age. Intracellular GSH levels are also diminished by several diseases including oncogenesis [2, 3], idiopathic pulmonary fibrosis [4] and HIV infection [5, 6, 7, 8].

Typically, the GSH dependence of a reagent's fluorescence is determined by analyzing cell populations differing in their GSH levels with both the FACS assay and a biochemical GSH assay. Such populations of cells can be easily prepared by chemically depleting the GSH levels of the cells with either an inhibitor of GSH synthesis (such as buthionine sulfoximine) [18], or with a substrate of GST transferase (such as chlorodinitrobenzene) to conjugate GSH [19]. Of the reported stains for GSH, only MCB is specific for GSH by the above criteria [19].

Many studies demonstrate that lowering intracellular GSH levels alters cell function. In lymphocytes, decreased GSH levels diminish antigen-triggered lymphocyte blast formation [9] and proliferation [10]. These changes in lymphocyte function are at least partially accounted for by changes in signal transduction pathways [11]. For example, decreased levels of GSH lead to inhibition of the TCR triggered calcium influx and augmentation of the phosphorylation events resulting from inflammatory cytokine stimulation. Low intracellular GSH levels may also affect the life/death decision of cells. The progression of programmed cell death is, at least in some cases, determined by the intracellular GSH levels [12, 13]. GSH, in its role as an antioxidant can also influence cellular survival following oxidant attack [14, 15, 16]. Therefore, the measurement of the intracellular GSH levels within lymphocytes, as well as other cells types, provides both a

### MCB structure and chemical properties

MCB is a heterocyclic compound that is relatively nonfluorescent but becomes highly fluorescent upon conjugation with thiols [20]. The halogen on MCB quenches the bimane fluorescence. Thiols react with MCB through a nucleophilic displacement reaction leading to the release of the halogen and a dramatic increase in quantum efficiency.

MCB and the GS-bimane conjugate possess the diffusion properties necessary for a quantitative intracellular stain. The substrate of the reaction, MCB, diffuses readily into the cell because of its uncharged nature [20]. The product of the reaction, the GS-bimane conjugate, is blocked from diffusion out of the cell because of its charge. Nevertheless, van der Ven et al recently

identified that an active transport system can export the GS-bimane conjugate from the cell [van der Ven, 1994 #2]. We have found that this transport of the GS-bimane conjugate can be blocked by an inhibitor of anion transport (see below).

#### Evolution of the MCB assay for GSH

The conditions used to stain cells with MCB typically have been chosen based upon one of two strategies. In the first strategy, concentrations of MCB and times of reaction are chosen such that essentially all of the cellular GSH becomes conjugated to MCB. With this strategy, the fluorescence of the cells reaches a plateau during the staining reaction because of exhaustion of the GSH substrate. To be useful, this requires that the concentrations of MCB necessary to reach the plateau are below the concentrations that result in significant MCB conjugation to other cellular constituents.

In the second strategy, conditions of staining are chosen which make the GSH level the rate-limiting step in the progression of the conjugation. For this strategy to be useful, the rate of conjugation of GSH to MCB must be solely dependent upon the intracellular concentration of GSH.

The relationship of the MCB fluorescence and the MCB assay was initially established in rodent cells stained with the plateau strategy. Under these conditions a good correlation was established between MCB stained fluorescence and biochemically measured GSH levels. However, the use of this assay has proven more problematical for the analyses of human cells.

#### GSH conjugation to MCB is catalyzed by GSTs

The specificity of the MCB assay for GSH results from its requirement of a constitutively expressed cellular enzyme for significant product formation. MCB and GSH are co-substrates for the enzyme GST transferase [21]. Although MCB can react slowly with all intracellular thiols, the rate of the GST catalyzed reaction with GSH is much greater. Only the GSH reaction with MCB is catalyzed by GST. The specificity of the MCB assay for GSH is governed by the ratio of these two reaction rates.

The rate at which MCB is conjugated to GSH and the resulting fluorescence formed is dependent upon the level and type of GST (isozyme, species) expressed within the cell. Currently, we rarely know the expression level of GST(s) within a given cell type. The cellular role of GST transferases is the removal of lipid peroxides and other toxic metabolic products. The cellular concentrations of these substrates fluctuate with growth conditions and with the encounter of external stimuli. For example, GST expression levels can be increased by both oncogenic transformation and oxidative stress [22, 23].

Wide variations are found among the  $K_m$ s and  $k_{cat}$ s of these enzymes for MCB. For example, rat hepatic cells express the neutral GST isozyme with a low  $K_m$  for MCB of 2.6  $\mu M$  [24]. In contrast, human liver cells express a neutral GST with a high  $K_m$  for MCB of 204  $\mu M$ . Consequently, almost two orders of magnitude separate the affinities of the rat and human neutral GSTs although they share similar  $k_{cat}$ . The concentration of MCB required to saturate the rat and the human GSTs therefore is quite different. GST enzymes can also vary in their catalytic efficiency for the MCB substrate. The  $k_{cat}$  of the human  $\pi$  GST isozyme found in human tumors and in peripheral lymphocytes is only 4% of that of the neutral GSTs found in the human and rat. The human  $\pi$  GST also possesses a high  $K_m$  of 264  $\mu M$ . Therefore,

MCB is a very good substrate for the GST isozymes found in rat liver cells but is a suboptimal substrate for the GST isozymes that are found in human tumor cells and in peripheral lymphocytes [25]. Thus depending upon the local concentration of MCB and the availability of the GSTs, the rate and extent of MCB conjugation in these various cell types could be quite different.

