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Original Contribution

REGULATION OF CELLULAR THIOLS IN HUMAN LYMPHOCYTES BY α-LIPOIC ACID: A FLOW CYTOMETRIC ANALYSIS

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Abstract—Modulation of cellular thiols is an effective therapeutic strategy, particularly in the treatment of AIDS. Lipoic acid, a metabolic antioxidant, functions as a redox modulator and has proven clinically beneficial effects. It is also used as a dietary supplement. We utilized the specific capabilities of N-ethylmaleimide to block total cellular thiols, phenylarsine oxide to block vicinal dithiols, and buthionine sulfoximine to deplete cellular GSH to flow cytometrically investigate how these thiol pools are influenced by exogenous lipoate treatment. Low concentrations of lipoate and its analogue lipoamide increased Jurkat cell GSH in a dose-dependent manner between 10 (25 μM for lipoamide) to 100 μM. This was also observed in mitogenically stimulated peripheral blood lymphocytes (PBL). Studies with Jurkat cells and its Wurzburg subclone showed that lipoate dependent increase in cellular GSH was similar in CD4+ and − cells. Chronic (16 week) exposure of cells to lipoate resulted in further increase of total cellular thiols, vicinal dithiols, and GSH. High concentration (2 and 5 mM) of lipoate exhibited cell shrinkage, thiol depletion, and DNA fragmentation effects. Based on similar effects of octanoic acid, the cytotoxic effects of lipoate at high concentration could be attributed to its fatty acid structure. In certain diseases such as AIDS and cancer, elevated plasma glutamate lowers cellular GSH by inhibiting cystine uptake. Low concentrations of lipoate and lipoamide were able to bypass the adverse effect of elevated extracellular glutamate. A heterogeneity in the thiol status of PBL was observed. Lipoate, lipoamide, or N-acetylcysteine corrected the deficient thiol status of cell subpopulations. Hence, the favorable effects of low concentrations of lipoate treatment appears clinically relevant. © 1997 Elsevier Science Inc.

Keywords—Bromobimane, Redox, Oxidative stress, Antioxidant, Glutathione, Apoptosis, Lipoamide, N-Acetylcysteine, Thioctic acid, Free radicals

INTRODUCTION

The oxidation-reduction state of cellular thiols define the redox *milieu* of cells. Thiol homeostasis determines critical aspects of cell function and response.[1,2] Oxidative stress results in a shift of intracellular thiols to disulfides. Such redox changes are implicated in the regulation of signal transduction processes known to be associated with pathogeneses of disease. In T lymphocytes, thiols regulate proliferation and programmed cell death.[3,4] In 1989, Eck et al.[5] were the first to show that human immunodeficiency virus infected (HIV+) individuals have decreased levels of cysteine and GSH in their plasma and leukocytes. Herzenberg and associates have shown that in HIV+ individuals, T-cell subsets are GSH deficient.[6–8] Several studies have indicated that restoration of cellular GSH levels by glutathione replenishing drugs may markedly modulate the effect of inflammatory cytokines and inhibit the stimulation of HIV in both acute and chronic infections. Cellular GSH replenishing drugs such as GSH itself, esterified GSH, oxothiazolidine carboxylate, cysteine, and its derivative N-acetylcysteine (NAC) have been proven to be beneficial in this respect.[9–16]

Flow cytometric determination of cellular thiols is a clinically applicable approach. One of the major advantages is that cellular responses from a considerably small sample amount can be detected on a subpopulation basis. Cell populations gated for defined parame-

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ters such as immunostaining or size can be followed using multiparametric flowcytometry. One major concern in the flow cytometric detection of cellular thiols, however, has been the thiol specificity of recorded signals and related data interpretation.[17–19]

Chloro or bromo derivatives of bimane are nonfluorescent in their native forms but emit strong fluorescence when reacted with thiols.[20] This property of bimanes has been utilized to establish several types of thiol-detecting methods based on HPLC,[21] electrophoresis,[22] and flow cytometry.[18,23] We have used a monobromobimane (MBB)-based modified flow cytometric method to study different cellular thiol pools. As previously reported, this type of assay can be also used for AIDS research because cells can be fixed with paraformaldehyde to inactivate HIV with no effect on any of the measured parameters.[24]

Recent studies from our laboratory[25] have shown that 100 μM α-lipoic acid treatment increases GSH levels of cultured T cells. Lipoic acid is safe for human use and has been clinically used particularly for the treatment of diabetic polyneuropathies for a considerably long time.[26] A modified flow cytometric technique has been implemented to show that α-lipoic acid and a related compound lipoamide may serve as very effective GSH replenishing agents in cultured Jurkat T cells and peripheral blood lymphocytes. These effects have been studied in different subsets of immunotyped T cells. Impaired cellular cystine uptake in HIV+ patients because of high plasma glutamate level has been suggested to be a causative factor of low leukocyte GSH level in these patients.[27,28] Thus, we have studied the effects of low doses of both lipoate and lipoamide on the glutamate inhibition of GSH synthesis. We have investigated the effect of lipoic acid on lymphocyte thiols with the objective to rationalize the selection of this drug for AIDS related clinical studies.

MATERIALS AND METHODS

Cell culture

Cell lines and culture conditions. Human Jurkat T cells were obtained from American Type Culture Collection (ATCC, Bethesda, MD). Wurzburg T cells, a subclone of the Jurkat T cells, developed by Dr. Patrick Baeuerle (Frieburg, Germany) was a generous gift of Dr. Leonard Herzenberg of Stanford University, CA.[16,29] For standard culture, Jurkat and Wurzburg cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 1% (w/v) penicillin/streptomycin, 110 mg/l sodium pyruvate and 2 mM L-glutamine (University of California, San Francisco) in humidified air containing 5% CO_2 at 37°C.

Isolation and culture of human peripheral blood lymphocytes. Blood drawn from healthy male volunteers was collected in heparinized tubes. Peripheral blood lymphocytes (PBL) were isolated by a standard density gradient separation on Ficoll-Hypaque (Pharmacia, Sweden). PBL were 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°C.

Cell treatment

Treatment of Jurkat and Wurzburg T cells with supplements. Cells were resuspended (10^6/ml) in the standard culture medium and seeded in 24-well plates at 10^6 cells/well. Sodium L-glutamate and L-glutamine stock solutions were prepared in phosphate-buffered saline (PBS), pH 7.4. As indicated in respective figure legends, cells were treated with an additional 5 mM L-glutamate or L-glutamine for 18 h. α-Lipoate (racemate mixture, ASTA Medica, Fankfurt, Germany) and N-acetyl-L-cysteine (Sigma, MO) stock solutions were prepared fresh in PBS, pH 7.4. Stock solution of lipoamide (DL-6,8-thioctic acid amide, Sigma, MO) was prepared in dimethylsulfoxide at concentrations such that the final concentration of the solvent in the cell suspension never exceeded 0.01%. Respective controls were treated with equal volume of dimethylsulfoxide. As indicated in respective figure legends cells seeded in 24-well plates were treated with different concentrations of lipoate, lipoamide, or NAC for 18 h. For each supplement-treated group four separate wells containing 1 ml of cell suspension were prepared.

Long-term treatment of Wurzburg cells with lipoate. Cells were resuspended (10^4 cells/ml) in 25 ml standard culture medium containing either no (control) or different concentrations (50, 100, or 150 mM) of α-lipoate. Cells were passaged as above once every 7 d, and in this way the cells were maintained in culture with or without lipoate for 16 weeks.

Treatment of Jurkat and Wurzburg T cells with thiol regulatory reagents. After 18 h of supplement treatment, cells from each of the four wells of a treatment-group were pelleted ($400 \times g \times 5$ min) and resuspended (10^6 cells/ml) in PBS. Each treatment group was subjected to the following four types of treatment: (a) control: not treated with any thiol regulatory agent so that the total thiol signal from these cells could be read; (b) GSH depleted: treated with 150 μM buthionine sulfoximine (BSO) for 18 h. Under conditions where BSO was added to supplement-treated cells, supplement and BSO addition was done simultaneously;

(c) vicinal dithiol blocked: treated with 20 μM phenylarsineoxide (PAO) for 10 min before treatment of the cells with bimane; and (d) total thiol blocked: treated with 250 μM N-ethylmaleimide for 10 min before bimane treatment.

Treatment of human peripheral blood lymphocytes with supplements. PBLs isolated and seeded as described above were mitogenically stimulated with phytohemagglutinin P (2 μg/ml; Grade B, Pharmacia, Sweden). Four hours after such stimulation PBLs were either treated or not (as mentioned in respective figure legends) with supplements prepared as described above for the cell lines. The supplement treatment time for PBLs was 48 h.

Treatment of human peripheral blood lymphocytes with thiol regulatory reagents. PBLs were treated with BSO, PAO, and NEM exactly as described for Jurkat and Wurzburg cells except that BSO treatment was for 48 h.

