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Radioprotection of human lymphoid cells by exogenously supplied glutathione is mediated by γ-glutamyl transpeptidase

(buthionine sulfoximine/γ-glutamylcysteine synthetase/radiation)

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ABSTRACT **Human lymphoid cells depleted of glutathione by treatment with buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase, may be partially repleted by adding glutathione to the medium. The mechanism of repletion involves the action of γ-glutamyl transpeptidase on exogenous glutathione, transport of products of glutathione metabolism, and intracellular synthesis of glutathione. Lymphoid cells, previously shown to export glutathione at rates proportional to intracellular glutathione levels, do not take up intact glutathione to an appreciable extent, even under conditions of marked glutathione deficiency. The role of glutathione in radioprotection was examined by subjecting cells to γ-radiation after modification of cellular glutathione levels. Glutathione-depleted cells exhibited increased radiosensitivity under aerobic conditions, as compared to the nondepleted controls. Partial repletion of cellular glutathione prior to irradiation led to radiosensitivity comparable to nondepleted controls. Cells were not protected by suspension in media containing glutathione just prior to irradiation; thus, protection appears to require intracellular glutathione.**

Thiols have long been thought to affect the sensitivity of cells to irradiation (see, for example, refs. 1–5), and there is increasing evidence that glutathione (GSH), usually the most prevalent intracellular thiol, is a major determinant of cellular radiosensitivity (6). The radioprotective functions that have been ascribed to GSH include destruction of radiation-induced radicals, donation of hydrogen to radiation-damaged molecules, and mediation of DNA repair, an effect which may reflect, at least in part, its role as a cofactor in deoxyribonucleotide synthesis (7–10). In the effort to elucidate the functions of GSH in radioprotection and in other biological processes, attempts have been made to deplete cellular GSH. The experimental procedures used for such depletion include application of compounds that oxidize GSH to glutathione disulfide (GSSG), and compounds that react with GSH to form GSH adducts (7, 11–15). Such methods have major disadvantages, including those associated with nonspecificity, rapid reversibility, and the production of high concentrations of GSSG. Depletion of GSH by inhibition of GSH synthetase would be expected, on the basis of studies of patients with a deficiency of this enzyme (16, 17), to be complicated by marked metabolic acidosis. In contrast, inhibition of γ-glutamylcysteine synthetase offers a satisfactory approach to the production of experimental GSH deficiency. Buthionine sulfoximine (*S-n*-butylhomocysteine sulfoximine) was developed in this laboratory (18–24) as a potent and selective inhibitor of γ-glutamylcysteine synthetase. This and similar agents are active *in vitro* (23–25) and *in vivo* (23, 26–33). Previous studies showed that treatment of human lymphoid cell lines with buthionine sulfoximine is an effective and relatively nontoxic method of decreasing GSH formation in these cells and that such depletion produces a marked increase in cellular radiosensitivity (25). The present communication describes additional studies on the effects of GSH depletion on the radiosensitivity of three human lymphoid cell lines. Partial repletion of cellular GSH has been achieved by addition of GSH to the culture medium, and the mechanism of such repletion has been examined.

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EXPERIMENTAL PROCEDURES

Materials. Medium RPMI 1640 (medium A), GSH- and cysteine-deficient RPMI 1640 (medium B), fetal calf serum, and Dulbecco's phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} were obtained from GIBCO. GSSG reductase, NADPH, GSH, glycine, L-glutamic acid, and L-cysteine were obtained from Sigma. (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. DL-Buthionine-*SR*-sulfoximine was obtained as described (24). CEM, a human T-cell line, was kindly supplied by Paul P. Trotta. RPMI 8226, a human myeloma cell line, and HSB, another human T-cell line, were obtained from the American Type Culture Collection and Associated Biomedic Systems (Buffalo, NY), respectively.