The amount of background fluorescence formed in the MCB FACS assay because of conjugation of MCB with non-GSH cellular thiols is dependent primarily upon the concentration of MCB. Since the range of MCB concentrations used in the reported MCB FACS assays varies by over a hundred-fold, the extent of labeling of protein thiols with MCB is expected to differ significantly between these protocols [24, 26].

It has been widely assumed that the vast majority of the fluorescent products formed in the MCB assay consist of the bimane conjugates of three types of species: GSH, cysteine, and proteins. Of these species, the GS-bimane conjugate has been reported to be almost the sole low molecular weight product formed [19, 27, 28]. Recently, another low molecular weight species has been found in the assay of human T-cells with MCB [29].

Rice first demonstrated that MCB stained rodent cells, the accumulated fluorescent product of the MCB staining reaction directly correlated with the amount of intracellular GSH [27]. In these studies, the reaction of GST with bimane reached a plateau in which no further product was formed. Therefore, the amount of fluorescent product formed in the MCB staining reaction was determined by the amount of available GSH substrate to be conjugated and not necessarily by the rate of product formation [27]. These observations have been essentially confirmed in several independent studies [21, 30].

The choice of rodent cells in the original studies was fortuitous in the establishment of MCB FACS assay. As mentioned earlier, rodent cells express GST transferases with both a high affinity and high turnover rate for MCB [24, 26]. Cells expressing these types of GST require only low micromolar concentrations (10 to 20  $\mu M$ ) of MCB to saturate fully the GST for substrate and to reach plateau MCB fluorescent staining levels. Use of these low concentrations of MCB in the staining reaction minimizes the uncatalyzed conjugation of MCB with cellular thiols.

When these MCB staining conditions were used with human cells, the maximal fluorescence levels obtained were much lower than those obtained with rodent cells [3, 24, 26, 31]. The GSH levels in human and rodent cell lines are similar but the human GSTs differ markedly from the rodent GSTs in their affinities for MCB. The  $K_m$  for MCB is approximately 100-fold higher in human GSTs than in rodent GSTs. Consequently, the human GSTs are mostly not bound by MCB substrate under the original MCB assay conditions.

Two different approaches have been reported to adapt the MCB assay to human cells. The first approach both increases the concentration of MCB substrate to approximately fourfold above the  $K_m$  of the human GSTs (100  $\mu M$ ) and increases the length of time of incubation with the MCB substrate (60 min). These modifications lead to almost complete conjugation of the GSH with MCB in at least some human cells [24, 30]. Analogous to the MCB assay of rodent cells, these modifications lead to MCB fluorescence values determined primarily by the amount of GSH substrate for conjugation. Unfortunately, using higher concentrations of MCB and increasing the length of staining led to higher

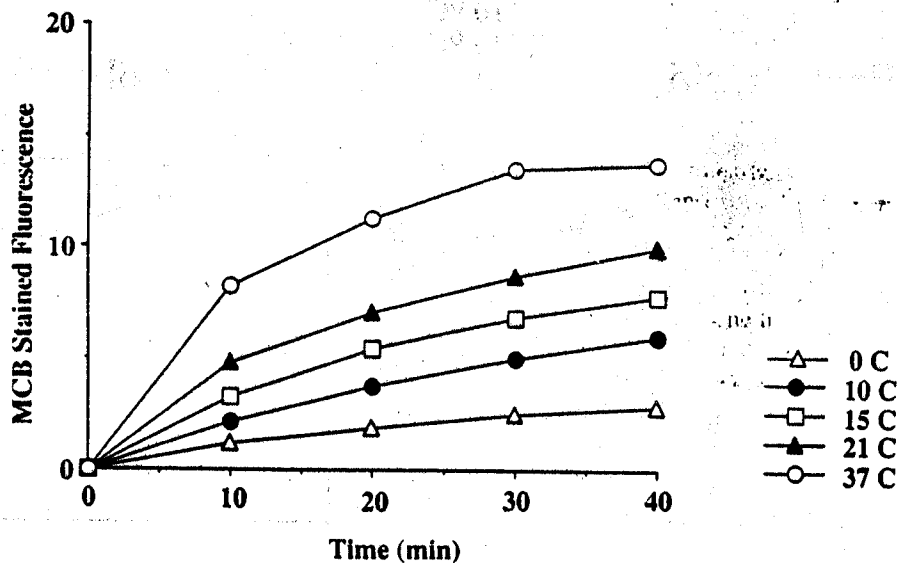


Fig. 54.1. Time and temperature dependence of MCB staining. Human peripheral mononuclear cells were stained as described in the MCB staining protocol except the time and temperature were varied as indicated in the figure. The MCB stained cells were analyzed as described in FACS analysis. Fluorescence values represent the median fluorescence of MCB stained cells after subtraction for autofluorescence.

levels of uncatalyzed thiol conjugation with MCB. With these assay conditions, the nonglutathione-dependent fluorescence is approximately 20 to 25% of the total. This approach establishes a loose quantitative correlation between the intracellular GSH levels and the MCB assay fluorescence in human cells [24, 26, 32].

