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Glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via the CD2 and CD3 antigens

(immunodeficiency/immunopotentiation/T-cell activation/buthionine sulfoximine/glutathione ester)

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ABSTRACT **Regulation of proliferation of normal human T lymphocytes (T cells) by glutathione (GSH) was explored with T-cell activation models that do not require accessory cell signals. L-Buthionine-(*S,R*)-sulfoximine (BSO), which inactivates γ-glutamylcysteine synthetase and therefore inhibits GSH synthesis, inhibited proliferation elicited by monoclonal antibodies directed at cluster designation 2 (CD2) and CD3 antigens, or by *sn*-1,2-dioctanoylglycerol and ionomycin. L-Buthionine-(*R*)-sulfoximine, which does not inactivate γ-glutamylcysteine synthetase, did not affect proliferation. BSO-induced inhibition of accessory cell-independent T-cell proliferation was not reversed by recombinant human interleukin 2, despite activation-dependent expression of interleukin 2 receptor α by T cells treated with BSO. However, BSO-associated inhibition of T-cell proliferation was reversed by GSH or GSH ester. These studies, which show that GSH can directly modulate proliferation of highly purified T cells, suggest that GSH is essential for steps close to or at DNA synthesis. The availability of methods for decreasing and for increasing GSH levels suggest therapies to produce (*i*) immunosuppression (of value in organ transplantation), and (*ii*) immunopotentiation (of potential value in treatment of immunodeficiency states such as AIDS).**

Glutathione (GSH) has a number of important functions in cellular physiology and metabolism (1–4). That GSH might be involved in the lectin-activated proliferation of T lymphocytes (T cells) was suggested by studies in which human peripheral blood lymphocytes were found to exhibit decreased proliferation after treatment with diamide (5) and with 2-cyclohexene-1-one (6). Diamide decreases GSH levels by oxidizing GSH (7), and compounds such as 2-cyclohexene-1-one react with GSH to form conjugates (8); however, these compounds act nonspecifically and thus may be expected to have effects other than depletion of GSH. Studies in this laboratory (4, 9) and subsequently in others (10–13) in which peripheral blood mononuclear cells were treated with L-buthionine-(*S,R*)-sulfoximine (BSO) (14–16), which selectively inhibits γ-glutamylcysteine synthetase and thus GSH synthesis, gave much stronger support to the view that GSH is required for proliferation.

Several questions about GSH regulation of T-cell functions remain: (*i*) Is the effect of BSO associated with a direct effect on GSH synthesis or is it related to another separate effect of this amino acid sulfoximine? (*ii*) If the effect of BSO is mediated via decreased GSH synthesis, is the inhibition of proliferation a consequence of GSH depletion in T cells (mediators of antigen-specific immunity), or is it due to GSH deficiency, with consequent decrease of function, of the antigen-presenting (accessory) cells that generate the obligatory and costimulatory signals for T-cell activation? (*iii*) Can the inhibitory effect of BSO be prevented or reversed by correction of the GSH deficiency, for example by application of GSH monoester, which is well transported into cells and converted intracellularly to GSH (17–21)?

In the present studies, we examined these questions by use of two models of T-cell activation in which highly purified normal human T cells are induced to proliferate, in the absence of accessory cell signals, by signaling with a synergistic combination of monoclonal antibodies directed at the cluster designation 2 (CD2) and CD3 antigens expressed on the T-cell surface, or with a complementary combination of *sn*-1,2-dioctanoylglycerol (diC_8) and ionomycin (22, 23). The use of these T-cell activation systems permits evaluation of direct effects of GSH on T cells.

EXPERIMENTAL PROCEDURES

Materials. BSO (14–16) and GSH monoethyl ester ½(H_2SO_4) (21) were prepared as described. The diastereoisomeric BSO product [a mixture of L-(*S*) and L-(*R*) forms] was crystallized from 80% (vol/vol) ethanol in 90% yield and then fractionally crystallized from water to obtain the less soluble diastereoisomer, L-buthionine-(*R*)-sulfoximine (24, 25). After two recrystallizations, L-buthionine-(*R*)-sulfoximine was obtained in ≈5% yield; the product contained <0.5% of the corresponding *S*-isomer as determined by HPLC analysis (26).