Flow cytometric determination of cellular sulfhydryls

Bimane loading of cells. Thiol probes monochlorobimane (MCB; Thiolyte®, Calbiochem, San Diego, CA) and MBB (Molecular Probes, Eugene, OR) were dissolved in acetonitrile to obtain a 8 mM stock concentration for each reagent. After the time required to incubate the cells with the supplements (i.e., 18 h for cell lines and 48 h for PBL) cells were pelleted (400 $\times$ *g* $\times$ 5 min) and resuspended in PBS (pH 7.4) at 10^6/ml, and if required treated with PAO or NEM for 10 min. Following this interval, MCB or MBB stock solution was added to the cell suspension such that the final concentration of the bimane reagent was 40 μM.[18] Bimane loaded cells were analyzed flow cytometrically.

Flow cytometric determination of thiols using bimane probe

Bimane-loaded cells were excited using a 20 mW powered UV line of a Innova 90-4 argon ion laser (Coherent, Palo Alto, CA) set at 350 nm in a EPICS Elite (Coulter, Miami, FL) flow cytometer. Fluorescent emission from cellular sulfhydryl reacted bimane was recorded using a 450 nm bandpass filter. A morophometrically homogenous cell population, typically representing $\approx$90% of the total population, was gated. Data were collected from at least 10,000 cells at a flow rate 250–300 cells/s. When regular-sized and shrunken cell populations were studied, two gates containing a representative population of each cell type was constructed, and bimane emission from the two gated cell population were collected simultaneously. Results are presented as mean $\pm$ SD of at least three experiments for cell lines. Human peripheral blood lymphocyte data represent results from two experiments.

Multiparameter flow cytometric analyses of immunotyped T cells

Multiparameter flow cytometric analyses were performed for detecting CD3 (mature T cells), CD4 (helper T cells), and CD8 (cytotoxic-suppresser T cells). Fluorochrome coupled monoclonal antibodies CD4-FITC/CD8-PE/CD3-PE-Cy5 (OptiClone™), and CD4-FITC were obtained from Immunotech, Westbrook, ME. Cells in culture were pelleted (400 $\times$ *g* $\times$ 5 min) for being immunostained. After this, cells were washed thrice and finally suspended in PBS at 10^6/ml for being loaded with MCB as described above. In a EPICS-Elite (Coulter, Miami, FL) flow cytometer, the fluorochromes were excited using a 488 nm argon ion laser and emission at the following wavelengths were recorded for the respective fluorochrome: 525 nm for fluorescein isothiocyanate, 575 nm for R-phycoerythrin, and 675 nm for R-phycoerythrin-cyanine5. Cellular MCB signal was detected from the same cells using a UV line of argon laser as described above.

Cell sorting

Flow cytometric sorting of lipoate-treated shrunken and regular cells. To maximize the yield of shrunken cells, Jurkat cells were treated with 5 mM lipoate for 18 h. Cultured cells were pelleted (400 $\times$ *g* $\times$ 5 min) and resuspended in cold PBS (pH 7.4) at 10 $\times$ 10^6 cells/ml. Before sorting (EPICS Elite, Coulter, Miami, FL), a forward scatter vs. 90° side scatter dot plot was obtained, and cells were gated on the basis of their forward scatter. Approximately 100,000 cells were sorted, and gated regular-sized and viable shrunken cells were collected in two sterile 15 ml Corning tubes containing 5 ml PBS (pH 7.4) in each. A postsort analysis (forward-side scatter plot) of the collected regular and shrunken cells was performed to test the homogeneity of each sorted population.

Cell viability

Plasma membrane integrity of all cells were determined flow cytometrically (EPICS Elite or XL, Coulter, Miami, FL) using the nonpermeant DNA intercalating dye propidium iodide (Molecular Probes, Eugene, OR) that is generally excluded by viable cells.[30] A 15 mW powered argon ion laser was used for excitation at 488 nm and emission signal was collected

at 575 nm. Propidium iodide negative cells were interpreted as viable cells.

Determination of DNA fragmentation in agarose gels

Cell suspension (5×10^6 Jurkat T cells/5 ml PBS) were lysed by addition of an equal volume of lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 2% sodium dodecyl sulfate, pH 8.0) containing 0.1 mg/ml proteinase K and 0.1 mg/ml RNase A. The mixture was incubated in 37°C for 30 min. DNA was extracted by NaI solution (6 M NaI, 13 mM EDTA, 0.5% sodium-N-laurylsarcosinate, 10 mg/ml glycogen, 26 mM Tris-HCl, pH 8.0) treatment and isopropanol extraction.[31,32] DNA concentration adjusted samples (18 μl = 5 μg) were treated with 2 μl loading dye (25% glycerol, 5 mM EDTA, 0.5% SDS, 0.8 mg/ml bromophenol blue) and electrophoresed for 3 h at 80 V through a 1% agarose gel in Tris-borate-EDTA buffer (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.0). A 1 kb DNA ladder was run as a standard. After the run DNA was stained with ethidium bromide, visualized using UV illumination, and photographed.

HPLC-EC determination of glutathione

Cells were pelleted ($400 \times g \times 5$ min) and deproteinized by treatment with 0.2 M monochloroacetic acid. Following the acid treatment, the mixture was snap-frozen in liquid nitrogen and stored in −80°C for the HPLC determination of GSH content. A HPLC system coupled with a gold-mercury electrode electrochemical detector (HPLC-EC) was used.[33] HPLC separation of GSH was done using a Alltech Altima C-18 (150×4.6 mm, 5 μM) column and a mobile phase consisting of 99% 0.1 M monochloroacetic acid and 1% methanol.

RESULTS

Monobromobimane and monochlorobimane fluorescence: kinetics and differential assessment

Fluorescence emission from UV laser excited cells that were loaded with either MBB or MCB was followed for 30 min. Mean fluorescence collected from 10,000 cells at every time point showed a rapid time-dependent increase (Fig. 1). The fluorescent signal due to MBB was fourfold higher compared to that of MCB (Fig. 1). Figure 2 illustrates the shift of bimane fluorescent emission from cells that were either not (a) or treated with (b–d) different thiol regulatory agents. Almost all (92–97%) of the MBB and MCB signal was quenched in cells in which thiols were blocked by 250

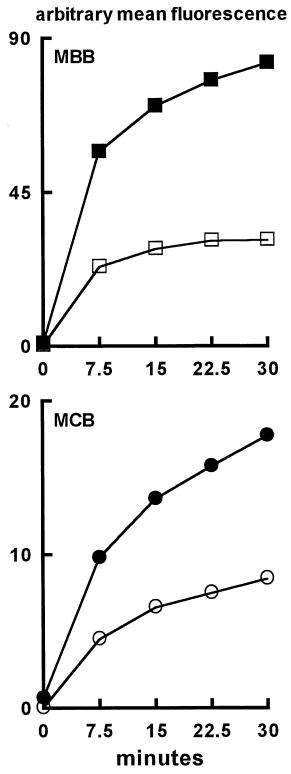

Fig. 1. Kinetics of monobromobimane and monochlorobimane fluorescence development in Jurkat T cells. Filled square and circle represent total sulfhydryl signal from respective bimane treated cells. Open square and circle represent the kinetics of GSH-dependent (BSO-sensitive) component of the total signal.

μM NEM treatment for 10 min before bimane treatment (Fig. 2). Table 1 illustrates the relative contribution of cellular GSH content, vicinal dithiols, and total thiols to the net fluorescent emission.

Pretreatment of cells with BSO decreased intracellular GSH level by 95% as estimated by HPLC-EC (not shown). In these GSH deficient cells both MBB and MCB staining showed a 40–50% decrease in fluorescent emission in response to UV excitation (Table 1 and Fig. 2). Under the given conditions, this component of the emission signal may be interpreted as an estimate of intracellular GSH. The kinetics of BSO-sensitive signal development was characterized by a rapid increase followed by a plateau (Fig. 1). Thus, for next experiments the 15 min time point result was selected for data collection.

The BSO-sensitive fluorescence signal was fourfold more intense for MBB compared to that for MCB (Fig. 1, compare y-axis scales). Thus, for the same amount of GSH in the cell the MBB emission signal was much stronger than that of MCB. This increases the signal:noise ratio, but also has a negative aspect with respect to multiparametric flow assays. Under conditions where FITC immunostained cells were studied for thi-

