[21] Flow Cytometric Determination of Cellular Thiols

By CHANDAN K. SEN, SASHWATI ROY, and LESTER PACKER

Introduction

The oxidation–reduction (redox) state of cellular thiols plays a central role in antioxidant defense and in the regulation of a large number of signal transduction pathways.[1–4] Vicinal dithiols have been identified to serve key cellular signaling and metabolic functions.[5] Redox signaling is implicated in the modulation of a number of signal transduction processes that are known to be associated with the pathogeneses of human disease. Oxidative stress results in the oxidation of intracellular thiols to disulfides. For example, in oxidative stress situations, cellular glutathione (GSH) is oxidized rapidly to glutathione disulfide (GSSG). When produced in excess, all of the intracellular GSSG cannot be recycled to GSH by NADPH-dependent glutathione reductase activity, and GSSG is effluxed from oxidatively challenged cells. Such efflux lowers cellular GSH levels. The intracellular redox state and amount of GSH in the cell may be influenced by a large number of factors.[6]

Flow cytometric determination of cellular thiols is a powerful tool with considerable clinical potential. In contrast to conventional biochemical assays where cell extracts are studied to obtain mean results, flow cytometry allows the collection of results from individual cells. As a result, cells may be immunostained on the surface for any specific marker and information may be obtained for a subpopulation of cells. For example, using appropriate antibodies, results can be obtained from $CD4^+$ and $CD4^-$ subpopulations of a lymphocyte population of cells.[7] In such cases, appropriate fluorophores should be selected and the instrument should be properly set up to ensure that the compensation is well adjusted such that emission from one fluorophore does not interfere with the emission of the other.

The heterocyclic molecule bimanes or 1,5-diazabicyclo[3.3.0]octadienediones react preferentially with thiol groups.[8] Halo (e.g., chloro or

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bromo) derivatives of bimane are nonfluorescent in their native forms but emit strong fluorescence when reacted with thiols. This property of bimanes has been utilized to establish several types of thiol-detecting methods based on high-performance liquid chromatography (HPLC),[9] electrophoresis,[10] and flow cytometry.[11] Monochlorobimane (MCB) has been used commonly in clinical studies for the detection of cellular GSH.[12–15] The reaction of MCB with GSH requires glutathione *S*-transferase activity. Because of a low affinity of human glutathione *S*-transferase isoenzymes for MCB, this thiol probe has been evaluated not to be ideal for human cell GSH measurements.[16–18] Thus, the focus has been shifted to probes that would not be dependent on the activity of glutathione *S*-transferase for their thiol reactivity. Replacement of the Cl atom of MCB by Br renders the bimane much more chemically reactive than MCB. Monobromobimane (MBB) specifically labels both protein and nonprotein thiols by a glutathione *S*-transferase-independent mechanism. The reaction of bromobimanes with thiols are second order and are dependent on the pH, the active nucleophile being the thiolate anion such as GS^-. The reaction of bromobimane with a thiolate converts the nonfluorescent agent into water-soluble fluorescent products[8] that can be detected flow cytometrically. Two other properties of the thiol-reacted MBB fluorophore have made it a quantitative analytical tool that has been used for the electrophoretic analysis of proteins. First, the quantum yield of each fluorescent adduct is identical and independent of the protein species. Second, MBB labels a known number of cysteine residues for each protein.[10]

Preparation of Cells

This technique is most suited for cells that grow in suspension. This article describes the measurement of cellular thiols from cultured Jurkat T cells and human peripheral blood lymphocytes.

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Jurkat T Cells

Human Jurkat T cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Wurzburg T cells, a subclone of the Jurkat T cells, was a kind gift of Dr. L. A. Herzenberg of Stanford University, California.[19] Both types of cell are grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 1% (w/v) penicillin/streptomycin, 110 mg/liter sodium pyruvate, and 2 m*M* L-glutamine (University of California, San Francisco) in humidified air containing 5% (v/v) CO_2 at 37°.