GSH Determinations. T cells were washed twice with phosphate-buffered saline (PBS) and lysed by addition of sulfosalicylic acid (final concentration, 4.31%) containing 0.2 mM EDTA and two cycles of freeze–thawing. After centrifugation, the supernatant solutions were assayed for total GSH [GSH + ½(GSH disulfide)] by the enzymatic recycling assay (27).

T-Cell Isolation. T cells were isolated from normal human peripheral blood mononuclear cells (from several normal volunteers) by a slight modification of the previously described sequential four-step procedure, which includes (*i*) depletion of adherent cells by Sephadex G-10 gel filtration; (*ii*) depletion of residual monocytes by treatment with 5 mM L-leucine methyl ester; (*iii*) depletion of HLA-DR-bearing cells and natural killer cells by treatment with anti-HLA-DR monoclonal antibody (mAb), anti-NKH-1A mAb, and prescreened rabbit complement; and (*iv*) use of the sheep erythrocyte rosetting technique (28). Fluorescence-activated cell sorter (FACS) analyses of a representative T-cell preparation gave the following data: 99% $CD2^+$, 98% $CD3^+$, 0.36% $NKH\text{-}1^+$ (CD56), 0.34% $HLA\text{-}DR^+$, 0.07% Leu-M3

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Abbreviations: GSH, glutathione; mAb, monoclonal antibody; diC_8, *sn*-1,2-dioctanoylglycerol; BSO, L-buthionine-(*S,R*)-sulfoximine; FACS, fluorescence-activated cell sorter; IL-2, interleukin 2.

(CD14), 0.05% Leu-12 (CD19), and 0.56% interleukin 2 (IL-2) receptor (CD25) cells.

Accessory Cell-Independent T-Cell Activation with Cross-linked Anti-CD2 and Anti-CD3 mAbs. T cells (10^6 cells per ml) suspended in RPMI 1640 medium (catalog no. 380-2400; GIBCO) supplemented with 5% heat-inactivated fetal bovine serum (catalog no. 380-6140; GIBCO) plus 0.1% gentamycin (complete medium) were incubated with 0.5 μg of anti-CD3 mAb per ml (OKT3; Ortho Diagnostics), and 0.5 μg of anti-CD2 mAb per ml (OKT11; Ortho Diagnostics), and the mAbs were crosslinked with 5.0 μg of affinity-purified goat antibodies specific for mouse IgG per ml (Southern Biotechnology Associates, Birmingham, AL). T-cell activation is dependent on signaling T cells with crosslinked anti-CD3 and anti-CD2 mAbs and is independent of additional antigenic or accessory cell signals (22).

Accessory Cell-Independent T-Cell Activation with *sn*-1,2-Dioctanoylglycerol (diC$_8$) and Ionomycin. T cells (10^6 cells per ml) were suspended in complete medium and then incubated with diC$_8$ (10.0 μg/ml) and ionomycin (1.0 μM). The proliferative response is dependent on signaling T cells with both ionomycin and diC$_8$ and is independent of additional antigenic or accessory signals (23). Ionomycin [*Streptomyces conglobatus* (ATCC31005); Calbiochem] and diC$_8$ (Sigma) were dissolved in ethanol and then diluted with PBS (Sigma) to obtain the appropriate concentrations.

Measurement of T-Cell Activation. T-cell activation was quantitated by determining [^{3}H]thymidine incorporation during 48–64 hr of culture, as described (22, 23). T-cell activation was also assessed by determining IL-2 receptor α expression by FACS analysis of cells labeled with anti-CD25 mAb as described (28).

RESULTS AND DISCUSSION

Effect of BSO on T-Cell Proliferation. Induction of proliferation of quiescent normal T cells is dependent on a multitude of coordinated signals including those that are initiated by the antigen(s) and those that are initiated by accessory cells (22, 29). In the recently designed T-cell activation model (22), the obligatory accessory cell signal is bypassed by mAbs directed at the CD2 antigen. In the present work, we used this accessory cell-independent T-cell activation system to directly examine the effect of GSH depletion in T cells.