Methods. The lymphoid cell lines were grown in medium RPMI 1640 containing 20% heat-inactivated fetal calf serum and 100 μg of streptomycin and 100 units of penicillin per ml. Flasks were maintained in an incubator at 37°C in 95% O_2/5% CO_2. Cells were used 24 hr after addition of fresh medium. Viability was at least 95% by trypan blue exclusion. Studies of the CEM cell cycle indicated that these cells are predominantly in G_1 phase (25).

Cells were collected by centrifugation for 10 min at 1,000 × *g* and then resuspended in fresh medium at 1×10^6 cells per ml. Cells were harvested for GSH analyses by centrifugation for 5 min at 1,000 × *g*. The cell pellets were washed twice with phosphate-buffered saline by centrifugation. The washed cell pellets were resuspended in 10 mM HCl, and complete cell disruption was achieved by freezing and thawing three times. Proteins were precipitated by adding sulfosalicyclic acid (final concentration, 3.3%). After centrifugation for 1.5 min at 8,740 × *g*, the clear supernatants obtained were assayed for GSH (34). GSH values are expressed on the basis of the number of cells. Data expressed on the basis of cell protein are qualitatively similar (35). Irradiation was performed with a cobalt-60 source (Gammacell 200, Atomic Energy of Canada). Postirradiation viability was monitored by trypan blue exclusion.

Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; AT-125, (α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid.

RESULTS

In the studies described in Fig. 1, three human lymphoid cell lines were depleted of GSH by suspension in media containing buthionine sulfoximine. After 24 hr, GSH was added to the medium in one set of experiments for each of the cell lines. This led to a substantial increase in the intracellular GSH level in all of the cell lines as compared to the cells that were treated with buthionine sulfoximine but not given GSH.

Depletion of GSH significantly decreased the postirradiation viability of all cell lines, but no such decrease was found in cells whose intracellular GSH levels were repleted (Table 1). When GSH-depleted cells were irradiated within 15 min after suspension in media containing GSH, no protection was found. Protection against irradiation was observed only with cells that exhibited an increase in the level of cellular GSH. An increase in cellular GSH was observed 18–48 hr after suspending the cells in media containing GSH. Cellular GSH levels also increased when the cells were placed in media containing glutamate, cysteine, and glycine. It seems notable that cells of the 8226 line exhibited a much more rapid repletion of cellular GSH than did the other cell lines. The rates of GSH repletion, estimated from the data given in Fig. 1 for the increase in cellular GSH found between 24 and 48 hr, were 15, 1.4, and 0.83 nmol per 10^7 cells, respectively, for cell lines 8226, CEM, and HSB. These estimated rates of repletion correlate fairly well with the γ-glutamyl transpeptidase activities of these cell lines—i.e., 10,000, 80, and 1 nmol/mg per hr (36), respectively.

The findings described above suggest that the repletion of cellular GSH found after addition of GSH to the medium is due to breakdown of the exogenous GSH by the action of γ-glutamyl transpeptidase and dipeptidase, absorption of the products formed, and resynthesis of intracellular GSH. Although repletion of cellular GSH after addition to the medium of amino acids or of GSH occurred in these studies in the presence of buthionine sulfoximine (initial concentration, 1 mM), such GSH repletion could be explained if inhibition of GSH synthesis were not complete under these conditions. To test this, studies were carried out with 5 mM buthionine sulfoximine; under these conditions, no repletion occurred. It also was noted that more rapid repletion of GSH occurred when the cells were removed from the medium containing 1 mM buthionine sulfoximine and resuspended in medium containing GSH.

To examine this question further, the buthionine sulfoximine-treated cells were transferred to fresh medium (B), which was fortified with GSH or with its amino acid constituents (Table 2). In these studies substantial cellular repletion of GSH occurred when either GSH or a mixture of the three amino acids was present in the medium. Notably, cysteine alone provided substantial GSH repletion. Addition of 1 mM buthionine sulfoximine inhibited GSH repletion in all cases.