Human Peripheral Blood Lymphocytes

Blood drawn from an antecubital vein is collected in heparinized tubes. Peripheral blood lymphocytes (PBL) are isolated by a standard density gradient separation on Ficoll–Hypaque (Pharmacia, Sweden). Peripheral blood lymphocytes are seeded at 1×10^6/ml in RPMI 1640 culture medium containing 10% heat-inactivated FCS and maintained in humidified air containing 5% CO_2 at 37°. Peripheral blood lymphocytes isolated and seeded as just described are stimulated mitogenically with phytohemagglutinin P (2 μg/ml; grade B, Pharmacia, Sweden). Four hours after such stimulation, PBL are either treated or not (as mentioned in the respective figure legend) with supplements. The supplement treatment time for PBL is 48 hr.

Differential Assessment of Cellular Thiols

As is clear from the principle of the reaction between MBB and thiols, the interaction is not specific for any particular cellular thiol. In the assay system reported here, the specificity of fluorescence signals for distinct thiol pools was ensured by using defined cellular thiol regulatory agents such as buthionine sulfoximine, phenylarsine oxide, and *N*-ethylmaleimide. This method exploits the ability of buthionine sulfoximine to selectively deplete cellular GSH, phenylarsine oxide to block vicinal dithiols, and *N*-ethylmaleimide to block all cellular free thiols. The differential assessment of MBB fluorescence of cells indicates that only around half of the total emission signal is GSH dependent (Fig. 1). The difference of results obtained from GSH-adequate cells and GSH-depleted cells provides a specific estimation of the intracellular GSH level.

Cells in culture are resuspended (10^6/ml) in the standard culture medium as described earlier and seeded in 24-well plates at 10^6 cells/well. For each treatment group of cells, four separate wells containing 1 ml of cell suspension are prepared. Cells from each of the four wells of a treatment

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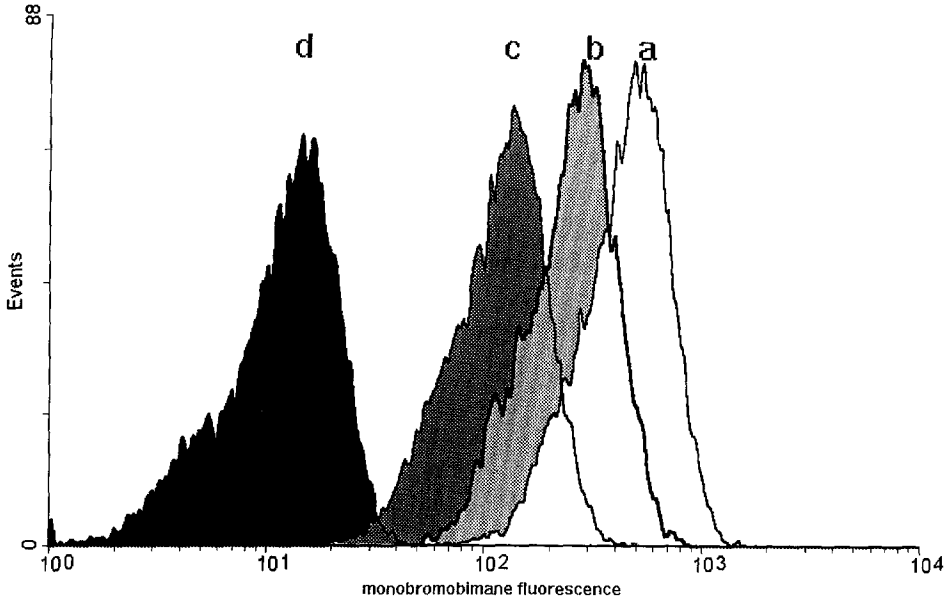