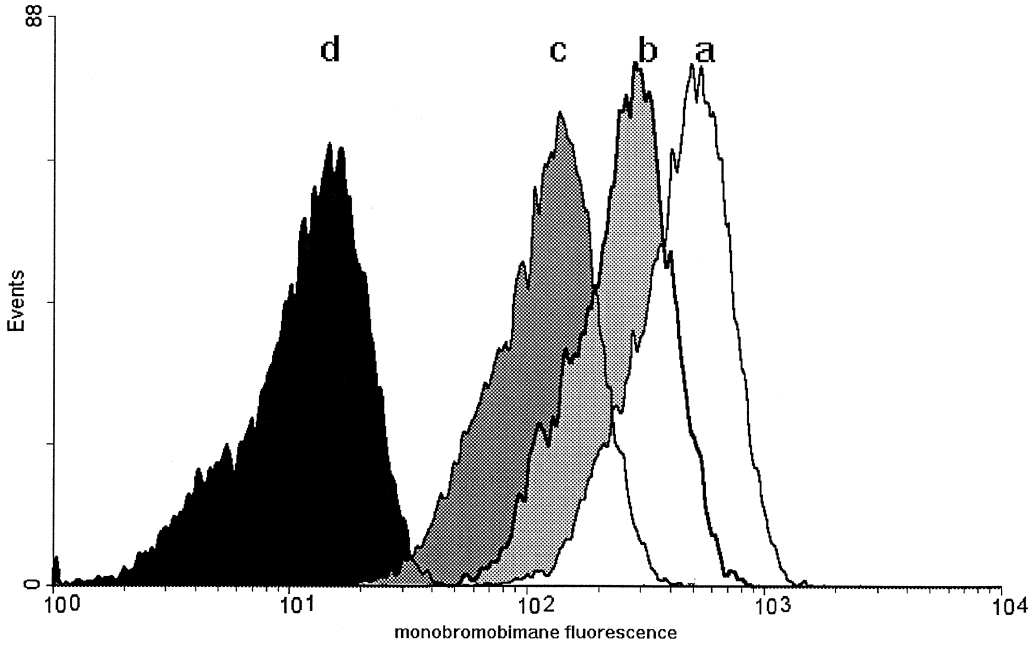

Fig. 2. Differential estimation of thiol-specific cellular monobromobimane fluorescence. Jurkat T cells were treated with 40 μM MBB for 15 min, then fluorescent emission from UV excited cells were collected. In this histogram, the x-axis represents relative cell number and the y-axis represents the intensity of thiol reacted bimane fluorescence in a four-decade log scale. (a) Total fluorescence; (b) buthioninesulfoximine-treated cells; (c) phenylarsineoxide treated cells; (d) N-ethylmaleimide treated cells. a minus b estimates intracellular GSH; a minus c estimates intracellular vicinal dithiols; and a minus d estimates total cellular thiols.

ols, MBB emission was so intense that it tended to overlap with the FITC emission. This limitation may be overcome by using the relatively weakly fluorescent MCB probe. Thus, all of our multiparameter flow assays were performed using MCB as the thiol probe.

Lipoic acid and lipoamide effects on cell glutathione

Using either MBB or MCB as the thiol probe we consistently observed that cells pretreated for 18 h with lipoate or lipoamide had increased GSH content. Even at 10 μM, lipoate resulted in a marginal but consistent increase in cellular GSH (Fig. 3). Using HPLC-EC de-

Table 1. Sensitivity of Cellular Monobromobimane and Monochlorobimane Fluorescence Signal to Different Thiol Regulatory Agents

	Estimates Cellular	T-Cell Lines (Jurkat/ Wurzburg)		PBL	
		MBB	MCB	MBB	MCB
BSO	glutathione	44	48	40	40
PAO	vicinal thiols	73	85	59	n.a.
NEM	total thiols	97	92	94	n.a.

Data are expressed as % inhibition of the total fluorescence signal in response to treatment of cells with the corresponding reagents. n.a., not available.

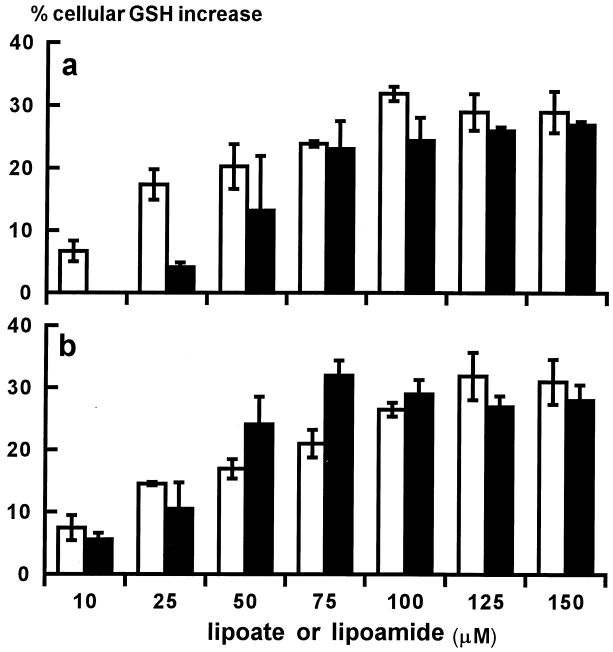

Fig. 3. Concentration-dependent effect of lipoate and lipoamide on intracellular glutathione content of Jurkat T cells. open bar, lipoate; filled bar, lipoamide. Intracellular GSH was estimated by MBB (a) or MCB (b). Results are expressed as % change compared to respective lipoate or lipoamide nontreated controls. All effects shown above were statistically significant at the level of $p < .05$.

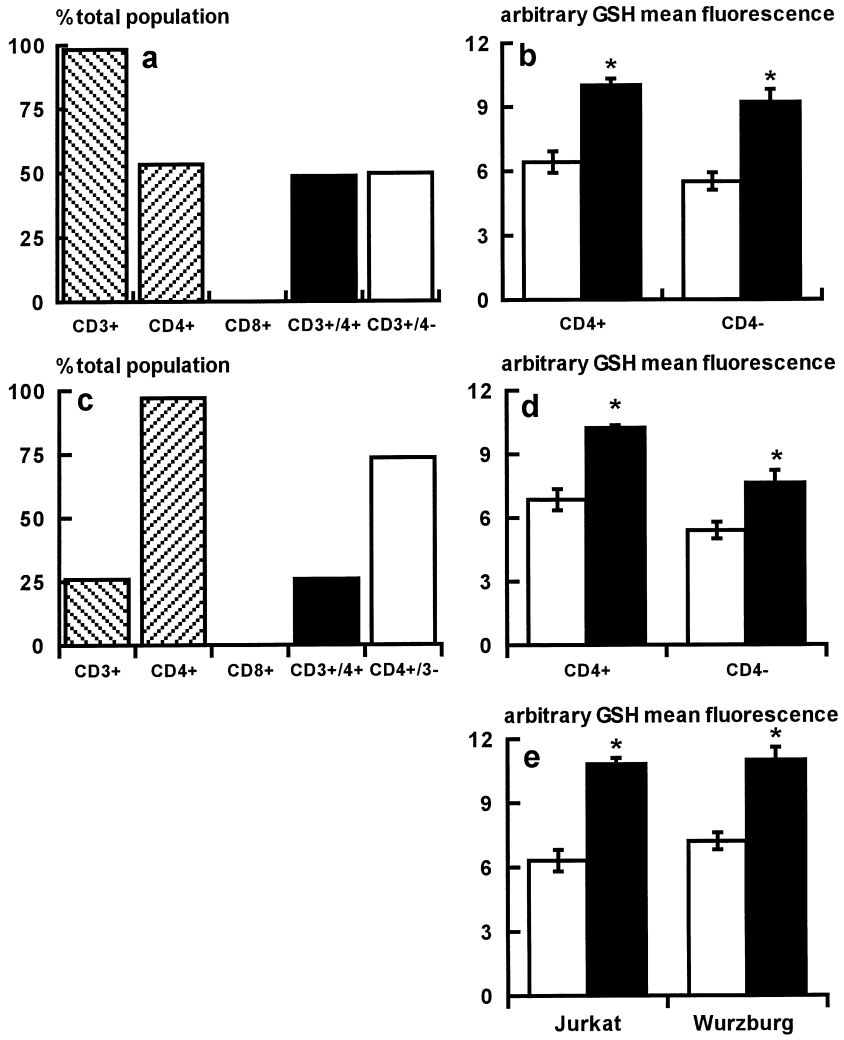

Fig. 4. Effect of lipoate on intracellular glutathione level of Jurkat and Wurzburg T cell subsets. Intracellular GSH was estimated by MCB from CD4-FITC immunostained cells. Relative distribution of CD3, CD4, and CD8 in Jurkat (a) and Wurzburg (c) cells. Effect of 18-h treatment with 100 μM lipoate on CD4+ and CD4− Jurkat (b) and Wurzburg (d) cells. The effect of a similar treatment of lipoate on the entire Jurkat and Wurzburg cell population is illustrated in e. Open bars, nontreated controls; filled bars, lipoate treated cells. Results are expressed as arbitrary fluorescence emission units. *$p < .05$ compared to respective non treated controls.

tection, 10 μM lipoate resulted in a 16 $\pm$ 13% increase of Jurkat T cell GSH. During biochemical determinations, such as with HPLC-EC, it is not possible to gate viable cells and eliminate cell debris. In addition, manual cell counting efficiency is certainly much less than the precise flow cytometric estimation. These factors may have contributed to the high variability of the effect detected by HPLC-EC.

With increasing concentrations of lipoic acid or lipoamide, cellular GSH level increased dose dependently until 100 μM. Both MBB and MCB methods consistently showed that a further increase in lipoate or lipoamide concentration failed to increase cellular GSH levels. A maximum of 30% increase in intracellular GSH was observed for both lipoate or lipoamide treatments (Fig. 3). The effect of lipoate and lipoamide to increase GSH as detected by HPLC-EC was 40–50% $\pm$ 7–10% (not illustrated). Both flow cytometric and HPLC-EC data consistently showed that NAC was not effective to increase Jurkat T cell GSH content at concentrations 100 μM or below (not shown).