Fig. 1 illustrates the effect of BSO on T-cell proliferation induced with crosslinked anti-CD2 and anti-CD3 mAbs. It is evident that T cells stimulated by a combination of anti-CD2 and anti-CD3 mAbs exhibit significant proliferation independent of any additional antigenic or accessory cell signals. It is notable that pretreatment with BSO inhibited the proliferative response that was achieved by crosslinking the CD2 and CD3 antigens on the T-cell surface. The marked and concentration-dependent inhibitory effect of BSO was significant ($P < 0.001$ by one-way analysis of variance).

T-cell proliferation initiated via the CD2 and CD3 antigens is dependent on activation of protein kinase C and the mobilization of calcium ions (22). Indeed, highly purified T cells proliferate in response to activation of protein kinase C by diC$_8$ and calcium mobilization with ionomycin independent of additional antigenic or accessory cell signals. Transmembrane signaling of T cells with diC$_8$ and ionomycin resembles physiological signaling of T cells with respect to transcriptional activation and translation of genes for the IL-2 system (23). This accessory cell-independent T-cell proliferation system was used here as an additional model for the direct evaluation of the effects of GSH depletion on T cells.

Fig. 2 shows the effect of BSO on T-cell proliferation induced with diC$_8$ and ionomycin. In accordance with earlier studies, significant proliferation was evident only when T cells were signaled with both diC$_8$ and ionomycin; either agent alone evoked only a minimal proliferative response. Notably, pretreatment of the T cells with BSO inhibited the proliferative response (Fig. 2; $P < 0.001$, analysis of variance).

The data given in Figs. 1 and 2 permit the interpretation that depletion of GSH in normal human T cells is associated with a profound inhibition of their proliferative potential due to a direct effect on T cells. Study of these T-cell activation models supports the conclusion that the cellular site of blockade (due to GSH depletion) is located after very early events linked to T-cell activation.

L-Buthionine-(*R*)-Sulfoximine Has Little if any Effect on T-Cell Proliferation. In previous studies on the effect of BSO on mice and on cells studied in suspension and culture, little evidence was obtained for toxicity that cannot be ascribed to GSH deficiency (see refs. 20, 30, and 31). However, it is difficult to completely exclude the possibility that BSO has an effect on cells in addition to inhibition of γ-glutamylcysteine synthetase. The possibility that impurities in the BSO preparations used produce toxicity needs to be considered but is

