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Glutathione export by human lymphoid cells: Depletion of glutathione by inhibition of its synthesis decreases export and increases sensitivity to irradiation*

(buthionine sulfoximine/cell membrane/transport/therapy)

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ABSTRACT **Glutathione (in the form of GSH) is transported out of cultured human lymphoid cells at rates proportional to the intracellular glutathione levels. Inhibition of glutathione synthesis by buthionine sulfoximine, a potent selective inhibitor of γ-glutamylcysteine synthetase, leads to exponential decrease in intracellular glutathione, a large fraction of which appears extracellularly, indicating that glutathione turnover is associated with its export. Although cells with 0.09 mM glutathione (4% of controls) were 85% viable, further decrease was associated with marked loss of viability. Cells with 4–5% of control glutathione levels were much more sensitive than control cells to the effects of γ radiation and of 5,5′-dithiobis(2-nitrobenzoate). Depletion of glutathione by use of buthionine sulfoximine has advantages over other reagents (such as diamide, other oxidizing agents, and diethylmaleate, which affect other cellular components and may increase glutathione disulfide levels) and therefore has potential usefulness in sensitizing cells to the effects of radiation and to therapeutic agents that are detoxified by reactions involving glutathione.**

Studies in which mice were given selective enzyme inhibitors indicate that glutathione is translocated out of renal and liver cells (1–4). Glutathione export, which has also been observed in a liver perfusion system (5) and in suspensions of fibroblasts (6) and lymphoid cells (7), is a discrete step in the γ-glutamyl cycle (3) and accounts for the delivery of intracellular glutathione to membrane-bound γ-glutamyl transpeptidase. Glutathione is translocated from liver into blood plasma (3, 8, 9) and bile (10–13). Transport of glutathione, an important step in glutathione utilization, is a property of many, perhaps most, cells and seems to reflect operation of a general mechanism that may function to protect and maintain the integrity of cell membranes, mediate amino acid transport, and facilitate recovery of the amino acid constituents of glutathione (2).

In the present work, in which we have studied some properties of glutathione export, we used buthionine sulfoximine, which specifically inhibits γ-glutamylcysteine synthetase (14, 15). Previous studies showed that administration of buthionine sulfoximine to animals inhibits glutathione synthesis in liver, kidney, and other tissues (1–4); such inhibition was subsequently observed *in vitro* in macrophages (16), and in trypanosomes (17). We report here that inhibition of glutathione synthesis in lymphoid cells leads to a marked decrease in intracellular glutathione, decline of the rate of its export, decreased cell viability, and increased sensitivity to irradiation.

EXPERIMENTAL PROCEDURES

Materials. Medium RPMI 1640, fetal calf serum, Dulbecco's phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}, and Hanks' balanced salt solution lacking Ca^{2+}, Mg^{2+}, and phenol red were obtained from GIBCO. [*glutamyl*-U-^{14}C]GSH, [*glycine*-2-^{14}C]GSH, and [*methyl*-^{3}H]thymidine were obtained from New England Nuclear. L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) was kindly supplied by L. J. Hanka of Upjohn (Kalamazoo, MI). DL-Buthionine-(*SR*)-sulfoximine was synthesized (15). Glutathione reductase, NADPH, 5,5′-dithiobis(2-nitrobenzoate), glutathione, L-γ-glutamyl-*p*-nitroanilide, oligomycin, ouabain, and glycylglycine were obtained from Sigma. RPMI 8226, a human myeloma cell line, was obtained from the American Type Culture Collection. CEM, a human T-cell line, was obtained from Paul P. Trotta, Sloan–Kettering Institute (New York). HSB, another human T-cell line, was obtained from Associated Biomedic Systems (Buffalo, NY).

Methods. Cells were grown at 35°C in medium RPMI 1640 containing 20% heat-inactivated fetal calf serum and 100 μg of streptomycin and 100 units of penicillin per ml (7); they were used 20–24 hr after fresh medium had been added. Viability of the cells used was at least 95% by trypan blue exclusion (18).