FIG. 1. Differential estimation of cellular thiols. Jurkat T cells were treated with 40 μM monobromobimane (MBB) for 15 min and then fluorescent emission from UV excited cells was collected. In this histogram, the y axis represents the relative cell number and the x axis represents the intensity of thiol-reacted bimane fluorescence in a four-decade log scale. (a) total fluorescence, (b) buthionine sulfoximine (BSO)-treated cells, (c) phenylarsine oxide (PAO)-treated cells, and (d) *N*-ethylmaleimide (NEM)-treated cells. a minus b estimates intracellular GSH, a minus c estimates intracellular vicinal dithiols, and a minus d estimates total cellular thiols. Analysis of data showed that almost all (97%) of the MBB emission was sensitive to the treatment of NEM. PAO and BSO treatment of cells blocked 73 and 44% of the total emission from MBB-reacted cells, respectively. Reprinted with permission from C. K. Sen *et al., Free Radic. Biol. Med.* **22,** 1241 (1997).

group are pelleted (400g for 5 min) and resuspended (10^6 cells/ml) in phosphate-buffered saline (PBS) at room temperature. Each treatment group is subjected to the following four types of treatment: (a) the control is not treated with any thiol regulatory agent so that the total emission from these cells can be read and (b) GSH-depleted cells are treated with 150 μM buthionine sulfoximine (BSO; aqueous solution) for 18 hr in culture. Pretreatment of cells with BSO decreased intracellular GSH level by ~95% as estimated by HPLC electrochemical detection (not shown). The concentration of BSO and time required to obtain maximum depletion of intracellular GSH without loss of viability should be optimized for each cell type. (c) Vicinal dithiol-blocked cells are treated with 20 μM phenylarsine oxide [PAO; in dimethyl sulfoxide (DMSO)] for 10 min before treatment of cells with bimane and (d) total thiol-blocked cells are treated with 250 μM

N-ethylmaleimide (NEM; aqueous solution) for 10 min before bimane treatment.

Monobromobimane Loading

Monobromobimane (Molecular Probes, Eugene, OR) is dissolved in acetonitrile to obtain an 8 m*M* stock concentration. Cells are pelleted (400*g*, at room temperature for 5 min) and resuspended in PBS (pH 7.4) at 10^6/ml and, if required, treated with PAO or NEM for 10 min as described in the previous section. Following this interval, the MBB stock solution is added to the cell suspension at room temperature such that the final concentration of the bimane reagent is 40 μM. Bimane-loaded cells are analyzed using a flow cytometer.

Flow Cytometry

Bimane-loaded cells are excited using a 20-mW powered UV line of a Innova 90-4 argon ion laser (Coherent, Palo Alto, CA) set of 350 nm in a EPICS Elite (Coulter, Miami, FL) flow cytometer. Fluorescent emission from cellular sulfhydryl-reacted bimane is recorded using cytometer settings, and filter arrangements as shown in Fig. 2. A forward scatter/side-