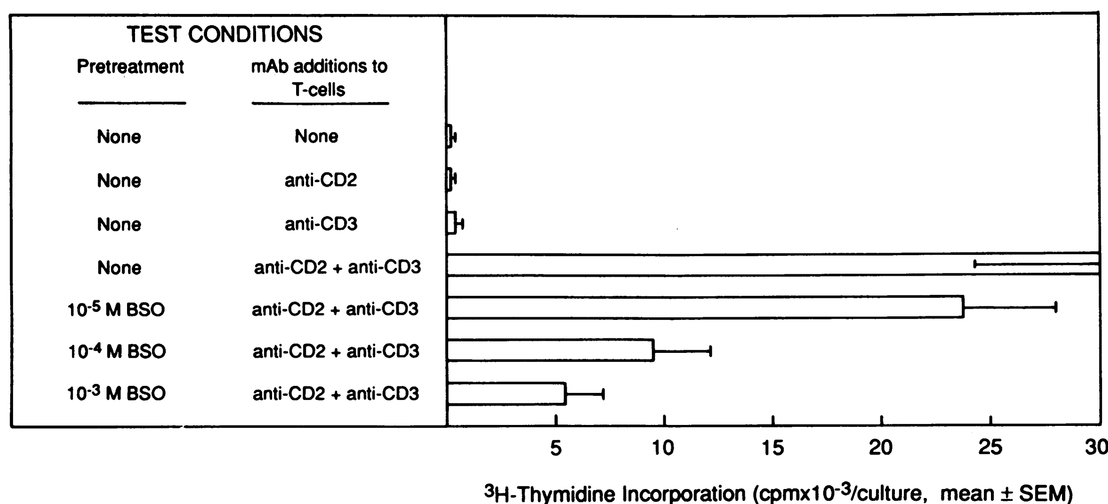

FIG. 1. Inhibition of anti-CD2 and anti-CD3 mAbs induced T-cell proliferation by BSO. T cells were pretreated with the indicated concentrations of BSO for 16 hr prior to stimulation with anti-CD2 (0.5 μg/ml), anti-CD3 (0.5 μg/ml), or anti-CD2 plus anti-CD3 mAbs. The mAbs were crosslinked with goat anti-mouse IgG (5.0 μg/ml). Proliferation was measured by determining incorporation of [^{3}H]thymidine into DNA during 48–64 hr of culture. Results are from consecutive studies on three normal individuals.

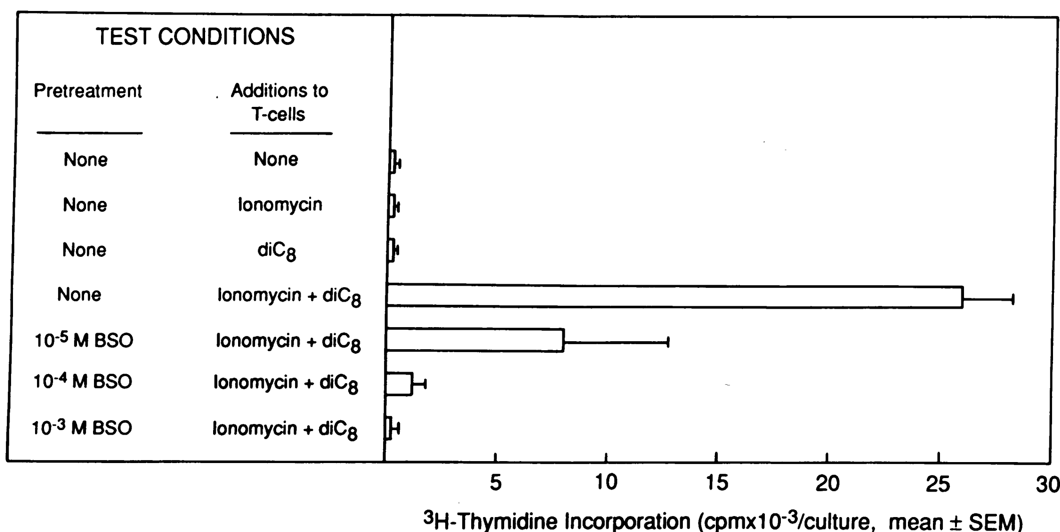

FIG. 2. Inhibitory effect of BSO on T-cell proliferation elicited with diC_8 and ionomycin. T cells were pretreated with different concentrations of BSO for 16 hr prior to stimulation with ionomycin (1.0 μM), diC_8 (10.0 μg/ml), or ionomycin plus diC_8. Results are from consecutive studies on three normal individuals.

rendered less likely by use of preparations that have been carefully recrystallized. BSO may competitively inhibit renal transport of γ-glutamyl amino acids (32) and has been reported to inhibit cellular uptake of cystine in an *in vitro* system (33). It is of interest that L-buthionine-(*R*)-sulfoximine like L-methionine-(*R*)-sulfoximine (34) does not inactivate γ-glutamylcysteine synthetase (16). The stereospecificity exhibited by the amino acid sulfoximine inhibitors of γ-glutamylcysteine synthetase is analogous to that of glutamine synthetase, which is inactivated by L-methionine-(*S*)-sulfoximine but not by L-methionine-(*R*)-sulfoximine (35), and reflects the specific geometry of the tetrahedral intermediates formed on these enzymes.

The present studies, which show that L-buthionine-(*R*)-sulfoximine has little or no effect on T-cell proliferation elicited with crosslinked anti-CD2 and anti-CD3 or diC_8 plus ionomycin (Table 1), strengthen the conclusion that inhibition of GSH synthesis is responsible for decreased proliferation of BSO-treated T cells.