Table 1. Viability of irradiated cells*

	Cell lines, % viable cells		
	HSB	CEM	8226
Controls	33	78	62
Buthionine sulfoximine	2	47†	48
Buthionine sulfoximine + GSH	45	75	63

*The cells were irradiated as indicated in Fig. 1. Viability was determined at 68 hr. The initial (42 hr) viabilities were 85–90%, 90–94%, and 83–86%, respectively, for HSB, CEM, and 8226 cells.
†The viability was 45–49% when GSH was added to the medium 15 min prior to irradiation.

The data given in Table 3 indicate that addition of γ-glutamyl transpeptidase inhibitors inhibited repletion by exogenous GSH. In these studies, we used AT-125, an irreversible inhibitor of transpeptidase (37), and a mixture of L-serine and borate (38), which inhibits the enzyme by another mechanism (39). Addition of AT-125 to the medium inhibited repletion by about 70%. It was noted, however, that this agent also inhibited GSH repletion when free cysteine was added to the medium, possibly because of inhibition of cyst(e)ine transport. In similar experiments with a mixture of L-serine and sodium borate, repletion of cellular GSH from exogenous GSH was inhibited by about 24%. Under these conditions, there was no inhibition of GSH repletion when free cysteine was added to the medium.

DISCUSSION

The repletion of intracellular GSH of human lymphoid cell lines by exogenously supplied GSH appears to require the activities of γ-glutamyl transpeptidase and the enzymes required for GSH biosynthesis. The findings may best be interpreted to indicate utilization of exogenous GSH by membrane-bound γ-glutamyl transpeptidase and dipeptidase, uptake of the products formed, and intracellular resynthesis of GSH. These interpretations are supported by the finding that repletion is blocked by inhibitors

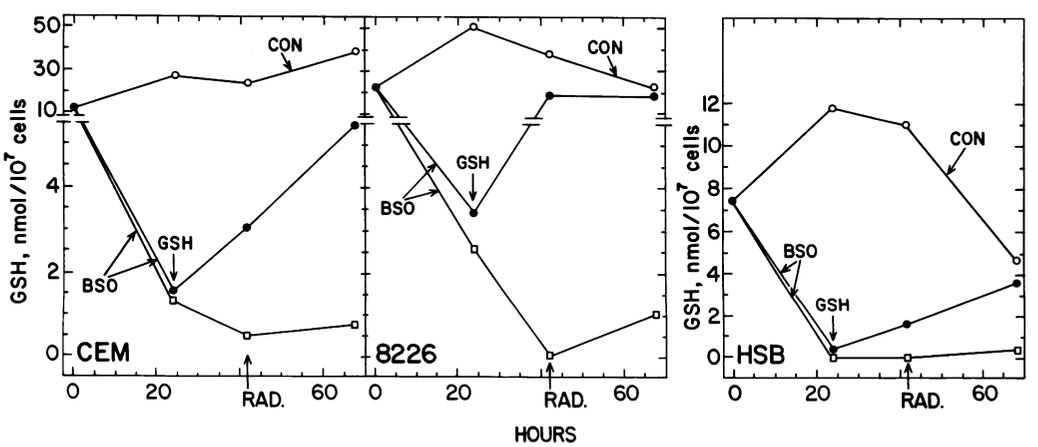

FIG. 1. Depletion and repletion of GSH in lymphoid cells of the HSB, 8226, and CEM lines. Cells were suspended (10^6 cells per ml) in medium A and grown in three sets as described. Two groups were grown in medium containing 1 mM buthionine sulfoximine (BSO); after 24 hr, one of these was brought to 10 mM with respect to GSH. These sets and the controls (CON) were exposed to 700 rads of γ-radiation (Rad. arrow) after 42 hr; cell viabilities are given in Table 1. The cells were harvested and assayed for GSH at intervals; data are the means of duplicate assays on cells from duplicate flasks in each group. ○, Controls; □, buthionine sulfoximine; ●, buthionine sulfoximine plus GSH.