The second approach used with human cells employs a kinetic strategy for the staining of cells with MCB [8, 19, 28]. With this strategy, the rate of formation of product for a given concentration of MCB is dependent both upon the GST level/type of expression and on the concentration of GSH. Under these conditions, the reaction will not approach a plateau due to the low concentrations of MCB, which are only approximately 1/5 of the  $K_m$  of the GSTs expressed. This second approach is more sensitive to the changes in either the level or types of GST transferases expressed within the cell, but avoids the high background levels of fluorescence resulting from the uncatalyzed conjugation of MCB with cellular thiols occurring with the first approach. With these assay conditions, depletion of GSH in peripheral lymphocytes with CDNB has been shown to yield MCB stained fluorescence values quantitatively correlated with GSH levels [19].

#### Improved MCB assay

We have significantly modified the procedures for MCB staining human lymphocytes. These modifications increase the reproducibility in the extent of MCB conjugation and lead to quantitative retention of conjugate product. Our assay is based upon a kinetic strategy described above for human cells and largely measures the rate of conjugate formation. We have also improved the normalization procedure permitting a more quantitative interassay comparison of MCB FACS values.

#### Temperature dependence of staining

We first examined the time and temperature dependence of GS-bimane product formation. Human peripheral lymphocytes were stained with 40  $\mu$ M MCB and both the time and temperature of the staining reaction were varied. Analysis of the values

obtained identified a nearly linear increase with time of the MCB stained fluorescence at temperatures of 21°C or less (Fig. 54.1). Thus, measurement of the accumulated fluorescence under these conditions is an indirect measure of the rate of generation of the products. At 37°C, the values deviated significantly from this linear relationship. We chose staining conditions of 21°C for 20 minutes, since the fluorescence formed was at least 10-fold above autofluorescence levels, but still within the relatively linear range of product formation.

#### Temperature and anion transport dependence of export

For the MCB FACS assay to be quantitative, the GS-bimane product should be completely retained within the cell. We measured the loss of fluorescence from MCB stained cells after they were incubated in a variety of temperatures for different lengths of time. As has been recently reported by van der Ven, we observed that the fluorescent product(s) of the MCB reaction is rapidly lost from the cell. This loss of product(s) increases with both time and temperature [29]. This problem is solved in our MCB staining conditions by adding the anion transport inhibitor, probenecid [29, 33] to the staining medium. The addition of probenecid to the medium completely blocked the loss of fluorescence from peripheral lymphocytes at 21°C and at lower temperatures (Fig. 54.2). Probenecid only partially blocks this loss at 37°C, as has been previously described by van der Ven [29]. These studies identify anion transport as the predominant means of export of the MCB fluorescent product(s) within human lymphocytes. Therefore, we have defined MCB staining conditions that quantitatively retain the MCB product(s) within the cell.

To further define conditions necessary for optimal and consistent MCB staining, we examined the influence of pH on the formation of fluorescence in the MCB staining reaction. A nearly linear increase in the fluorescence of the MCB stained cells was observed as the pH of the media was increased in the staining reaction. These changes in pH resulted in an almost two-fold difference between the most acid pH tested (pH 6.8) and the most