Glutathione export was determined on cell suspensions in phosphate-buffered saline containing 5 mM L-serine and 5 mM sodium borate at 25°C and pH 7.2. After incubation, the cells were separated by centrifugation in a mixture containing mineral oil, dibutyl phthalate, and chloroform (19). The upper aqueous layer was removed and its glutathione content was determined by the glutathione reductase recycling procedure (20). Export was proportional to cell concentration over the range 10^6–10^7 cells per ml. The packed cells were suspended in 0.1 M HCl; after freezing and thawing in dry ice/acetone three times and centrifugation, the glutathione present in the supernatant was determined (20). In some experiments, the cells were disrupted by vigorous mixing in 0.5% trichloroacetic acid. The recycling method (20) measures both GSH and glutathione disulfide (GSSG); about 95% of the glutathione found in these studies extracellularly and intracellularly was shown to be GSH by the 2-vinylpyridine method (21). Cell volume was estimated by centrifuging a known number of cells in a calibrated Corning micropipette. The values obtained for the CEM, HSB, and 8226 cell lines were, respectively, 1.0×10^6, 0.7×10^6, and 1.3×10^6 cells per μl.

CEM cells were synchronized by a modification of the procedure of Kwok and Litwin (22). γ-Glutamyl transpeptidase activity is expressed as nmol/mg of protein per hr (7). Protein

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Abbreviation: AT-125, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid.

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was determined (23) on samples to which 1% sodium dodecyl sulfate was added. Irradiation was done with a cobalt-60 source (Gammacell 200; Atomic Energy of Canada, Limited). The irradiated cells were diluted with medium (to yield 10^6 cells per ml), placed at 35°C for 10 hr, and then examined for viability.

RESULTS

Some General Properties of Glutathione Translocation. Previously, intracellular glutathione levels and export rates were found to vary with different batches of lymphoid cells of the same line (7). To investigate this, we determined the glutathione levels of cells synchronized at several stages of the cell cycle. The glutathione concentrations of CEM cells in the S, G_2 + M, and G_1 phases were, respectively, 5.3, 5.5, and 1.8 mM. These findings are consistent with data on nonprotein sulfhydryl levels in other cells (24). We found that lymphoid cells, 20–24 hr after addition of fresh medium, had constant glutathione levels and export rates; such cells (80%, 16%, and 4% in the G_1, S, and G_2 + M phases, respectively) were used here.

Glutathione export from 8226, CEM, and HSB cells was examined (Fig. 1); the respective apparent export rates were 2.7, 1.9, and 0.6 nmol per 10^7 cells per hr in the presence of serine + borate, a competitive inhibitor of transpeptidase (25, 26). In the absence of serine + borate, no glutathione was found in the medium of 8226 cells, which have high transpeptidase activity (12,000 nmol/mg per hr). CEM cells, which have much less transpeptidase (80 nmol/mg per hr) exhibited about a 20% decrease in the apparent rate of export in the absence of serine + borate. Increasing the level of serine + borate to 40 mM each gave a result equivalent to that found with 5 mM (serine + borate). HSB cells, which have very low levels of transpeptidase (1 nmol/mg per hr) exhibited similar export rates in the presence and absence of serine + borate.

Addition of or pretreatment for 30–60 min with 1 mM oligomycin, 10 mM ouabain, and 100 mM K^+ (as KCl) had no effect on glutathione export. Addition of L-glutamate, L-methionine, or glycylglycine in 1–10 mM concentrations in the presence or absence of 5 mM (serine + borate) did not affect the export rate. Export was not significantly affected by adjustment of the pH of the medium to 5.5 or to 8.5. The rate of export increased slightly as the temperature was increased from 0°C to 15°C, and a more substantial increase occurred at higher temperatures

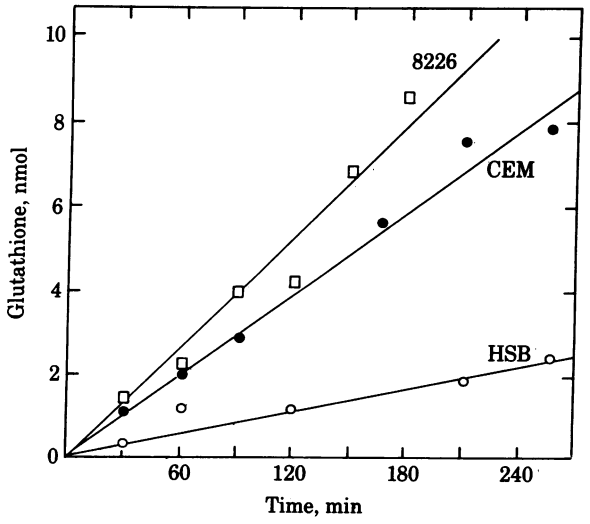

FIG. 1. Export of glutathione from lymphoid cells. The cells were suspended (10^7 per ml) in phosphate-buffered saline. Ordinate, glutathione export per 10^7 cells.