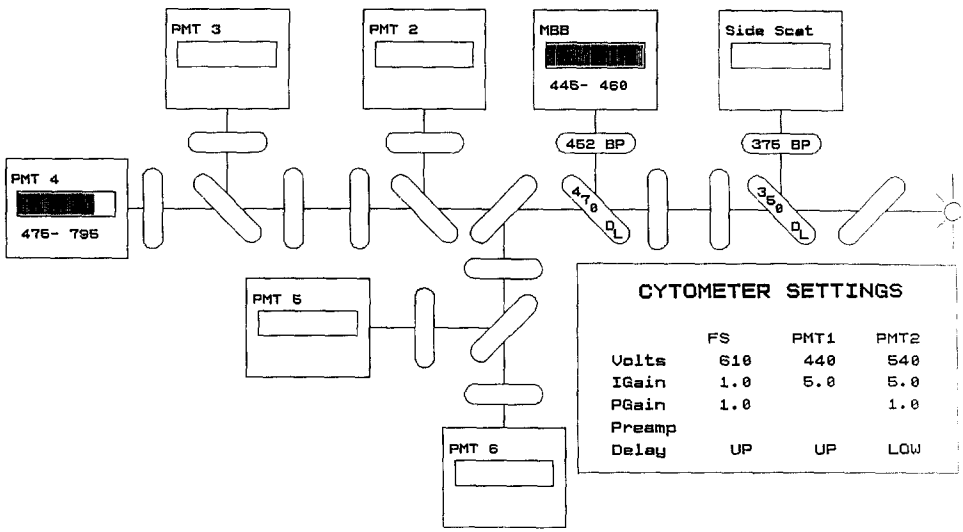

FIG. 2. Flow cytometer filter arrangements (OPTICAD, Coulter Corporation, FL) and a typical example of cytometer settings used for the measurement of emission from monobromobimane (MBB)-reacted cells. A 20-mW powered UV line of a Innova 90-4 argon ion laser (Coherent, Palo Alto, CA) set at 350 nm was used for excitation. The open circle at the right represents the laser source. PMT, photomultiplier tube. Generated using the standard Coulter Elite software version 4.02 (Coulter Corporation).

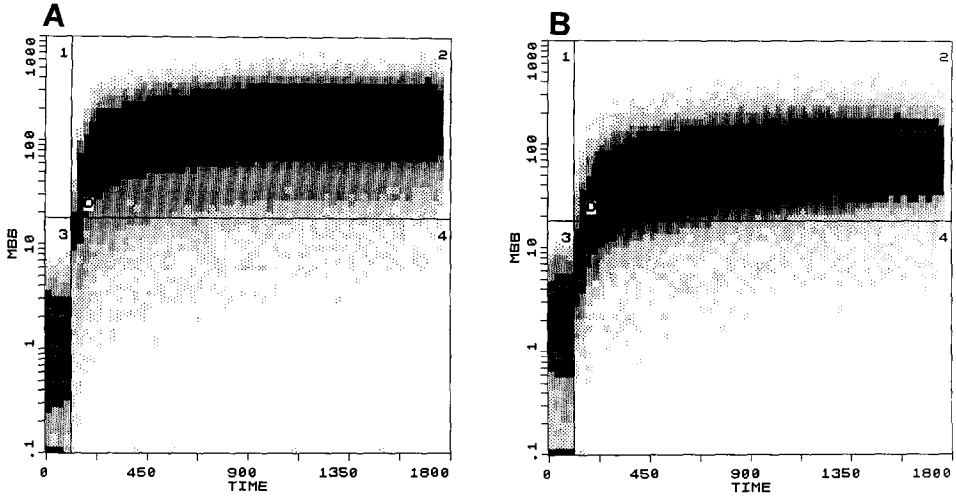

FIG. 3. Kinetics of monobromobimane (MBB) fluorescence development in Jurkat T cells. Autofluorescence (see quadrant D3, bottom left) was collected for 2 min from cells (10^6/ml) suspended in phosphate-buffered saline (pH 7.4). After this, monobromobimane (40 μM) was added to the cell suspension, and the kinetics of fluorescence development was followed for a total of 30 min. Time plotted on the x axes is represented in seconds. The y axes represent the MBB fluorescence plotted in log scale. Data were collected at a flow rate of 300–400 cells/sec for 1800 sec. (A) Control nontreated cells and (B) GSH-depleted cells (treated with 150 μM buthionine sulfoximine for 18 hr).

scatter dot plot is obtained from which a morophometrically homogeneous viable cell population is gated. Data are collected from at least 10,000 cells at a flow rate 250–300 cells/sec.

Multiparameter flow cytometric analyses may be performed with surface immunostained cells using fluorochrome-coupled monoclonal antibodies. For example, T cells may be immunotyped for the presence or absence of CD3 (mature T cells), CD4 (helper T cells), and CD8 (cytotoxic suppressor T cells), and thiol data can be collected from a subpopulation of cells split on the basis of their immunotyping.[7] Precaution should be taken to ensure that the antibodies used for the staining process do not influence cellular thiols. For example, treatment of T cells with certain antibodies may result in GSH efflux from the cell. This must be avoided or controlled for.