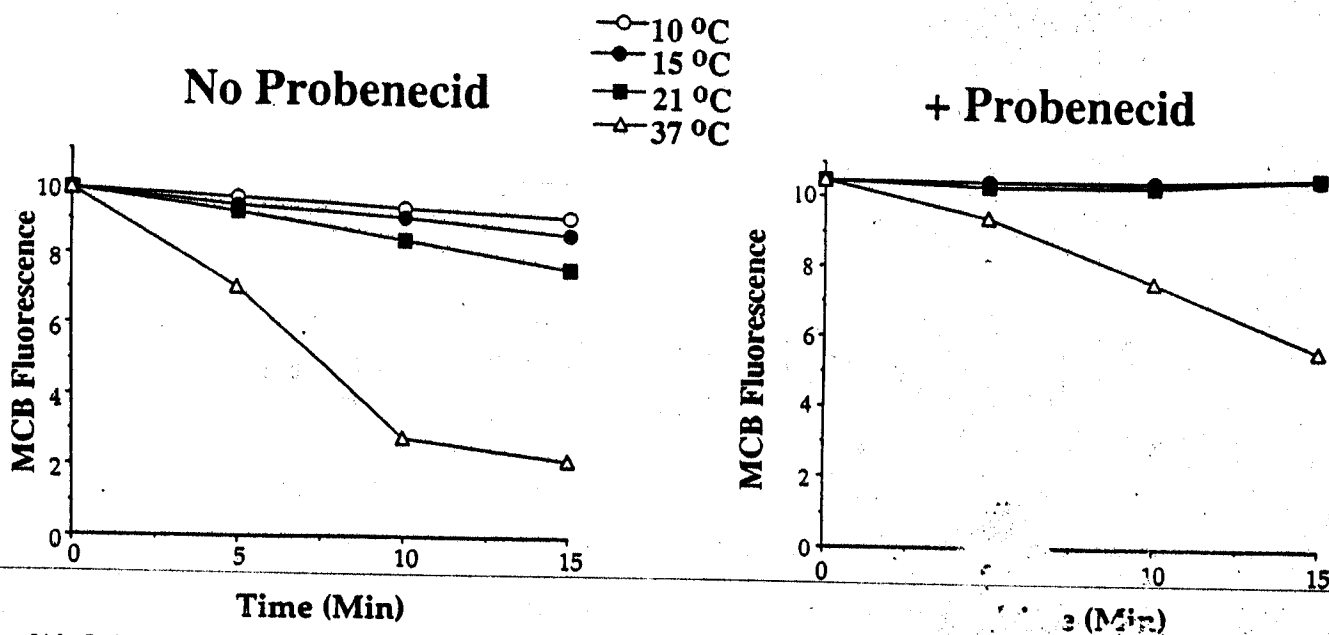


Fig. 54.2. Probenecid blocks the export of the fluorescent product(s) of MCB staining. Human peripheral mononuclear cells were stained as described in the MCB staining protocol except hRPMI was substituted for hRPMI + P. The MCB stained cells were split into two aliquots. One aliquot was resuspended in hRPMI (no probenecid) and the other aliquot was resuspended in hRPMI + P (plus probenecid). The stained cells were incubated at the temperatures and for the lengths of time indicated. After these incubations, the cells were placed on ice and analyzed as described in FACS analysis. Fluorescence values represent the median fluorescence of MCB stained cells after subtraction for autofluorescence.

alkaline pH tested (pH 8.4). The mechanism which confers the pH sensitivity to the reaction is not yet known. We have not pursued further the reactions occurring at the more alkaline pHs, since the rate of bimane-GST export is also increased at the more alkaline pH. Therefore, we have chosen the nearly physiological pH of 7.4 for use in the MCB staining reaction.

The MCB staining medium should also maintain both the cellular redox state and cell viability. Typically, we culture our lymphocytes in RPMI 1640 to maintain both of these parameters, and therefore chose a HEPES buffered formulation of this media, HEPES RPMI 1640, for the MCB staining reaction. The standard RPMI 1640 is not sufficiently buffered to maintain a constant pH during exposure to atmospheric CO<sub>2</sub>. Therefore, we have omitted the carbonate from the RPMI 1640 media in order to maintain a constant pH during the staining reaction.

#### PBMC standard

In working with this assay, we find that small changes in the conditions of MCB staining and FACS settings will alter the endpoint fluorescence measured. This interassay variation can be reduced by inclusion of a reference standard among the samples stained. The MCB fluorescence values obtained for samples are then expressed as relative values to the reference standard [32]. This works well to reduce the interassay variation as we will show below.

The use of a continuously cultured cell line as a reference standard presents potential difficulties. We have found that the MCB stained fluorescence levels of cell lines are significantly dependent upon their culture density (Anderson, unpublished observations). This potential source of variation makes cell lines not sufficiently constant for their use as a standard. Therefore, we have chosen instead to employ peripheral lymphocytes stored as

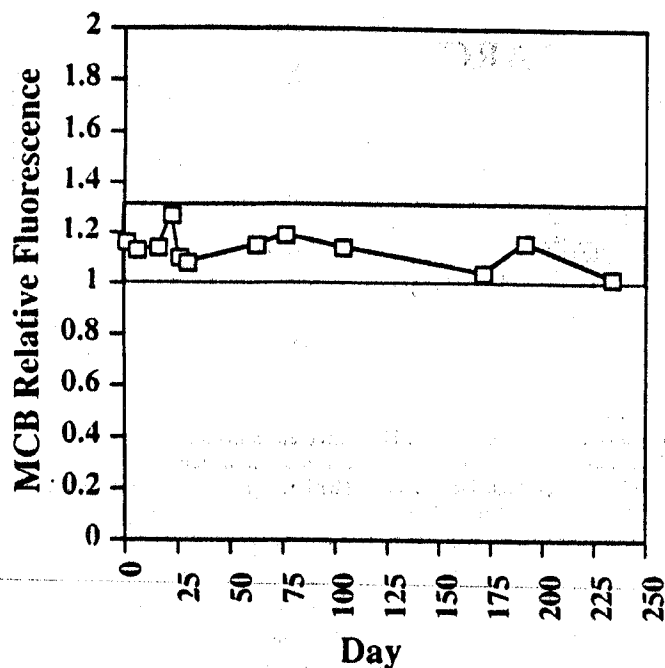
frozen aliquots as our reference standard. Inclusion of the peripheral lymphocyte standard in our MCB FACS assay typically leads to interassay variation of less than 10% for MCB fluorescence values of a given individual's human T cells stained on different days (Fig. 54.3).