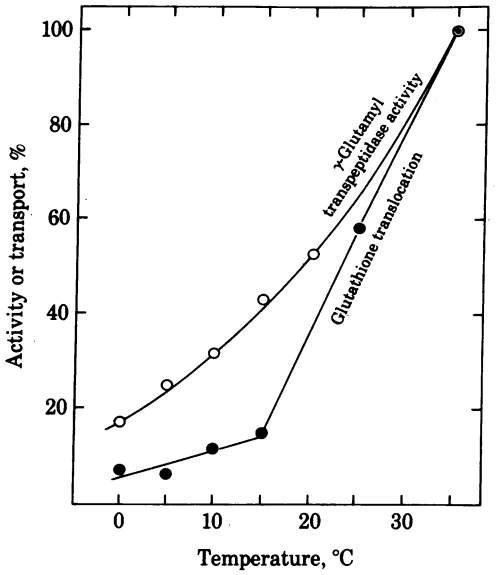

FIG. 2. Effect of temperature on glutathione export and γ-glutamyl transpeptidase activity. Suspensions of CEM cells (10^7 per ml) in phosphate-buffered saline. Ordinate, percent of the activity and glutathione transport observed at 35°C.

(Fig. 2). Similar temperature effects have been observed in other transmembrane transport and diffusion systems (27, 28).

We obtained no evidence for transport of glutathione into cells, but such transport cannot be definitely excluded. When CEM cells were suspended for 15 min in phosphate-buffered saline containing 100 mM glutathione, there was no detectable increase in the intracellular glutathione level. In studies in which normal CEM cells and CEM cells containing 0.1 mM glutathione (see below) were suspended in solutions containing labeled glutathione, the uptake of label by the cells was equivalent to less than 0.5 nmol of glutathione per 10^7 cells, and such uptake was markedly inhibited by 5 mM (serine + borate).

Effects of Inhibition of Glutathione Synthesis by Buthionine Sulfoximine. When cells of the 8226, CEM, and HSB lines were incubated at 35°C in media containing 1 mM buthionine sulfoximine, the glutathione levels decreased linearly on a logarithmic scale with time (Fig. 3); about 50% disappeared after 5.5 hr, and more than 97% disappeared after 30 hr. Glutathione levels and export rates of CEM cells were determined on cells incubated in the presence of buthionine sulfoximine (Fig. 4). The findings indicate a direct relationship between export rate and intracellular glutathione level. When CEM cells were grown in medium containing buthionine sulfoximine and serine + borate, about half of the glutathione that disappeared intracellularly was found extracellularly (Fig. 5). This indicates that the extent of glutathione transport is substantial; it is probably greater than indicated by this study, because it is likely that the residual transpeptidase activity utilized an appreciable amount of the transported glutathione. However, occurrence of other types of glutathione utilization cannot be excluded.

Effects of Glutathione Depletion on Cell Viability and Sensitivity to Irradiation. The viability of control CEM cells grown on standard medium and that of cells grown on the same medium containing 1 mM buthionine sulfoximine were compared (Table 1). Cells grown in the presence of buthionine sulfoximine were 85% viable when the glutathione content was about 4% of the control. After additional depletion of glutathione, the viability of the cells decreased substantially.

When CEM cells grown for 24 hr on media containing buthionine sulfoximine were subjected to γ radiation there was