Effect of Treatment with BSO on the IL-2 System. Crosslinking of CD2 and CD3 antigens on the T-cell surface or stimulation of T cells with diC_8 and ionomycin leads to expression of IL-2 receptor α; this is an early T-cell activation event essential for T-cell proliferation (36). FACS analyses for IL-2 receptor expression by T cells, untreated or pretreated with BSO, and then stimulated with diC_8 and ionomycin, are shown in Fig. 3. These findings indicate that (*i*) stimulation of T cells with diC_8 and ionomycin leads to expression of IL-2 receptor α, and (*ii*) IL-2 receptor α expression is minimally affected by pretreatment of the T cells with BSO (Fig. 3; compare *A* with *B*, *C*, and *D*). The percentage of T cells that expresses IL-2 receptor α after activation, as well as the IL-2 receptor α antigenic density (determined by fluorescence intensity of IL-2 receptor-positive cells) are similar in T cells that were untreated or pretreated with BSO and then stimulated with diC_8 and ionomycin. These findings on purified T cells confirm and extend earlier observations that BSO pretreatment does not affect IL-2 receptor α expression as studied in unfractionated peripheral blood mononuclear cells stimulated with concanavalin A (12).

Effect of Recombinant IL-2 on BSO-Associated Inhibition of T-Cell Proliferation. IL-2 is a vital growth hormone for T cells and signals T cells via the IL-2 receptors (37). Since BSO pretreatment does not prevent the expression of IL-2 receptor α, we examined whether recombinant human IL-2 can reverse BSO-associated inhibition.

Table 2 summarizes six consecutive experiments in which the effects of recombinant IL-2 and BSO on accessory cell-independent T-cell proliferation were examined. Recombinant IL-2, as expected from its T-cell growth-promoting properties, augmented the proliferative response found by stimulating T cells with diC_8 and ionomycin or by crosslinked anti-CD2 and anti-CD3 mAbs. Recombinant IL-2, however, did not reverse BSO-associated inhibition of T-cell proliferation. Thus, recombinant IL-2 supplemented cultures exhibited similar levels of inhibition with BSO as compared with cultures established in the absence of exogenous IL-2. Earlier observations indicate that GSH regulates the binding and internalization of IL-2 by mouse cytotoxic T cells (38). The

Table 1. Effect of L-buthionine-(*R*)-sulfoximine on T-cell proliferation

Pretreatment	Proliferation (mean ± SEM), cpm: diC_8 + ionomycin	Proliferation (mean ± SEM), cpm: anti-CD2 + anti-CD3
None	26,100 ± 192	53,200 ± 1380
L-Buthionine-(*R*)-sulfoximine	25,800 ± 948	40,300 ± 336
L-Buthionine-(*S*,*R*)-sulfoximine	931 ± 40	1,070 ± 64

T cells were pretreated with 1 mM sulfoximine for 16 hr at 37°C and then stimulated as indicated. Proliferation was quantitated as cpm per culture. Results are representative of two similar consecutive studies.

Table 2. IL-2 does not reverse BSO-associated inhibition of T-cell proliferation

T-cell stimuli	IL-2	Proliferation, cpm per culture: Control	BSO	% inhibition
diC_8 + ionomycin	−	20,900 ± 3830	270 ± 36	89
	+	25,600 ± 4440	211 ± 44	99
anti-CD2 + anti-CD3 mAb	−	24,100 ± 4990	3000 ± 1370	88
	+	30,600 ± 5950	3320 ± 1440	89

T cells (10^6) were pretreated without or with 1 mM BSO for 16 hr prior to addition of the agents shown and 2.5 nM recombinant IL-2. Results are the means (±SEM) of studies on six normal individuals.