#### GSH depletion studies

Having optimized the MCB assay, we examined whether the method used for GSH depletion alters the relationship between the intracellular GSH and the MCB stained fluorescence levels of the human T cell line, Jurkat. Two methods have mainly been used to deplete GSH: CDNB, a substrate of GST which thereby depletes reduced GSH stores, and BSO, an inhibitor of GSH synthesis. Incubation of the Jurkat T cell line with either of these reagents led to decreased intracellular GSH as measured by the Tietze assay. As previously described for Jurkat T cells, the CDNB-decreased GSH levels correlate linearly with the MCB stained fluorescence. In contrast, the decreased GSH levels because of the inhibition of GSH synthesis with BSO led to a nonlinear monotonic relationship between the MCB stained fluorescence values and the intracellular GSH levels (Fig. 54.4). Therefore, in GSH depletion studies, the MCB fluorescence levels are determined not only by the intracellular GSH levels, but also by the method used to deplete the GSH.

CDNB and BSO deplete GSH with different time courses. In our experiments, both the CDNB depletion of GSH and the MCB assay occurred within a single hour. In contrast, the depletion of GSH mediated by BSO occurs over 24 hours, after which the cells are stained with MCB. Therefore, Jurkat cells are only briefly exposed to oxidative stress with CDNB treatment but experience a prolonged oxidative stress with the BSO treatment.

The exact mechanism which operates to specifically modify the



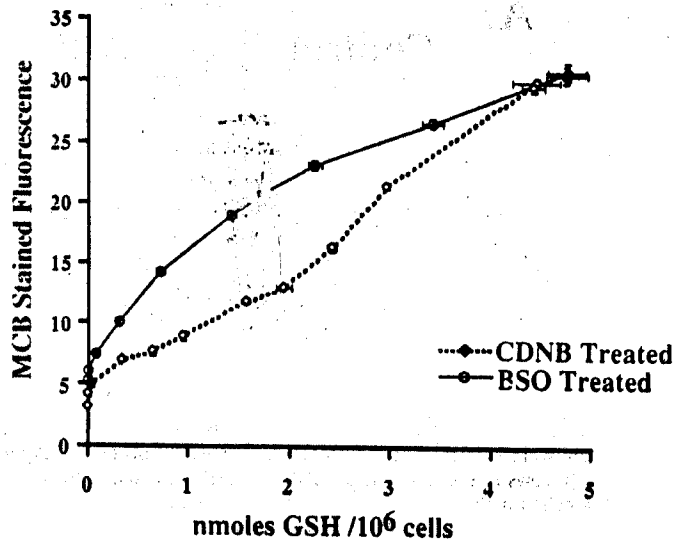
**Fig. 54.3.** Stability of normalized clinical MCB stained values. On twelve different occasions extending over a period of several months, the peripheral mononuclear cells of a single healthy adult were isolated by Ficoll Hypaque centrifugation. These mononuclear cells were then both stained with MCB as described in the MCB assay protocol and immunophenotyped with fluorescently conjugated antibodies for CD3 and CD4. The cells were analyzed as described in the FACS analysis and these analyses were gated for CD4+ CD3+ lymphocytes. The relative fluorescence values represent the median MCB stained fluorescence minus background autofluorescence of these CD4+ CD3+ gated cells divided by the median fluorescence with background autofluorescence subtracted of the frozen standard.

correlation between MCB stained fluorescence and intracellular GSH levels is unknown. One plausible explanation is that the GST levels are increased within cells as a cellular compensatory mechanism for low cellular GSH levels [22]. Higher levels of GST would then increase the GS-bimane product formation beyond that made in uninduced cells for the same GSH concentration. The final fluorescence formed would represent the product of the rate of GS-bimane conjugate formation at a particular GSH concentration and the level of GST expression within the cell. GST enzymes, including the  $\pi$  GST found within Jurkat and peripheral T cells, are regulated by redox sensitive elements (the ARE and EpRE) [34, 35]. Simply depleting the GSH levels has already been shown to be sufficient to increase the transcription directed by these elements [22].

In conclusion, using our modified protocol, the levels of MCB stained fluorescence generally reflect the intracellular GSH levels. However, the fluorescence levels obtained are also potentially influenced by the expression levels of the GSTs as well as by the non-GSH products formed [29].

#### GSH levels in human lymphocyte subsets

Different T lymphocyte subsets show characteristically different levels of MCB staining. For example, human CD4 T cells are delineated by the presence of two distinct subpopulations possessing different levels of MCB stained fluorescence. (Fig. 54.5). We



**Fig. 54.4.** Jurkat T cells treated with BSO or CDNB. Jurkat T cells were either cultured overnight in various concentrations of BSO (0  $\mu$ M to 160  $\mu$ M) or treated for 30 minutes with various concentrations of CDNB (0  $\mu$ M to 100  $\mu$ M). These reagents were washed twice from these cells by centrifugation and resuspension of the cells in fresh hRPMI. The treated Jurkat T cells were stained with MCB as above and analyzed on the FACS as above. MCB stained fluorescence is equal to the median fluorescence minus the autofluorescence. The amount of GSH was determined with the Tietze assay as modified by Baker et al [38].

have shown that the higher MCB staining CD4 T cell population is selectively lost during the course of HIV infection. Hence, the MCB stain provides an additional phenotypic marker that we have already shown to be useful in our studies of HIV infection and CD4 T cells.