Fluorescence Emission from Monobromobimane-Treated Cells

Emission from UV laser-excited cells that were loaded with MBB was followed for 30 min. Mean fluorescence was collected continuously from 300 to 400 cells/sec. The kinetics of increase in fluorescent emission is illustrated in Fig. 3. The sensitivity of the emission to the different thiol

regulatory agents is shown in Fig. 1. Almost all (97%) of the emission from MBB-reacted cells was quenched when thiols were blocked by 250 μM NEM treatment for 10 min before bimane treatment. This suggests that almost the entire emission originated from the reaction of MBB with thiols and that the thiol nonspecific response is minimal. Analysis of data obtained from Jurkat T cells showed that 44% of the total emission is contributed by cellular GSH and that vicinal dithiols in the cell account for 73% of the total emission. Based on these results, the different pools of intracellular thiol may be quantitated as indicated in the legend of Fig. 1.

Low Concentration of α-Lipoic Acid Increases Cellular Thiols

From concentrations as low as 10 μM, α-lipoic acid is effective in increasing the cellular GSH level. A dose-dependent effect of α-lipoic acid in the concentration range of 10–125 μM was observed (Fig. 4). Using HPLC electrochemical detection we observed that 10 μM lipoate resulted in a 16 ±13% increase of Jurkat T cell GSH (not shown). We have observed that in cultured cells there is almost always a small population (up to 10%) of cells that are either nonviable or smaller in size compared to the main population (>90% of all cells). If these cells are studied jointly with the main population of cells, the obtained results may be expected to have a

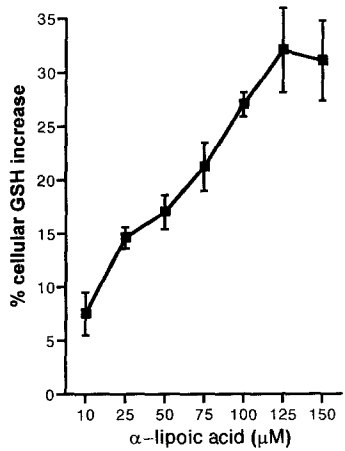

FIG. 4. Flow cytometric determination of the effect of α-lipoic acid on cellular GSH content. Intracellular GSH was estimated from monobromobimane-reacted Jurkat T cells as described in Fig. 1. Cells were treated with a racemic mixture of α-lipoic acid for 18 hr either in the presence or in the absence of 150 μM of buthionine sulfoximine. Results are expressed as percentage change compared to respective α-lipoic acid nontreated controls. Data are mean ± SD.

high standard deviation. Elimination of these cells by gating of the main population allows more consistent results to be obtained. During biochemical measurements, these outlying cells contribute to total protein measurements, but because their thiol status is much lower than that of the main population, the net result suffers from high standard deviation. For results that are expressed on a per cell basis it should be noted that manual cell-counting efficiency is certainly much less than the precise flow cytometric estimation. The effect of short-term (18 hr) and long-term (16 weeks) α-lipoic acid treatment on different intracellular thiol pools is illustrated in Fig. 5. Results show consistently that the effect of α-lipoic acid on intracellular thiols is more pronounced in response to chronic treatment compared to overnight treatment.

The lymphocyte population isolated from human peripheral blood may consist of a heterogeneous mixture of cells as indicated by the forward/side scatter dot plot (Fig. 6a). Typically, this mixture consists predominantly of lymphocytes, but may also contain some other cell types. The homogeneous lymphocyte population is gated as shown in Fig. 6a. That this cell population actually consists of lymphocytes may be verified separately by immunostaining. Monobromobimane emission detected from this gated cell population showed that the thiol content of the majority (>90%) of nontreated resting cells spanned across the M3 domain, indicating that the majority of the population had thiols ranging over the upper 90 percentile. One small subpopulation was clearly separated from the majority population with respect to thiol content. The thiol content of these cells in the M1 domain was in the lowest 1 percentile range. It would be predicted that this cell population should be more vulnerable to oxidative stress and be functionally impaired. α-Lipoic acid, lipoamide, or *N*-acetyl-L-cysteine (NAC) treatment corrected the thiol pool of this subpopulation. Treatment of lymphocytes with excess glutamate decreased the thiol pool of the main cell population[7,20,21] and also resulted in a shift of cells from the regular thiol M3 domain to the low thiol M1 domain population. α-Lipoic acid, lipoamide, or NAC prevented such a shift. This effect should be of remarkable significance with respect to improving the physiological functioning of cells that are thiol deficient under physiological or pathological conditions.[15]