Effect of lipoic acid on cellular glutathione in Jurkat and Wurzburg T cell subsets

A remarkable difference in surface receptor profile of Jurkat T cells and its subclone, the Wurzburg cells was observed. Almost all Jurkat T cells were CD3+,

whereas only about 25% of Wurzburg T cells tested positive for CD3 on their surface. Another difference observed was that although almost all of the Wurzburg cells were CD4+, only half of the Jurkat cells were CD4+. The presence of CD8 surface protein was not detected in either cell type. Lipoate was able to increase cellular GSH content of both CD4+ and CD4− cells by a comparable magnitude. Lipoate induced relative GSH increases in Jurkat and Wurzburg cells were comparable further supporting the observation that the effect of lipoate on increasing cellular GSH in these cultured cells were not CD4 subset dependent (Fig. 4).

Effect of lipoate on cellular thiol pools: short- and long-term treatments

Treatment of Wurzburg T cells with 50, 100, or 150 μM of lipoate for 18 h increased cellular thiol content as determined by N-ethylmaleimide–sensitive MBB fluorescence. Compared to 18-h treatment, this effect was markedly enhanced when cells were treated with lipoate for 16 weeks (Fig. 5a). Among the intracellular thiols, lipoate treatment clearly increased GSH and other vicinal thiols. Lipoate effects on these thiol pools were more pronounced following long-term (16 week) exposure compared to 18-h treatment (Fig. 5b and c).

Cellular thiols in response to high concentrations of lipoate exposure

Several previous lipoate and dihydrolipoate studies have used high (>2) mM concentrations of the thiol supplement.[34–36] The effect of high concentration of lipoate on cell thiols was thus investigated. A regular untreated Jurkat or Wurzburg population typically consists of 5–6% (gated as R2, Fig. 6, upper left panel) of shrunken cells although >98% of the total population tests positive for viability. Treatment of cells with high concentration of lipoate resulted in cell shrinking that could be visualized as a shift of cells from gate R1 to R2 (Fig. 6, upper left panel). This effect of lipoic acid was also seen with the structurally related octanoic acid that is similar to lipoic acid with respect to the 8-C fatty acid structure but does not have the dithiolane ring (Fig. 6, upper right panel; Fig. 7a).

A gated study of the two different populations, regular (in red) and shrunken (in green), of lipoate and octanoic acid-treated cells revealed that the shrunken cells were characterized by a marked decrease in cellular GSH, vicinal dithiols, and total thiols (Fig. 7). In the regular-sized cell population enclosed in gate R1 of Fig. 6, 2 mM octanoate but not lipoate decreased GSH, vicinal dithiols or total thiol levels. At higher concentration (5 mM) this effect of octanoic acid was more

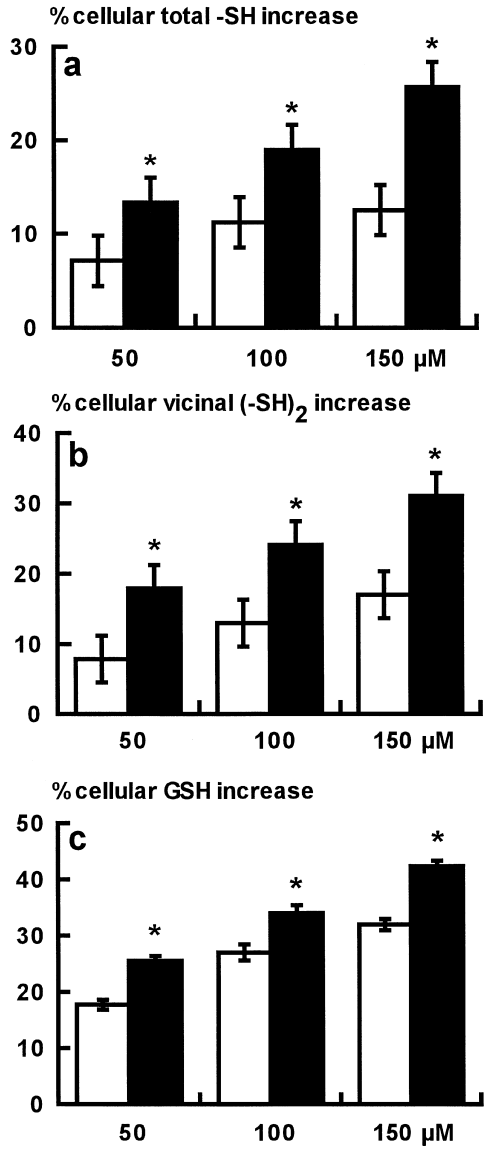

Fig. 5. Effect of long-term lipoate exposure on intracellular thiols of Wurzburg cells. Wurzburg cells were maintained in culture medium containing either 0 (control), 50, 100, or 150 μM lipoate for 18 h (open bars) or 16 weeks (filled bars). Cellular total protein sulfhydryls (a), vicinal dithiols (b), and GSH (c) were estimated in MBB-treated cells as described in Fig. 2. Results are expressed as % change compared to respective lipoate or lipoamide nontreated controls. *$p < .05$ represents effect of long-term exposure compared to respective 18-h treatment.

pronounced, certainly more than the corresponding effect of lipoate at that concentration. GSH levels of 5 mM lipoate-treated morphometrically regular cells were considerably higher compared to the corresponding cells treated with the same concentration of octanoic acid (Fig. 7b).

Viable regular and shrunken cells were sorted on the basis of their forward scatter. A postsort analysis of

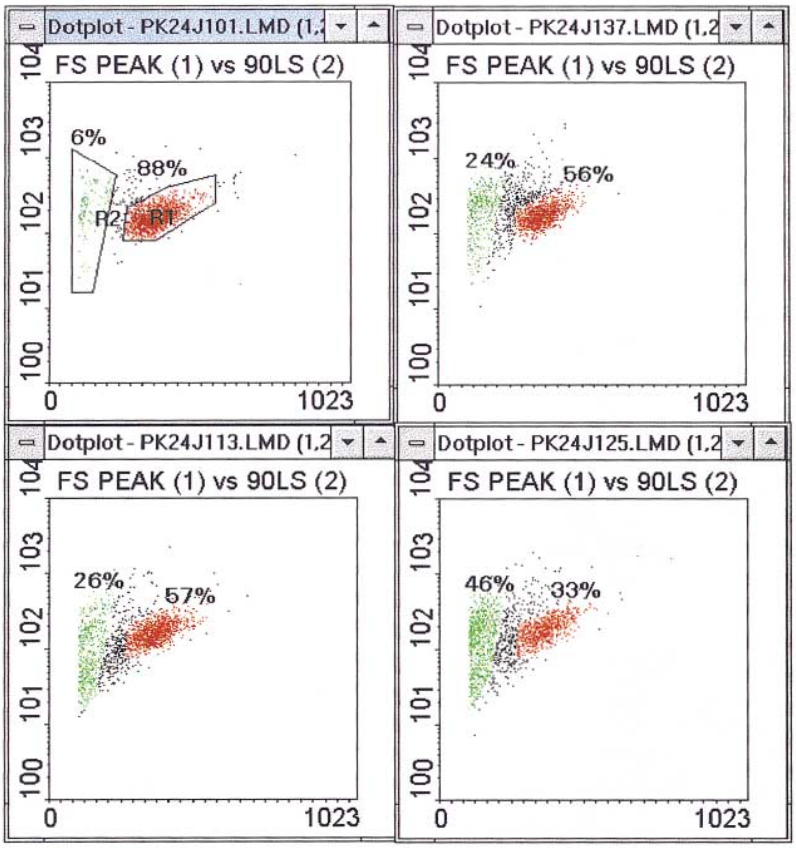

Fig. 6. Regular and shrunken cell population in control or high concentration lipoate treated Jurkat cells. X-axis represents forward scatter (cell size) peak and the Y-axis represents side scatter (90LS) or cell surface granularity. A shift of the signal towards the origin in the X-axis represents cell shrinking. Upper left panel, control cells. The main regular population and shrunken population are gated as R1 (red) and R2 (green), respectively. Numbers adjacent to each subpopulation represent the fraction of total cells included in the respective gates. To provide a better definition to each of these two population, the gates R1 and R2 are slightly spaced apart. A nongated (black) population is therefore visible. In the other three panels, the gates are not visible but the color code (based on the gates constructed in the control cells) represents the regular and shrunken cell population distribution. upper right, 2 mM octanoic acid treated; lower left, 2 mM lipoate treated; and lower right, 5 mM lipoate treated. All treatments were for 18 h.

separated cell populations revealed a 20% inclusion of shrunken cells in the regular cell population. The collected shrunken cell population had, however, only a 2% contamination of regular-sized cells (not shown). The failure to obtain a more homogenous population of each cell type was related to instrumental limitations that is routinely encountered in this type of a procedure.

High-concentration lipoate exposure resulted in DNA laddering (Fig. 8, lane 3). When DNA integrity was tested in sorted regular and shrunken cells, it was evident that the regular (nonshrunken) cells had intact DNA. A slight smearing of the DNA band in these cells could be mainly attributed to the 20% shrunken cell contamination in these cells during the sorting process. The shrunken cells did not have any intact DNA, and the presence of fragmented DNA was evident.