We are currently in the process of determining whether these MCB staining patterns reflect different GSH levels or other mechanisms such as differences in GST gene expression. The determination of MCB stained fluorescence levels is relatively easy to obtain. In contrast, the isolation of pure unperturbed lymphocyte populations of sufficient quantity for the biochemical determination of GSH levels is technically difficult and may be impossible if sufficient material for these type of studies is unavailable. Unfortunately, only upon the completion of these type of studies, can an unambiguous statement of the intracellular GSH levels of these populations be made.

#### Application of the improved MCB stain to clinical samples

We have applied the improved MCB staining protocol described above to 131 clinical samples drawn from HIV infected individuals. Rank ordering each individual's mononuclear subpopulation's median MCB fluorescence levels identifies a global regulation influencing the MCB stain (Fig. 54.6), i.e., the MCB stained fluorescence levels in each PBMC subset strongly correlates with the MCB stained fluorescence of the other subsets. We do not yet know whether this global regulation reflects the intracellular GSH levels or some other parameter affecting the MCB staining.

The global regulation detected with the MCB stained fluorescence levels correlates with the disease state of the HIV infected individuals. The median MCB stained fluorescence levels of the CD4 T cells are significantly positively correlated with the CD4

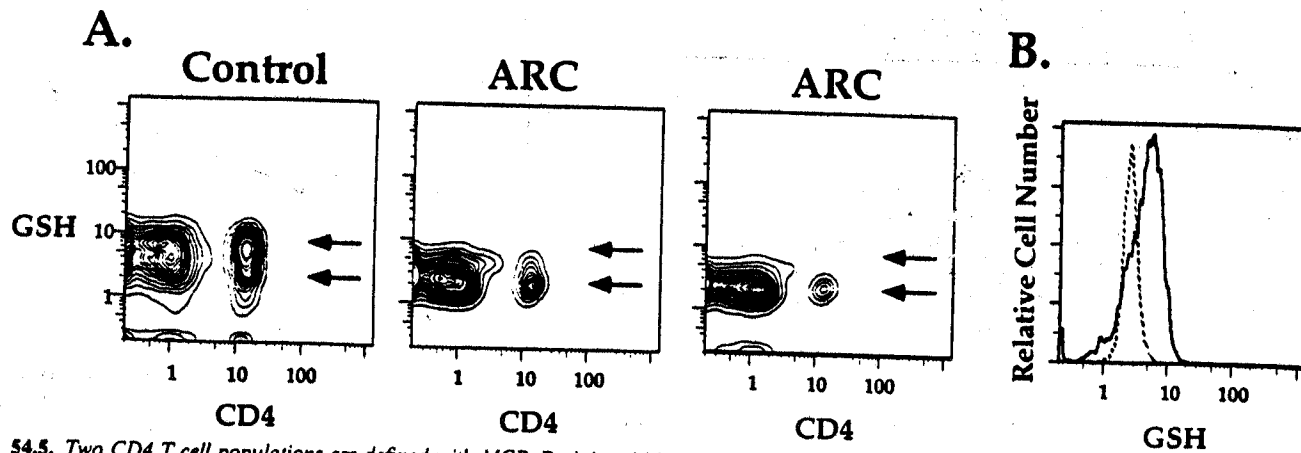


Fig. 54.5. Two CD4 T cell populations are defined with MCB. Peripheral blood cells from control and HIV-infected individuals (ARC=AIDS related complex) were stained as in Figure 3. (A) T cells in normal healthy individuals have a heterogeneous GSH distribution, including low- and high-GSH T cells (arrows in the leftmost panels). The HIV-infected individuals selectively lose the high-GSH T cells. (B) Histogram of the GSH distribution in CD4 T cells for the uninfected individual (solid line) or the first HIV-infected individual (hatched line).

cells/mm<sup>3</sup> in the peripheral blood of HIV infected individuals. Since, the median MCB fluorescence of CD4 T cells is a composite of both the high and the low MCB staining populations, this correlation at least partially reflects the progressive loss of the high MCB staining CD4 T cell population during the course of HIV infection. The loss of the high bimane population is at least partially explained by the loss of the naive CD4 T cells which contain the majority of the high bimane T cells [36]. However, statistical analysis shows that this is not the predominant factor in the decrease of MCB fluorescence levels observed during the progression of HIV infection.

### Methods

#### Reagents and solutions

MCB was purchased from Molecular Probes (Eugene, Oregon). A 10-mm stock solution is prepared by dissolving the MCB (Molecular Probes) powder into 100% ethanol. We aliquot 100  $\mu$ l of the 10-mm MCB into brown Eppendorf tubes and store these in the dark at 10°C for up to several months.