Summary

Several biochemical techniques are based on chromatography or electrophoresis for the determination of thiols from biological samples. These

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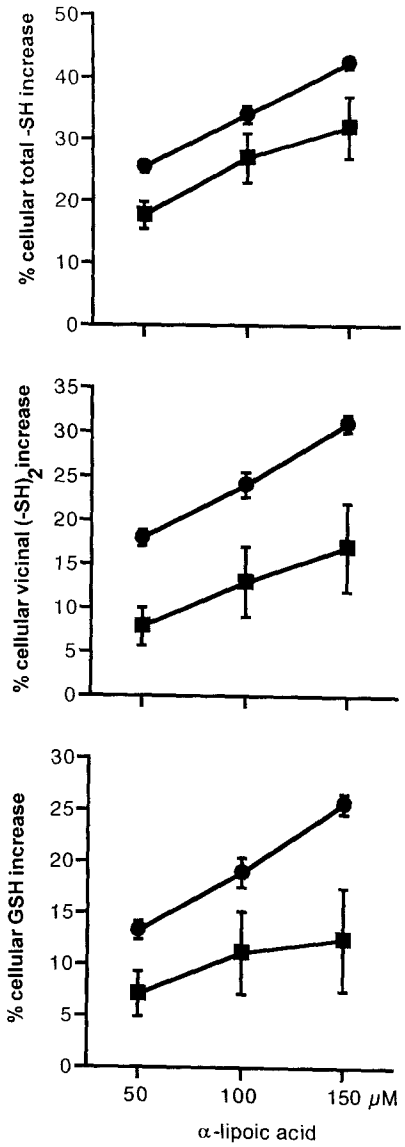

FIG. 5. Effect of treatment duration on α-lipoic acid-dependent changes in different pools of intracellular thiols. Wurzburg cells were maintained in culture medium containing 0 (control), 50, 100, or 150 μ*M* α-lipoic acid for 18 hr (■) or 16 weeks (●). Cellular total protein sulfhydryls (top), vicinal dithiols (middle), and GSH (bottom) were estimated from monobromobimane-treated cells as described in Fig. 1. Results are expressed as percentage change compared to respective α-lipoic acid nontreated controls. Data are mean ± SD.