Effect of lipoate and lipoamide on cellular glutathione in the presence of high concentration of extracellular glutamate

Exposure of cells to high concentration of extracellular glutamate resulted in a marked decrease of cellular GSH (Fig. 9). This remarkable effect, however, was not seen with 5 mM L-glutamine treatment of cells. Glutamine treatment slightly lowered cellular GSH level (Fig. 10). Both lipoate and lipoamide treatment (75 μM) was able to effectively circumvent glutamate dependent lowering of cellular GSH. This effect of lipoate and lipoamide was dose dependent with a significant beneficial effect on cell GSH being visible from 25 μM. At 100 μM lipoate or lipoamide-induced increase in cellular GSH level was comparable to the effect that is seen in the absence of excess extracellular glutamate (Fig. 10). Consistent with

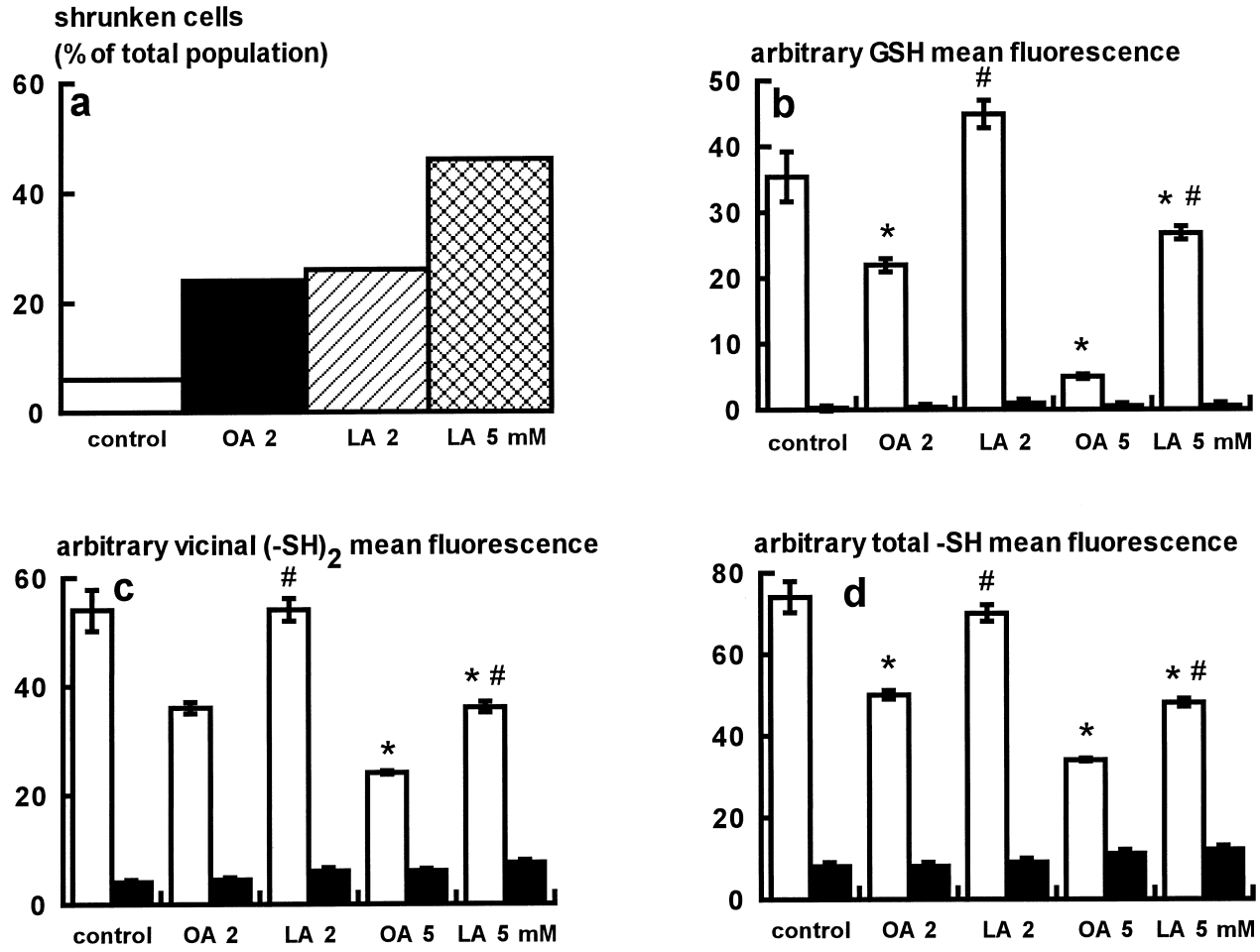

Fig. 7. Effect of high concentrations of lipoate or octanoic acid on cellular thiols. (a) Shrunken cells as a fraction of the total Jurkat T cell population following high concentration (2 or 5 mM) octanoic acid (OA) or lipoate (LA) treatment. Cells, either not treated or treated with octanoic acid or lipoate, were gated to obtain a regular (open bars) and shrunken (filled bars) population (as illustrated in Fig. 6). These gated cell populations were individually studied for their thiol profiles. Cellular GSH (b), vicinal dithiols (c), and total thiols (d) were estimated by MBB. Results are expressed as arbitrary fluorescence emission units. Thiol levels (a, b, and c) in shrunken cells were significantly lower than the respective regular cells. *$p < .05$ compared to respective nontreated controls. #$p < .05$ compared to the corresponding octanoic acid treated cells.

our observation in previous independent experiments, the effect of both lipoate and lipoamide on enhancing cellular GSH content did not increase with a further rise in their concentration from 100 to 125 or 150 μM (Fig. 9). The presence of additional 5 mM of extracellular glutamine was observed to significantly decrease the effect of 100 μM lipoate on increasing cellular GSH level of Jurkat T cells (Fig. 10).

Human peripheral blood lymphocyte glutathione: effects of lipoate, lipoamide, and N-acetylcysteine

Treatment of isolated and mitogenically stimulated PBL with different concentrations (25, 50, and 100 μM) of lipoate, lipoamide, or NAC for 48 h increased cellular GSH level (Fig. 11a). BSO depleted >95% cell GSH of these lymphocytes as detected by HPLC-EC. At rest, cell GSH content was quantitated by HPLC-EC to be 0.23 $\pm$ 0.03 nmol/10^6 cells. After 100 μM lipoate treatment for 2 d, GSH level increased to 0.34 $\pm$ 0.08 nmol/10^6 cells (not illustrated).

A study of the different subsets (i.e., CD4/CD8 positive and negative) showed that the lipoate-induced GSH increase tended to be more pronounced for CD4+ cells compared to the CD4− types. No such CD8 dependent effect was observed (Fig. 11b).

Human peripheral blood lymphocyte thiols: effects of lipoate, lipoamide, and N-acetylcysteine on cell subpopulations

The cell population isolated from peripheral blood consisted of a morphometrically heterogenous mixture

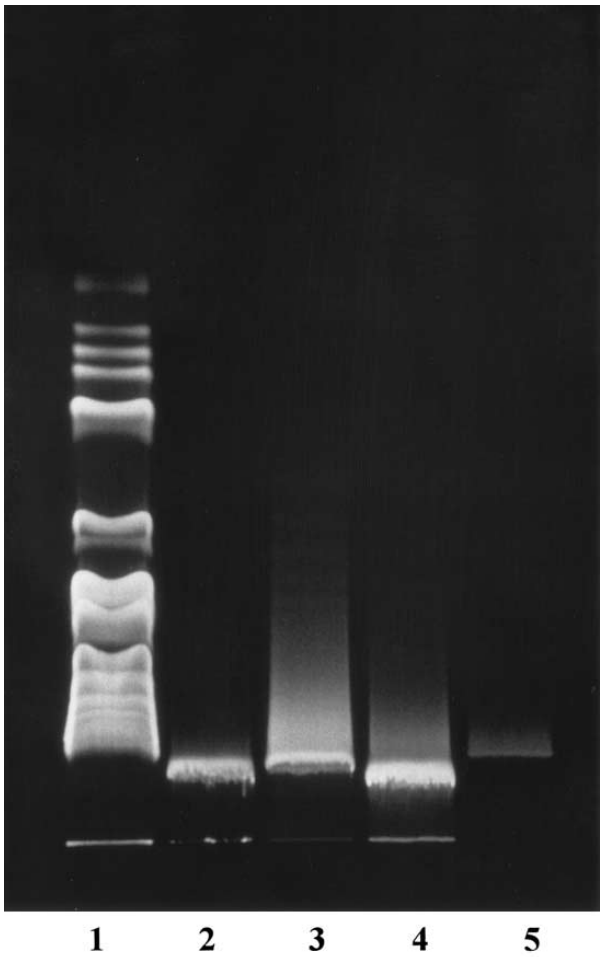

Fig. 8. Integrity of DNA in high-concentration lipoate-treated Jurkat T cells. Jurkat T cells were either not (control) or treated with 5 mM lipoate for 18 h. Cells treated with lipoate was subjected to flow cytometric sorting of regular and shrunken cells. Regular cells (as shown in Fig. 6) and viable shrunken cells were separated. A postsort analysis showed that the separated regular cell population contained 20% shrunken cell contamination, and that the separated shrunken cells contained 2% regular cell contamination. DNA was isolated from control (from left to right: lane 2), lipoate treated whole cell population (lane 3), lipoate treated sorted regular cells (lane 4); lipoate treated sorted shrunken cells (lane 5) and electrophoresed in 1% agarose gel. Lane 1 represents a 1 kb standard DNA ladder.

of cells. This mixture predominantly consisted of lymphocytes, but also contained some other cell types. We gated the morphometrically homogenous lymphocyte population (Fig. 12a) for our thiol studies, and excluded the other populations on the basis of their distinctly separate size category. Typically, 65–75% of the total isolated cells represent the morphometrically homogenous population that have been gated in the dotplot Fig. 12a.