**Media.** Complete RPMI 1640 (hRPMI) was prepared from HEPES-RPMI 1640 supplemented to 0.02% for sodium azide, 4% fetal calf serum, 290  $\mu$ g/ml glutamine, 100 units/ml penicillin, and 70  $\mu$ g/ml streptomycin. Complete RPMI with probenecid (hRPMI + P) was prepared by adding probenecid to hRPMI to a final concentration of 2.5 mM and then adjusting the pH to 7.4 with NaOH. The hRPMI + P was used for all steps in the staining of the cells with MCB as well as all subsequent steps for immunofluorescence staining of cells. The carbonate buffer is omitted from this medium to eliminate the carbonate induced pH changes that occur due to equilibration with atmospheric gases. Probenecid solubilizes more rapidly at 37°C than at room temperature. Therefore immediately after addition of the probenecid, the medium's pH was adjusted to approximately pH 7.4 and then incubated at 37°C until the probenecid was in solution. For studies that include immunofluorescence staining with biotin conjugates, we substitute a formulation of RPMI lacking biotin but prepared as above.

**Heat sink aluminum blocks.** These blocks are drilled out to spatially mirror a 96-well ELISA plate. The aluminum blocks are

chilled by partially embedding them in ice. Placement of the ELISA plate on the aluminum block ensures each well of the ELISA plate is surrounded by a cold conductor. Specifications for the aluminum heat sink block are available from the Herzenberg laboratory.

#### Monochlorobimane staining protocol

1. Resuspend cells in 1.1 ml of hRPMI + P. Exactly 1.0 ml is transferred with a 1-ml pipette to a 15-ml conical centrifuge tube, and placed in the 21°C water bath for five minutes. The remaining approximately 100  $\mu$ l of cells are set aside on ice to quantitate autofluorescence.
2. Add 900  $\mu$ l of (hRPMI + P) to a predispensed 100- $\mu$ l MCB tube. The concentration of the diluted MCB is 1 mM. Use immediately and discard the leftover reagent.
3. Use a stopwatch. Add 40  $\mu$ l of the diluted MCB to a sample. Finger vortex the sample briefly after adding the MCB, and then put it back into the waterbath. Whenever possible from this step on, cover the samples with aluminum foil to block exposure of MCB and its reaction products to light.
4. After 20 minutes incubation, quench the reactions by the addition of 10 ml of ice-cold (hRPMI + P) and immediately transfer the conical tube of MCB stained cells onto ice. Underlay each sample with about 1 ml of ice cold newborn calf serum. Note, the combination of a 10-fold dilution of the MCB staining reaction and chilling of the reaction mixture, essentially stops the MCB reaction.
5. Centrifuge cells at 4°C. Centrifugation of the cells through the underlying serum separates them from the now diluted bimane reaction mix.
6. Aspirate the supernatant well. All subsequent steps are to be carried out at 0°C; all solutions should be at 0°C. Resuspend the cells at  $20 \times 10^6$ /ml of (hRPMI + P) 4°C and aliquot 50  $\mu$ l into the wells of a 96-well tray. Add conjugated antibodies to the wells and incubate with the cells for 15 minutes. We keep the plastic tray in the aluminum block heat sink on ice at all times except for centrifugations.
7. After 15 minutes have elapsed, add 150  $\mu$ l of cold hRPMI to each well.