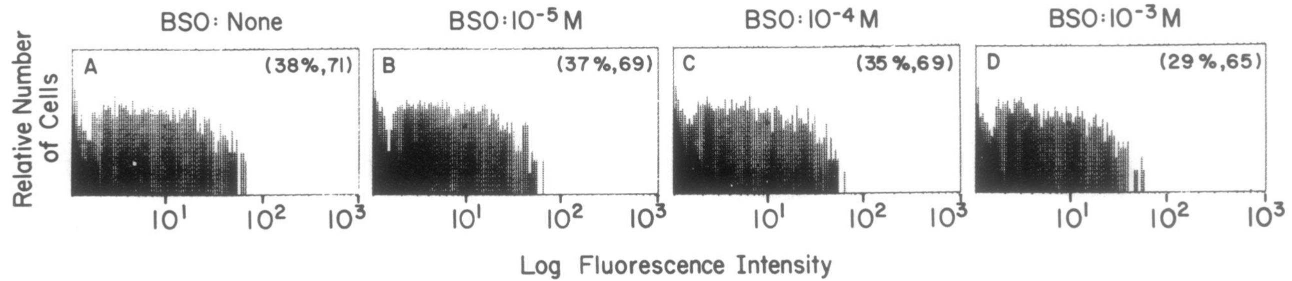

FIG. 3. Lack of effect of BSO on IL-2 receptor α expression. T cells were pretreated with indicated concentrations of BSO for 16 hr prior to stimulation with diC_8 (10.0 μg/ml) and 1.0 μM ionomycin for an additional 24 hr. The T cells were then labeled with phycoerythrin (PE)-conjugated anti-IL-2 receptor α (CD25) mAb or PE-IgG1 mAb (isotypic control) and analyzed by FACS. The percentage of T cells positive for IL-2 receptor α and the log mean channel fluorescence intensity of IL-2 receptor α-positive T cells (numbers in parentheses) were determined by analysis of immunofluorescence histograms with Epics C multidata acquisition and display systems, software version 3.1 (23). Results are representative of three similar studies.

failure of GSH-depleted purified T cells to proliferate in response to IL-2 may also be related to additional factors.

Effect of GSH and GSH Ester on BSO-Induced Inhibition of Accessory Cell-Independent Proliferation. When purified T cells were treated with BSO there was a marked inhibition of proliferation (Table 3, compare Exps. 1 and 2). Proliferation in the absence of BSO was moderately increased by treatment with GSH or GSH ester (Exps. 1, 3, and 4). Addition of GSH or GSH ester to BSO-treated T cells led to a substantial restoration of proliferation (Exps. 5 and 6 compared to Exp. 2). Results similar to these were obtained in studies on T cells from four other normal donors. Typically, treatment with BSO for 16 hr led to 10–40% decreases in T-cell GSH, and addition to the BSO-treated T cells of GSH or GSH ester led to increases (25–100%) in T-cell GSH levels. Although the GSH levels were increased by treating the cells with GSH or GSH ester, there was no apparent linear correlation between the absolute GSH level and the values for proliferation. In a series of experiments similar to Exps. 1, 3, and 4 (Table 3), the GSH levels increased from 0.70 ± 0.26 to 1.42 ± 0.67 and 2.14 ± 1.02 nmol per 10^6 cells after treatment with GSH ester and GSH, respectively. Increased GSH levels were also found after incubation of BSO-treated T cells with GSH and GSH ester; thus, in experiments similar to Exps. 2, 5, and 6 (Table 3), the GSH levels increased from 0.63 ± 0.14 to 0.88 ± 0.21 and 1.41 ± 0.48 nmol per 10^6 cells, respectively. That the values for T-cell GSH are lower after treatment with GSH ester than with GSH may reflect more rapid oxidation of GSH ester than of GSH in medium (M.E.A., unpublished observation). It is relevant that GSH ester and its disulfide, in contrast to GSH and its disulfide, are not substrates of γ-glutamyl transpeptidase (19). The increase of intracellular GSH levels found after treatment of the T cells with GSH may probably be ascribed to extracellular breakdown of GSH, uptake of products, and GSH synthesis.[§] Evidence for this pathway of extracellular GSH utilization has been obtained in studies on human lymphoid cell lines (31, 41), bovine arterial endothelial cells (42), and lung (43, 44). Although it is possible that the isolated T cells can take up intact GSH, no evidence for this was found in studies on mouse peripheral lymphocytes *in vivo* (43), human lymphoid cell lines (31, 41), and human peripheral lymphocytes (6, 11). It seems evident that intracellular GSH is required for proliferation of T cells, but GSH may also have extracellular effects (e.g., on the cell membrane, by reduction of cystine) in addition to serving as a source of cysteine moieties. Thus, 2-mercaptoethanol has been found to augment proliferation (see, for example, ref. 11).