Investigation of this gated cell population showed that the thiol content of lymphocytes may considerably vary between each other. At least 6% of the total

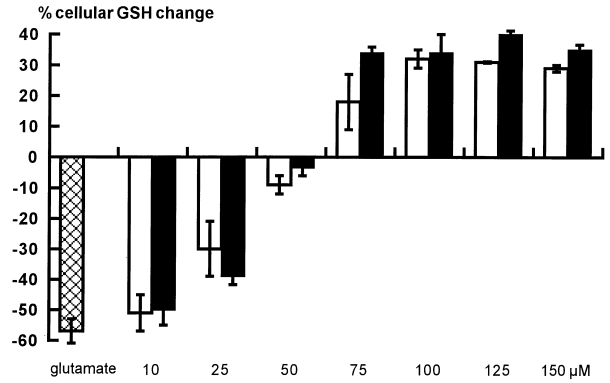

Fig. 9. Dose-dependent effect of lipoate and lipoamide on correcting glutamate dependent downregulation of cellular glutathione level. Jurkat T cells were incubated with 5 mM L-glutamate for 18 h either in the absence or presence of different concentrations (10 to 150 μM) of lipoate (open bars) or lipoamide (filled bars). Cell GSH was estimated by MBB. Results are expressed as % change compared to values from cells that were not treated with either glutamate, lipoate, or lipoamide.

cell population had thiol levels that had a MBB emission signal two orders of magnitude lower than most (93%) of the remaining population (Fig. 12b). All cells had bimane emission signals that were on or below the third decade of the log scale. Lipoate, lipoamide, or NAC increased the thiol content of the major

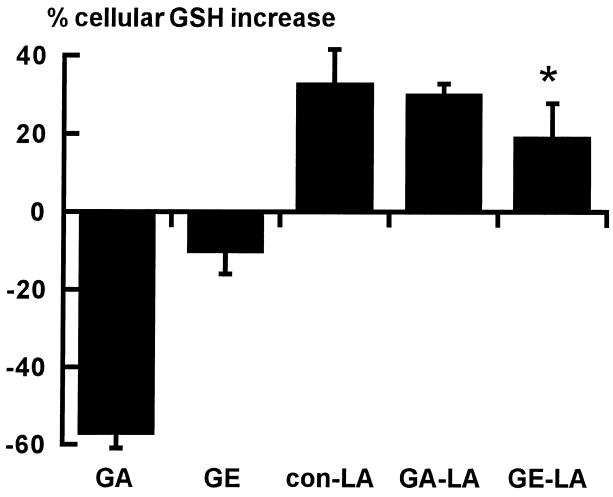

Fig. 10. Regulation of cellular glutathione by glutamate and glutamine. Jurkat T cells were either not treated with additional supplements (control), treated with 5 mM L-glutamate alone (GA), or in the presence of 100 μM lipoate (GA-LA), treated with 5 mM L-glutamine alone (GE), or in the presence of 100 μM lipoate (GE-LA), or treated with 100 μM lipoate only (con-LA). All treatments were for 18 h. Cell GSH was estimated by MBB. Results are expressed as % change compared to values from control cells that were not treated with either glutamate, glutamine, or lipoate. All effects shown above are significant ($p \leq .05$) when compared to nontreated controls. *$p = .053$ (Student t-test) for con-LA vs. GE-LA, i.e., lipoate-dependent increase in cellular GSH level tended to be less in the presence of supplemented L-glutamine.

cell population as evidenced by the nature of the histogram in the M3 domain, and the number of cells in the M4 domain (Fig. 12c–f). A more pronounced effect was that at 100 μM all three supplements remarkably improved the thiol status of the subpopulation of cells that were localized in the M1 domain of the control cells (Fig. 12). Treatment of the cells with an excess of L-glutamate resulted in reverse shift of a population of cells from the thiol-adequate M3 to the thiol-deficient M1 population. This adverse effect of glutamate was not observed in the presence of 100 μM lipoate (Fig. 12g and h).

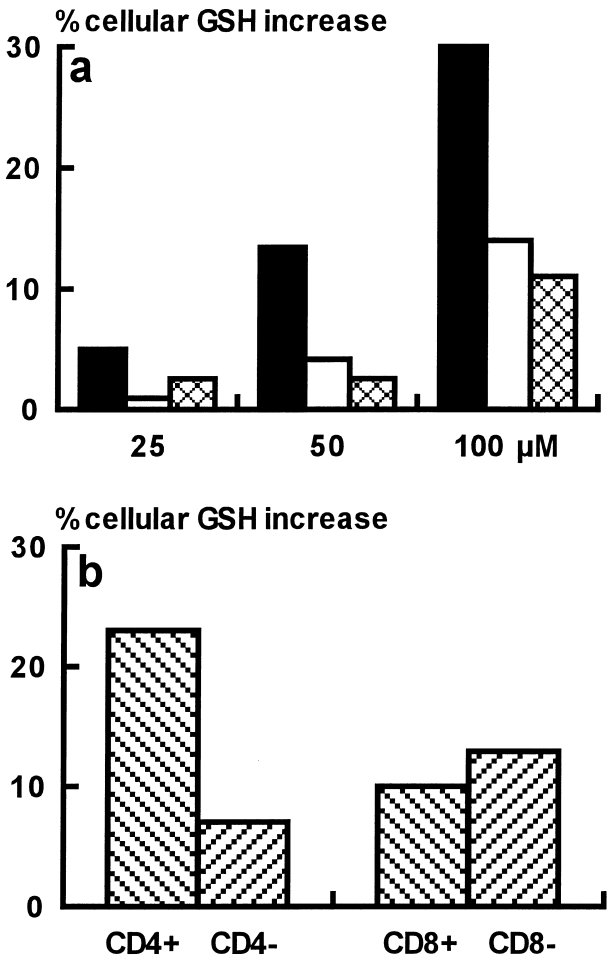

Fig. 11. Dose-dependent effect of lipoate, lipoamide, and N-acetylcysteine on human peripheral blood lymphocyte glutathione content. (a) Mitogen-activated PBL were treated with either no (control) or varying concentrations (25, 50, or 100 μM) of lipoate (filled bars), lipoamide (open bars), or N-acetylcysteine (hatched bars) for 48 h. Cellular GSH was estimated by MBB. (b) Lymphocytes treated with either no (control) or 100 μM lipoate for 48 h were immunostained for the multiparametric detection of CD3, CD4, and CD8. Cellular GSH was estimated by MCB. Results are expressed as % change compared to values from control cells that were not treated with any supplement.

DISCUSSION

Monochlorobimane and monobromobimane as probes for the differential estimation of cellular thiols

Although both MCB and MBB have been used for flow cytometric estimation of cellular GSH, MCB requires glutathione S-transferase activity. However, because human glutathione S-transferase isoenzymes have a low affinity for MCB, this thiol probe has been evaluated not to be ideal for GSH measurements in human cells.[17–19] MBB seems to be the thiol probe of choice because it specifically labels both protein and nonprotein thiols by a glutathione S-transferase independent mechanism.[37] The reaction of bromobimanes with thiols are second order and pH dependent where the active nucleophile is a thiolate anion such as GS^-.[20] Other properties of the MBB fluorophore make it a quantitative analytical tool, for example, 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.[22]

The reaction between bimanes and thiols is nonspecific. Hence, in our assay system, measurement of fluorescent signals for specific thiol pools was accomplished by using defined membrane permeable thiol regulatory agents such as BSO, PAO, and NEM. Our assay system was directed towards the distinct estimation of vicinal dithiols as they serve key signaling and metabolic functions.[38] Differential assessment of MBB and MCB emission signals indicate that around half of the total cell fluorescence is GSH dependent. Thus, data collected from GSH-adequate controls and GSH-depleted cells allowed for a specific estimation of cellular GSH.