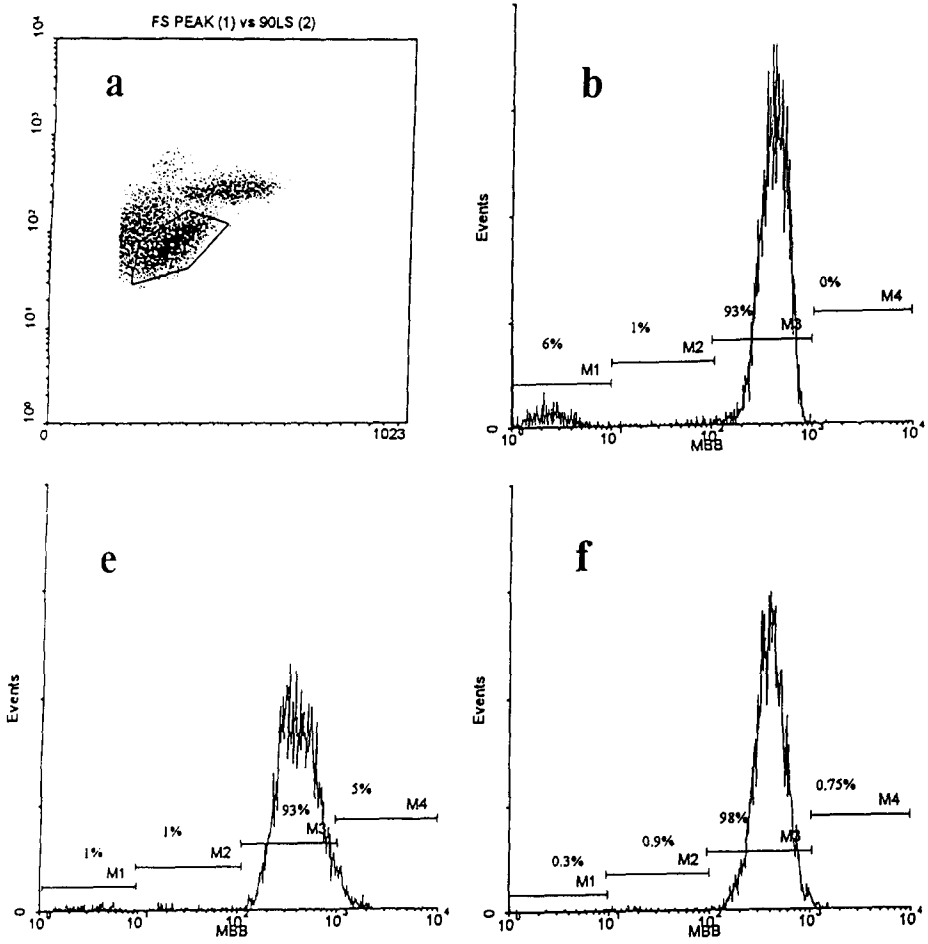

FIG. 6. Effect of lipoate, lipoamide, and *N*-acetylcysteine on human peripheral blood lymphocyte subpopulations. (a) Forward scatter/side scatter dot plot profile of PBL after 48 hr of mitogen-activated culture. A morphometrically homogeneous population of the predominant type of cells was gated for the study of cellular thiols. For the rest of the histograms (b–h) the *x* axis is a four-decade-log scale representing total monobromobimane fluorescent emission (estimates total cellular thiols, see Fig. 1). Each decade of the log scale is marked as follows: $10^0 \rightarrow 10^1$ = M1; $10^1 \rightarrow 10^2$ = M2; $10^2 \rightarrow 10^3$ = M3; $10^3 \rightarrow 10^4$ = M4. The fraction of the total population of cells present in each of the four marked domains is annotated just adjacent to the corresponding zone. The *y* axis represents the relative cell number (events) in each zone of the *x* axis, (b) nontreated control, (c) 50 μM lipoate treated, (d) 100 μM lipoate treated, (e) 100 μM lipoamide treated, (f) 100 μM *N*-acetylcysteine treated, (g) 5 mM L-glutamate treated, and (h) cotreated with 5 mM glutamate and 100 μM lipoate. Reprinted with permission from C. K. Sen *et al., Free Radic. Biol. Med.* **22,** 1241 (1997).