[§]The observations that intraperitoneally administered GSH augmented the activation of cytotoxic T lymphocytes in mice (39) and that dietary GSH enhanced T-cell-mediated immune responses in aging mice (40) are probably related to such utilization of extracellular GSH.

Implications in Immunosuppression and Immunopotentiation. Inhibition of GSH synthesis by administration of BSO may have usefulness in experimental work on immunosuppression and possibly for short-term immunosuppression therapy in connection with organ transplantation.

The role of GSH in T-cell function has been emphasized recently by the finding that human immunodeficiency virus (HIV)-seropositive individuals have markedly decreased levels of GSH in blood plasma and lung epithelial lining fluid, suggesting that systemic GSH deficiency might contribute to the immune deficiency of AIDS (45). Such patients have low levels of plasma cysteine and of GSH in blood mononuclear cells (46, 47). The synthesis of GSH by peripheral lymphocytes seems to depend on transport of cysteine moieties. The liver is the major source of plasma GSH, and AIDS infections are commonly associated with liver abnormalities (48). It is relevant that patients with severe generalized GSH deficiency due to inborn deficiency of γ-glutamylcysteine synthetase or GSH synthetase, though markedly impaired, do not typically exhibit signs or symptoms of AIDS; however, two patients with GSH synthetase deficiency had apparent increased susceptibility to bacterial infections and one of these had neutropenia and defective granulocyte function (49, 50). Although GSH deficiency does not seem to account for all of the findings in AIDS, it appears probable that GSH deficiency contributes to the observed morbidity.

It is therefore reasonable to think that treatment that increases cellular GSH might be beneficial. Utilization of

Table 3. Effect of GSH and GSH ester on BSO-associated inhibition of T-cell proliferation

	Treatment			Proliferation (mean ± SEM), cpm	
Exp.	A	B	C	No stimulation	diC_8 + ionomycin
1	None	None	None	148 ± 17	61,300 ± 501
2	BSO	None	BSO	197 ± 38	446 ± 132
3	None	GSH ester	None	220 ± 117	69,600 ± 2040
4	None	GSH	None	109 ± 28	74,700 ± 1500
5	BSO	GSH ester	BSO	141 ± 37	44,800 ± 999
6	BSO	GSH	BSO	126 ± 55	48,000 ± 2610

T cells (10^6 cells per ml) were suspended in complete medium with or without 1 mM BSO for 16 hr at 37°C (treatment A). The mixtures were centrifuged and the cells were resuspended in fresh medium containing 2.6 mM GSH or GSH ester as indicated (treatment B). After 3 hr, 1 mM BSO was added as indicated (treatment C). The cells were then incubated for 64 hr in the presence or absence of diC_8 and ionomycin. Proliferation was measured by incorporation of [^{3}H]-thymidine into DNA (22, 23).

administered GSH for formation of intracellular GSH, as stated above, involves degradation, transport, and intracellular synthesis, a pathway that requires several enzymes and cellular energy. Treatment with compounds that supply cysteine or γ-glutamylcysteine such as L-2-oxothiazolidine-4-carboxylate (51, 52), *N*-acetylcysteine (51, 53), or γ-glutamylcyst(e)ine (32), might also be of value, provided that γ-glutamyl transpeptidase and the two synthetases required for GSH synthesis are themselves not impaired in this disorder. However, treatment with GSH monoesters (or other GSH delivery agents) would be preferred over treatment with GSH and its precursors. Treatment with GSH ester leads to direct increase of intracellular GSH (without expenditure of cellular energy or requirement for the synthetases) since the esters are efficiently transported and hydrolyzed to GSH intracellularly by many cell types [including lymphocytes (43)] (17–21).¶

¶GSH esters protect cells against toxicity produced by a number of drugs and might for this reason also be valuable for therapy of patients with AIDS who are receiving toxic antiviral agents.

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