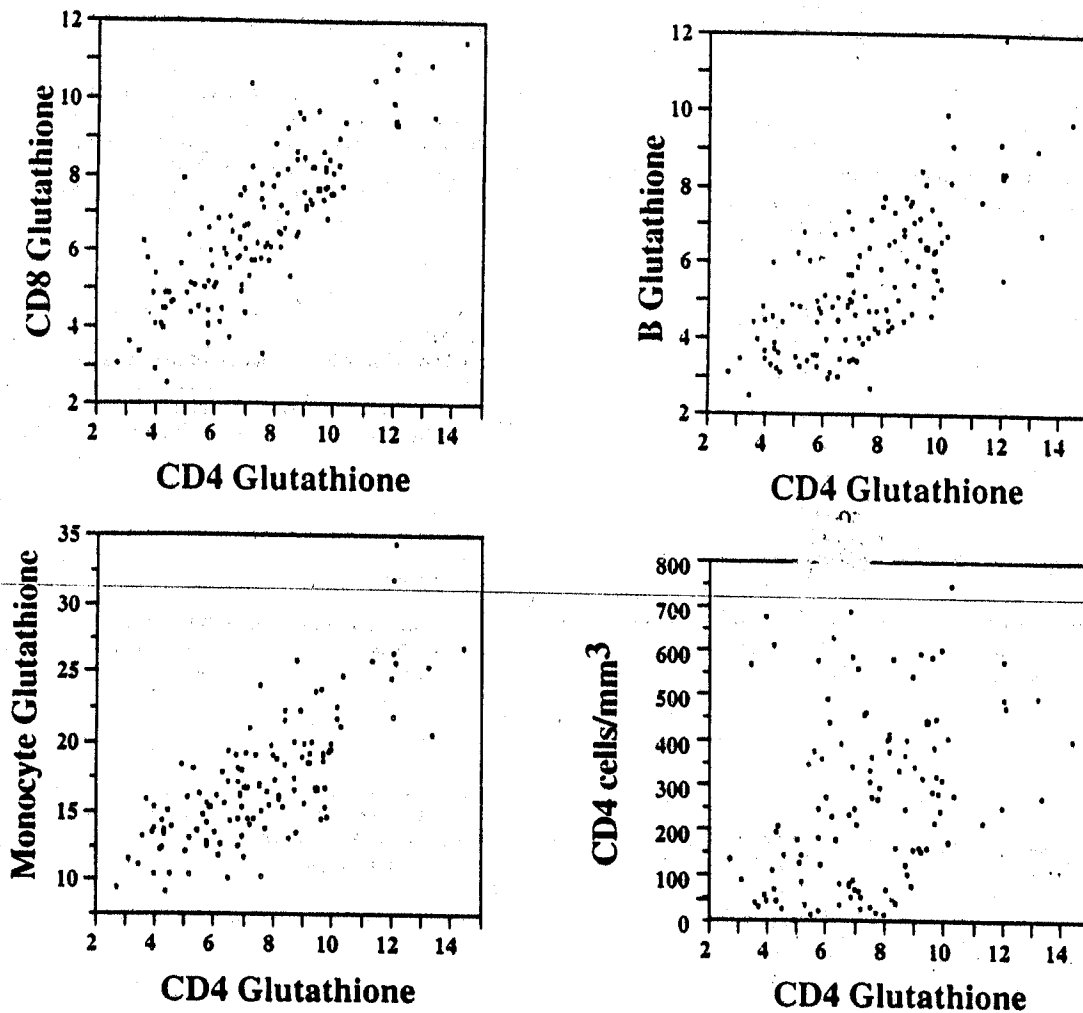


Fig. 54.6. Global regulation of MCB fluorescence and correlation to CD4 cell numbers in HIV infected individuals. Peripheral blood from 131 HIV infected individuals was obtained. The cells were stained and processed as in Figure 54.3 except aliquots were immunophenotyped with: CD4 and CD3 antibodies (CD4 cells), CD8 and CD3 antibodies (CD8 cells), CD20 antibodies (B cells) or CD14 antibodies (monocytes). The relative fluorescence values represent the median MCB stained fluorescence minus background autofluorescence of these gated cell populations divided by the median fluorescence with background autofluorescence subtracted of the frozen standard. The number of CD4 cells/mm<sup>3</sup> in peripheral blood were determined by the Immune Diagnostics Laboratory, San Francisco. The median normalized MCB stained fluorescence values of the subpopulations and number of CD4 cells/mm<sup>3</sup> were statistically analyzed using JMP (obtained from the SAS Institute Inc., Cary, North Carolina). Correlations were obtained using Kendall-Tau statistics.

8. Centrifuge for 3 to 5 minutes. Always transfer the ELISA plate immediately from the centrifuge to an aluminum block placed on ice.

9. Aspirate wells; add 200  $\mu$ l of cold hRPM. Repeat spin.

10. Repeat step 8 once more, for a total of 3 centrifugations.

11. After the final aspiration, resuspend cells in 200  $\mu$ l of (hRPMI + P). Transfer to FACS tubes; keep under aluminum foil until all samples are analyzed.

#### Preparation of human PBMC standard

##### Freezing of human PBMC Standard

1. Prepare lymphocytes by Ficoll-Hypaque and monocyte deplete them by adherence at 37°C for one hour in RPMI 1640 plus 10% fetal calf serum.

2. Place tube containing lymphocytes on ice and resuspend the

lymphocytes to  $10 \times 10^6$  cells/ml with 0°C RPMI 1640 with 20% fetal calf serum.

3. Add with swirling, an equal volume of 0°C RPMI 1640 with 20% DMSO.

4. Aliquot 0.5 ml each, into chilled freezing vials.

5. Freeze the vials in a CryoMed freezing chamber or other controlled freezing chamber set for 1°C decrease in temperature/min. Transfer the vials to liquid nitrogen.

##### Thawing of PBMC standard

1. Transfer a frozen standard vial from liquid nitrogen onto dry ice for transport.

2. Thaw the vial immediately by gentle agitation at 45°C until only a small piece of ice remains, then transfer the vial to an ice bath.

3. Add 1 ml of ice-cold (hRPMI) to the vial. Pipette up and down with 1-ml pipette 3 times. Transfer to 15-ml tube containing 10 to 12 ml of ice-cold (hRPMI). Invert 3 times to mix.
4. Centrifuge at 0°C at 300 × g to pellet lymphocytes for five minutes.
5. Aspirate supernatant; resuspend pellet in 1 ml of 0°C hRPMI and add 10 to 12 ml 0°C hRPMI. Invert tube to mix.
6. Centrifuge again at 0°C for five minutes.
7. Remove supernatant by aspiration; resuspend in 1.5 ml of cold hRPMI. Remove aliquot for assay of viability. Count live and dead cells. We typically obtain 85 to 95% viable cells following this protocol.

#### FACS analysis

We use a FACStarPlus (Becton Dickinson) equipped with two argon ion lasers for the measurements of our MCB FACS assay. The primary laser is tuned to 488 nm (130 mW) and is used for forward and orthogonal scatter measurements and for excitation of fluorescein (collected with a 515 to 545 nm band-pass filter), phycoerythrin (575 to 595 nm), and Cychrome™ (Phar Mingen, San Diego, California) (678 to 696 nm). The secondary laser is tuned to 351/361 nm (50 mW) and is used to measure bime fluorescence (515 to 545 nm). Data analysis (including gating, display, and statistical analyses) is performed with FACS-DESK software [37].

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