Thiol replenishing drugs in therapy

Some fundamental criteria that the use of such drugs should satisfy for clinical use are: (a) safety, i.e., nontoxic on long-term use; (b) elevate cell GSH; and (c) favorably modulate molecular responses that are implicated in disease pathogeneses,[39] for example, inhibition of NF-κB in HIV infection. Both NAC and lipoate meet the above-mentioned criteria. With respect to the regulation of redox sensitive molecular mechanisms, lipoate has proven concentration/effect advantage over NAC.[39] One other major advantage is that cells recognize lipoate as a substrate for bioreduction. In this way, the active dithiol form of lipoate can be metabolically recycled at the expense of the cellular reducing equivalents. Thus, lipoate can function as a ''metabolic antioxidant.''[26,40]

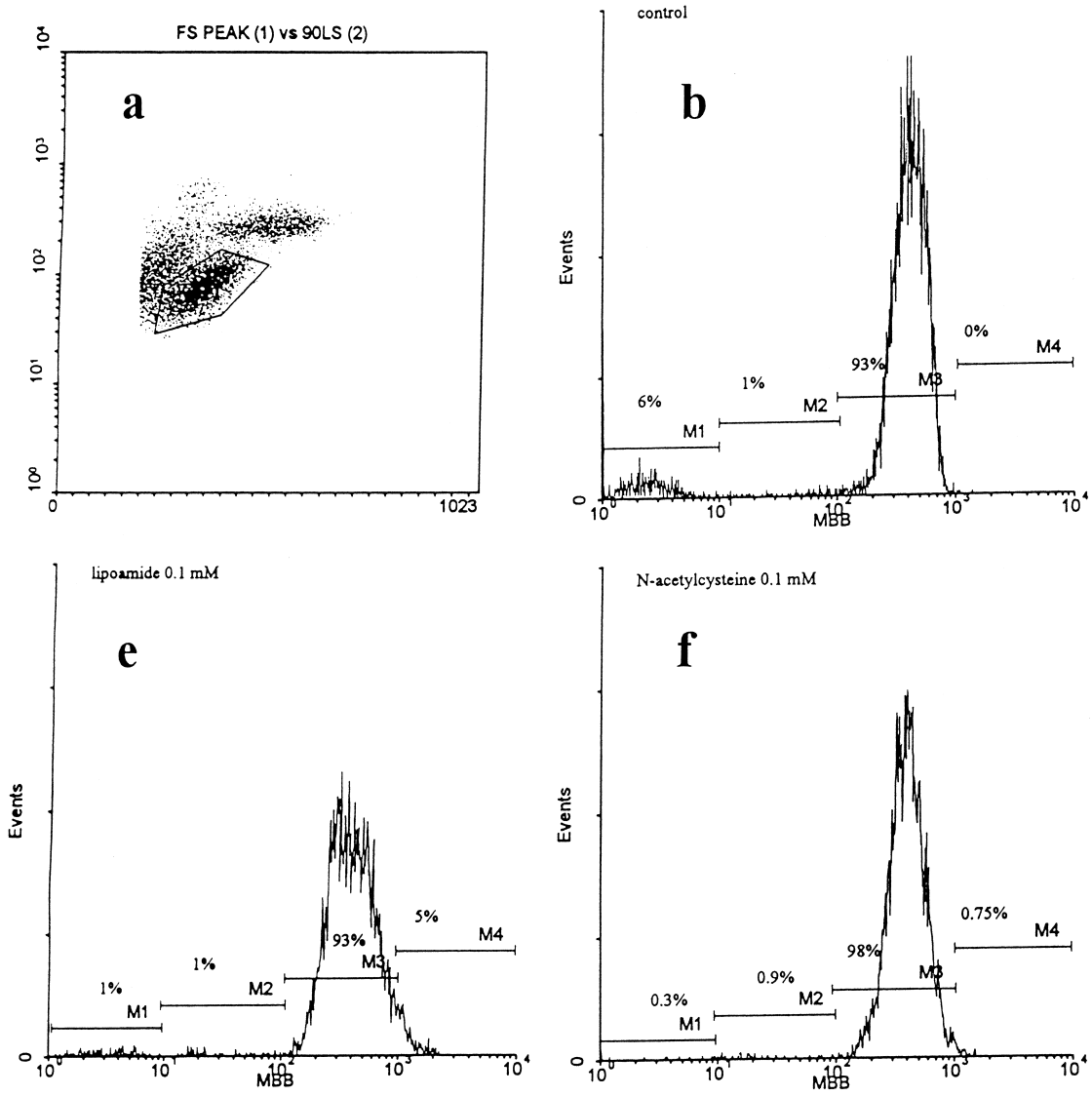

Fig. 12. Effect of lipoate, lipoamide, and N-acetylcysteine on human peripheral blood lymphocyte subpopulations. (a) Forward scatter-side scatter dotplot profile of PBL after 48 h of mitogen-activated culture. A morphometrically homogenous population of the predominant type of cells was gated for the 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. 2). 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 are 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; (h) cotreated with 5 mM glutamate and 100 μM lipoate.

Lipoate has been used clinically in the prevention and treatment of diabetic complications, neurodegenerative diseases, and radiation injury.[26] Lipoate influences redox-regulated transcription[39,40] inhibiting NF-κB activation in response to a wide range of stimuli.[40] Calcium signaling is involved in oxidant-induced NF-κB activation.[29,39] Disturbance in intracellular calcium homeostasis caused by thapsigargin stimulated HIV expression.[41] Lipoate treatment of Jurkat cells decreased oxidant-induced perturbation of intracellular calcium homeostasis.[29,39] In experimental[42] and clinical[43] studies lipoate was found to be potent in inhibiting HIV production and appears useful in the treatment of HIV infection. A recent HIV-related study directly compared the efficacy of NAC and lipoate with respect to NF-κB-mediated gene expression.[44] The study was performed using THP-1 cells that were stably transfected with a plasmid bearing a hygromycin B resistance gene under the control of HIV-1 LTR promoter. At 200 μM lipoate treatment resulted in a 40% decrease in

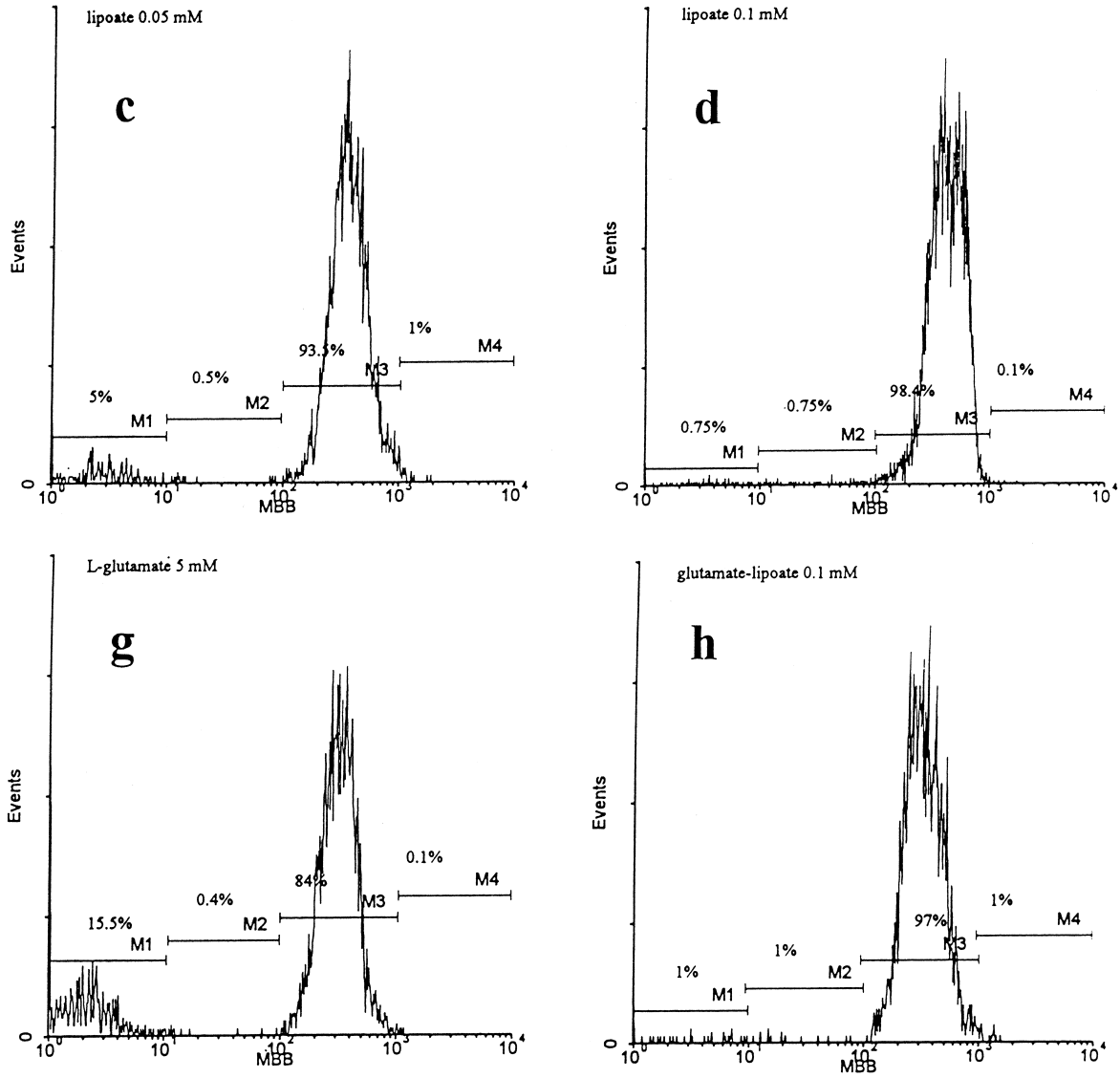

Fig. 12 (*Cont'd*)

HIV-1 p24 antigen expression in TNFα stimulated OM 10.1 cells latently transfected with HIV1. In contrast, 10 mM NAC was required to produce comparable effects.