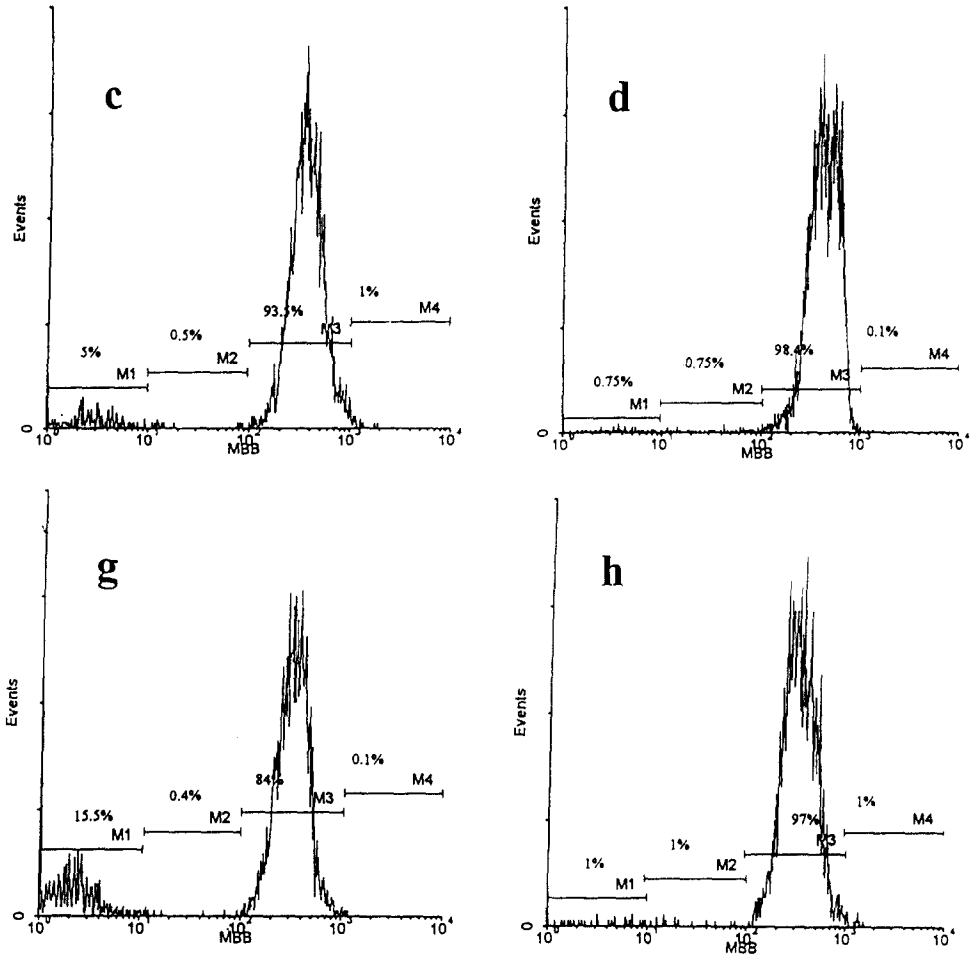

FIG. 6. (*continued*)

techniques are indispensable for the accurate and sensitive detection of specific thiols. Flow cytometric determination of cellular thiols is a powerful technique that is perhaps best suited for clinical application, particularly for cells in blood or other body fluids. Information can be obtained from a small sample amount with a relatively little and quick sample treatment. This technique offers an unique advantage to study the thiol status of a subset of cells because data are collected from individual cells. Multiparameter flow cytometry allows the study of different subsets of immunotyped cells. A major drawback of the flow cytometric method is the lack of specificity for the determination of distinct thiols. The reaction between

MBB and thiols is not specific for any particular intracellular thiol, although almost all of the entire thiol-reacted bimane emission is specific for thiols in general. This limitation can be partly overcome by the treatment of cells with known thiol regulatory agents as described in the section on the differential assessment of cellular thiols.

Acknowledgments

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[22] Measurement of Glutathione, Glutathione Disulfide, and Other Thiols in Mammalian Cell and Tissue Homogenates Using High-Performance Liquid Chromatography Separation of *N*-(1-Pyrenyl)maleimide Derivatives

By LISA A. RIDNOUR, ROGER A. WINTERS, NURAN ERCAL, and DOUGLAS R. SPITZ

Introduction

As glutatione and cellular thiol-related research progresses, the role of these thiols has been extended from acting only in the maintenance of steady-state redox equilibrium, to a proposed involvement in many stress-induced metabolic and bioregulatory functions, including signal transduction and gene expression.[1-3] Glutathione depletion has been identified in various disease processes, including Parkinson's disease and human immunodeficiency virus (HIV) infection.[4,5] Thiols are also known to protect against cytotoxicity associated with exposure to radiation and chemotherapeutic agents.[6,7] Therefore, the evaluation of cellular thiol status may pro-

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