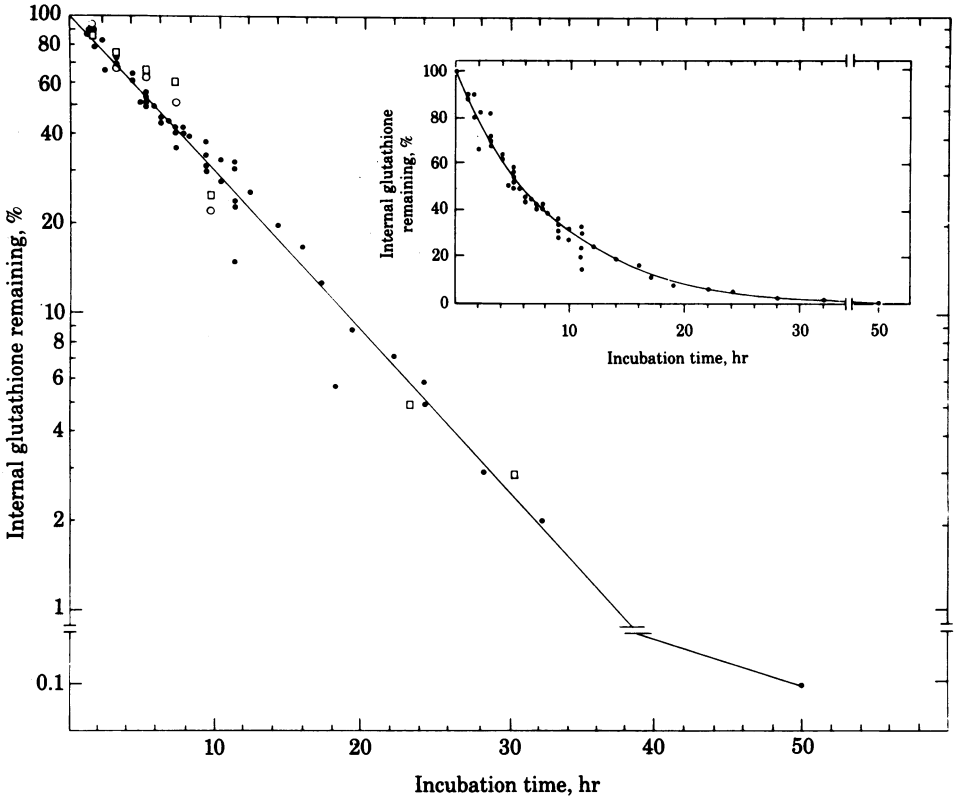

FIG. 3. Effect of inhibition of glutathione synthesis by buthionine sulfoximine on the intracellular glutathione concentration. Cell suspensions (10^6 per ml) of the CEM (●), 8226 (□), and HSB (○) lines were grown in standard medium containing 1 mM buthionine sulfoximine. Intracellular glutathione was determined in samples removed at various intervals. The ordinate is logarithmic. The data given in the *Inset* were obtained with CEM cells.

a marked and dose-dependent decrease in viability (Table 2). Such glutathione-deficient cells also exhibited significantly decreased viability when suspended in media containing 5,5'-dithiobis(2-nitrobenzoate).

DISCUSSION

The presence of membrane-bound γ-glutamyl transpeptidase constitutes an experimental difficulty in the determination of export rates. This problem can be overcome with CEM cells by adding serine + borate to the medium. Although other inhibitors of transpeptidase may also be useful, we noted somewhat less inhibition than found with kidney transpeptidase (29, 30), and apparent inhibition of glutathione transport from 8226 cells. When 8226 cells were incubated with [^{3}H]AT-125, there

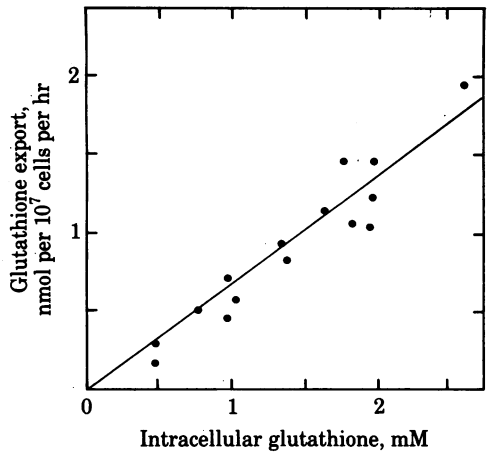

FIG. 4. Export of glutathione by CEM cells grown in the presence of buthionine sulfoximine. CEM cells were grown as described for Fig. 3, and samples were removed at intervals. The cells were centrifuged and resuspended (10^7 cells per ml) in phosphate-buffered saline and assayed for intracellular glutathione and the rate of glutathione export.

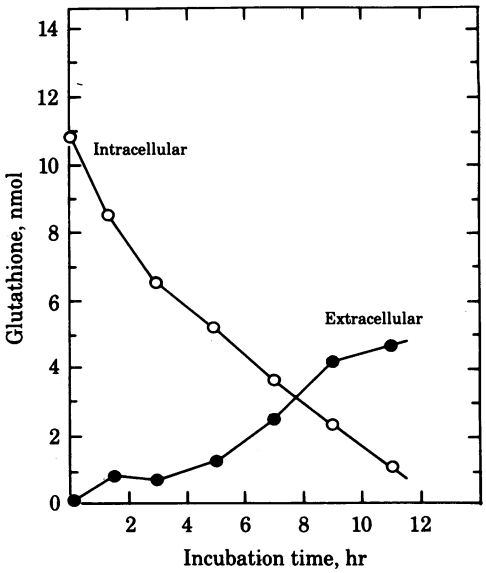

FIG. 5. Appearance of glutathione in the medium of CEM cells grown in the presence of buthionine sulfoximine. The cells (10^7 per ml) were grown in standard medium lacking glutathione and cysteine and containing 10 mM buthionine sulfoximine and 5 mM (borate + serine) at 25°C. The intracellular and extracellular levels of glutathione were determined.