HIV infected patients have depressed cysteine and GSH levels in the sera, peripheral blood mononuclear cells, and lung lining fluid.[24,27] Decreased T cell thiol status may result in loss of T cell function and adversely affect T cell colony formation.[11] Staal et al.[8] found that a decrease in GSH content of peripheral blood mononuclear cells is mainly confined to T cells in HIV-infected individuals. Most of the studies directed towards the correction of GSH decrease in HIV infection have tested the efficacy of the pro-GSH thiol antioxidant NAC.[27,45] AIDS patients improved substantially with NAC treatment.[27] The beneficial effect of NAC in this respect is thought to be mediated by its ability to increase cellular GSH levels.[45] Lipoate is clinically safe, and is also known to be very potent in increasing cellular GSH levels. Our previous studies with 50–500 μM lipoate show that at 100 μM lipoate can increase Jurkat T cell GSH content by over 40% in 12 h.[25] In mice, GSH increases in the lung, liver, and kidney after lipoate injection have been reported.[46] Our flow cytometric study of a debri-excluded gated population of Jurkat cells showed that at low concentrations lipoate increases cellular GSH.

In T cells, a limiting factor in GSH synthesis is bioavailability of cysteine. In the extracellular compartment, 90% of the cysteine is estimated to be present as

oxidized cystine.[27] In tissue culture media all of cysteine is present as cystine. T cells have a weak membrane x_c^- transport system for cystine. However, the cysteine transporting ASC system is estimated to be ten times more efficient than x_c^-.[47] Thus, delivery of the amino acid in its reduced form outside the cell should facilitate the availability of this GSH precursor inside the cell.

Lipoate functions as the prosthetic group for several redox reactions catalyzed by cellular α-keto-acid-dehydrogenases such as the pyruvate dehydrogenase complex. When treated to cells, lipoate is rapidly reduced to dihydrolipoate and released outside.[36] Lipoamide dehydrogenase is the main enzyme that at the expense of NADH catalyzes the cellular reduction of lipoate and lipoamide to the corresponding dithiols. Our studies showed that dihydrolipoate released from cells reduces extracellular cystine to cysteine, and thus promotes cellular cysteine uptake via the ASC system.[40,48] The dihydrolipoate/lipoate redox couple has a reduction potential estimated to be -0.32 V.[49] Because of this, dihydrolipoate can chemically reduce cystine to cysteine (reduction potential -0.22 V) and even glutathione disulfide to GSH (reduction potential -0.24 V).[50]

Our results show that at low concentrations, both lipoate and lipoamide increase cellular GSH in a dose-dependent manner. At 100 μM or below NAC was unable to increase cellular GSH of Jurkat cells. Using a MBB-based HPLC method, Witschi et al.[51] did not observe any effect of NAC supplementation (1.8 g daily $\times$ 2 weeks) on lymphocyte and plasma GSH of AIDS patients. Also, a single large dose of NAC did not increase GSH in peripheral blood mononuclear cells of AIDS patients.[52] However, low concentrations of lipoate, lipoamide, or NAC increased GSH of mitogenically activated PBL. This is the first evidence that low concentrations of lipoate or lipoamide increase GSH of PBL in a dose-dependent manner.

Lipoate and cell subsets

Decreased cellular GSH of CD4+ and CD8+ T cells is a hallmark of HIV infection. In AIDS patients, GSH levels 63% of normal in CD4+ T cells and 62% of normal in CD8+ T cells are found.[8] Although Wurzburg T cells are a subclone of Jurkat, it possesses markedly different CD3 and CD4 populations compared to the parental line. However, lipoate was effective in enhancing GSH content of both CD4+ and CD4− subsets and no CD4 subset specific difference was observed. In PBL, the effect of lipoate on the GSH content of CD8+ and CD8− cells was similar. However, lipoate appeared to be more effective in enhancing the GSH content of CD4+ cells than that of CD4− cells. Further experiments are necessary to clarify the significance and mechanism involved in this CD4-dependent effect.

Long-term lipoate treatment

It is evident that lipoate not only enhances cellular GSH pool, but may also increase non-GSH thiol pools. Long-term exposure to low concentrations of lipoate-enhanced cellular thiol pools more than that following a brief 18 h exposure. Intracellular dihydrolipoate, formed as a result of the cellular reduction of lipoate, may contribute in part to the lipoate-induced increase in vicinal dithiols. Dihydrolipoate is a potent reductant and may reduce cellular disulfides. Both dihydrolipoate and dihydrolipoamide are known to reduce thioredoxin.[26,53,54]

Effect of high concentrations of lipoate

Sato et al.[4] showed that in T cells oxidation of cellular sulfhydryls to disulfides induces apoptosis. At high mM concentrations lipoate induced thiol oxidation and apoptosis in a subpopulation of cells. Cell shrinkage in response to high concentration lipoate exposure appeared to be dependent on the fatty acid structure of lipoate because similar effect was also observed in cells that were treated with the analogous fatty acid octanoate. The dithiolane ring of lipoate appears to protect the nonshrunken cells against thiol depletion observed in octanoate treated cells.

Lipoate prevents glutamate-induced downregulation of cellular GSH

Glutamate competitively inhibits cellular cystine uptake.[55] Because under physiological conditions or in cell culture media almost all of extracellular cysteine is present, as cystine[27] elevated extracellular glutamate may cause cyst(e)ine deficiency in cells, which is manifested as lowered cell GSH. Patients in certain cases of advanced malignancies have markedly elevated plasma glutamate and a decreased lymphocyte reactivity.[56,57] Elevated plasma glutamate levels have been found in HIV+ patients, and also in individuals considered to be at risk of developing AIDS and, thus, has been suggested to cause lowering of GSH in peripheral blood cells of these individuals.[28] Low concentrations of either lipoate or lipoamide were able to bypass glutamate-dependent cell GSH lowering. Lipoate and lipoamide are reduced by cells to their respective dithiols. Dihydrolipoate released to the extracellular compartment reduces cystine to cysteine,[40,48] hence allowing to bypass glutamate inhibition of cystine uptake

because cysteine taken up by the ASC system is glutamate insensitive. This explains why lipoate not only bypasses glutamate blockade of GSH synthesis but can also increase cellular GSH by a magnitude that is comparable to the effect seen in the absence of glutamate.

Excess glutamine competetively retards cysteine uptake by cells.[58] Elevated extracellular glutamine resulted in only a slight decrease in cellular GSH content, because only a minor[27] fraction of the total cyst(e)ine uptake is contributed by cysteine uptake. Lipoate dependent increase in cellular GSH is cysteine uptake dependent[40,48] and glutamine competes with cellular cysteine uptake mechanisms. Thus, the magnitude of lipoate-induced GSH increase in Jurkat cells was observed to be less in the presence of elevated extracellular glutamine compared to that under regular culture conditions.

Thiol status of peripheral blood lymphocyte population

The thiol content of the majority (>90%) of the 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 functionally impaired.[3,11] Lipoate, lipoamide, or NAC treatment corrected the thiol pool of this cell subpopulation. This effect should be significant with respect to improving physiological functioning and antioxidant defenses. Treatment of lymphocytes with excess glutamate decreased the thiol pool of the main cell population, and also resulted in a shift of cells from the regular-thiol M3 domain to the low-thiol M1 domain population. Lipoate, lipoamide, or NAC prevented such shift. This observation, in addition to the results that lipoate or lipoamide increased cell GSH even in the presence of elevated concentration of extracellular glutamate lends credence to the hypothesis that these substances may protect against glutamate-induced cytotoxicity.

The possible mechanisms involved in the down-regulation of T cell GSH level in HIV+ individuals have been explained by HIV-related oxidative stress leading to enhanced cellular GSH consumption,[59,60] and decreased availability of cysteine for GSH synthesis.[61,62] The dihydrolipoate/lipoate couple may favorably influence both aspects by virtue of antioxidant and redox properties.[26,40] At clinically relevant concentrations lipoate increased cellular GSH in T cell lines as well as in PBL. Elevated plasma glutamate levels are believed to contribute to AIDS-associated decrease in blood cell GSH. Lipoate clearly bypassed glutamate inhibition of cellular GSH synthesis. Previous studies have reported that lipoate treatment favorably influences several molecular mechanisms involved in HIV replication[26,39,40] and the concentration of lipoate required to obtain such gene regulatory effects is much less than that required for NAC. Thus, the rationale that supports the use of NAC for AIDS therapy[27,45] adequately justifies the selection of lipoate for AIDS related clinical studies.

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ABBREVIATIONS

BSO—buthionine sulfoximine
Cy5—cyanine 5
EC—electrochemical detector
FCS—fetal calf serum
FITC—fluoroscein isothiocyanate
GSH—glutathione
GSSG—glutathione disulfide
HIV—human immunodeficiency virus
HPLC—high-performance liquid chromatography
MBB—monobromobimane
MCB—monochlorobimane
NAC—N-acetylcysteine
NEM—N-ethylmaleimide
PAO—phenylarsine oxide
PBL—peripheral blood lymphocyte
PBS—phosphate-buffered saline
PE—phycoerythrin
ROS—reactive oxygen species
UV—ultraviolet