Table 1. Viability of cells grown in the presence of buthionine sulfoximine

Time, hr	Viable cells, %	
	Control	Buthionine sulfoximine
0	100	100
24	100	85
48	93	68
72	93	28
96	93	0

CEM cells suspended (10^6 per ml) in standard growth medium (control) and in the same medium supplemented with 1 mM buthionine sulfoximine were incubated at 35°C. Portions were removed at intervals and the viability of the cells was determined by the trypan blue exclusion assay. The intracellular glutathione levels of the control and experimental cells were, respectively, 2.1 and 0.09 mM at 24 hr. The values given are the averages from three experiments.

was substantial labeling of the plasma membrane proteins, a result different from that found (31) in studies on the renal brush border. Serine + borate did not inhibit transport of glutathione in HSB or CEM cells. The observed rate of glutathione export by 8226 cells must be taken as a minimal value because serine + borate does not inhibit all of the transpeptidase activity present in these cells.

The lymphoid cell lines studied here differ significantly in their rates of glutathione export; these differences are associated with differences in γ-glutamyl transpeptidase activity. Thus, 8226 cells exhibit the most rapid export rates and have the highest transpeptidase activity, whereas HSB cells exhibit very little transpeptidase and have lower rates of export. Although the apparent association between transpeptidase activity and glutathione export may have metabolic significance, there is as yet no clear evidence indicating a mechanistic connection between transpeptidase activity and glutathione export. Earlier studies in this laboratory showed that the rate of decline of intracellular glutathione in the kidney after administration of an inhibitor of glutathione synthesis was decreased by about 20–30% after inhibitors of transpeptidase had been given (1–4). A definitive explanation of this effect is not yet evident. It is possible that the inhibitors of transpeptidase employed have an effect in some cells on the transport of glutathione in addition to their effect on transpeptidase activity.

Table 2. Effect of radiation and of 5,5′-dithiobis(2-nitrobenzoate) on viability of cells depleted of glutathione

Treatment	Viable cells, %	
	Control	Buthionine sulfoximine
None	100	85
Radiation		
100 rads	100	67
250 rads	100	54
500 rads	100	49
Dithiobis(nitrobenzoate)		
3 μM	100	73
30 μM	100	70
150 μM	100	62
300 μM	100	38

Cells (CEM) grown in standard medium (control) and cells grown in standard medium containing 1 mM buthionine sulfoximine for 24 hr were then exposed to either γ radiation from a cobalt-60 source or 5,5′-dithiobis(2-nitrobenzoate) as indicated (1 rad = 0.01 gray). The percent viable cells was determined 10 hr after such treatment. The intracellular glutathione concentrations of the control and experimental cells were 2.2 and 0.11 mM, respectively. The values given are the averages from three experiments.

It is notable that there is appreciable cell viability even when glutathione levels are depressed to 4% of the controls. However, depletion to lower levels led to substantially decreased cell viability and to markedly increased sensitivity to γ radiation. It seems probable that the effects of radiation observed here are due to cell membrane damage, which is assessed by the trypan blue procedure. The decrease in viability found after treatment with 5,5′-dithiobis(2-nitrobenzoate) is probably associated with destruction of essential protein sulfhydryl groups.

These results illustrate the usefulness of buthionine sulfoximine in studies on the effects of glutathione deficiency. Depletion of glutathione has been achieved previously by the use of oxidants such as hydroperoxides (32, 33) and diamide (34). These reagents have certain disadvantages. The effects of such compounds may be rapidly reversible, associated with increased glutathione disulfide formation and with oxidation of other cellular components. Similarly, the use of such compounds as diethylmaleate and 1-chloro-2,4-dinitrobenzene may be accompanied by other types of chemical interactions. Buthionine sulfoximine is a relatively nontoxic amino acid whose effects are apparently restricted to inhibition of γ-glutamylcysteine synthetase. In other studies in this laboratory this compound was given to mice for several weeks without effects other than those referable to glutathione depletion. Buthionine sulfoximine and other good inhibitors of glutathione synthesis therefore have potential value for the sensitization of cells to the effects of radiation and to therapeutic agents that are detoxified by reactions involving glutathione (35).

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