Table 2. Repletion of intracellular GSH by exogenous GSH and by amino acids

Additions	GSH, nmol per 10^7 cells
None	0.23
GSH	5.44
Glutamate + cysteine + glycine	6.36
Glutamate	0.63
Glycine	0.46
Cysteine	5.31

CEM cells (10^6 cells per ml; 5.5 nmol of GSH per 10^7 cells) were cultured in medium A containing 1 mM buthionine sulfoximine for 48 hr at which time the GSH content was 0.44 nmol per 10^7 cells. The cells were resuspended in medium B (2.25×10^6 cells per ml). Six hours later, GSH (0.5 mM) and equivalent concentrations of the amino acids were added as indicated to separate cultures. At the time of the additions, the GSH content was 0.47 nmol per 10^7 cells. The cells were harvested 36 hr later and analyzed for GSH. The values are means of duplicate assays from at least two flasks in each group. Cell viability was 86–96% throughout. In separate experiments, 1 mM buthionine sulfoximine was added at the time of resuspension in medium B; in these studies cellular GSH levels were less than 0.3 nmol per 10^7 cells.

of both transpeptidase and γ-glutamylcysteine synthetase. The findings are in accord with the previous observation that little label from exogenously supplied GSH is taken up by CEM cells and that this small amount was decreased in the presence of a mixture of L-serine and sodium borate (25).

The findings on the three human lymphoid cell lines reported here may not necessarily be applicable to other types of cells; thus, it cannot be excluded that intact GSH is taken up by some cells. However, there is to our knowledge no published unequivocal evidence for transport of intact GSH into cells. Although intraperitoneal administration of GSH to mice leads to increased levels of GSH in the kidney, a greater increase in kidney GSH levels was observed after administration of γ-glutamylcysteine disulfide and of γ-glutamylcystine (40), and the findings suggest that the administered GSH is used for formation of γ-glutamylcystine and related compounds, which are transported. Although GSH disappears in the basolateral circulation of rat kidney (41, 42), there is no convincing evidence for basolateral uptake of intact GSH. In fact, there is excellent evidence that the apparent "uptake" or "extraction" of GSH in the basolateral circulation of rat kidney is actually the result of cleavage of GSH to its constituent amino acids (43). It has been reported that Chinese hamster ovary cells depleted of GSH by treatment with diethylmaleate can be repleted by addition of 3 mM GSH to the culture medium; the mechanism by which repletion occurs was not determined (44).

Table 3. Effect of inhibitors of γ-glutamyl transpeptidase on GSH repletion by exogenous GSH

Additions	GSH, nmol per 10^7 cells	
	AT-125	Serine/borate
None	0	0.23
GSH	3.28	4.33
GSH + inhibitor	0.92	3.37
Inhibitor	0	0.19

CEM cells were cultured (10^6 cells per ml) in medium A containing 1 mM buthionine sulfoximine for 48 hr and then resuspended in medium B containing 5 mM AT-125 or 5 mM L-serine/5 mM Na borate as indicated. GSH (0.25 mM) was added 2 hr after addition of the inhibitors. The initial cellular GSH levels were 9.12 (AT-125) and 7.20 (serine/borate) nmol/10^7 cells. Cell counts at 48 hr were 2.48×10^6 cells per ml (AT-125) and 1.84×10^6 cells per ml (serine/borate); GSH levels were <0.1 nmol per 10^7 cells. Cells were harvested and assayed for GSH at 72 hr; values are the averages of duplicate assays on duplicate cultures. Cell viabilities were >90% throughout.

The present studies, which are in accordance with earlier findings that GSH-depleted cells exhibit increased radiosensitivity as compared to nondepleted controls, also indicate that partially repleted (about 50%) cells exhibit viabilities comparable to those of nondepleted controls. Other recent studies also indicate that GSH plays a major role in the determination of cellular radiosensitivity (45–49). It has been reported that depletion of GSH alters radiosensitivity of V79 fibroblasts only under hypoxic conditions (48). In the present and earlier studies (25), radiosensitivity of lymphoid cells was found under aerobic conditions. The reported differences in the radiosensitivities of different cell types under aerobic and hypoxic conditions require further study; evidently other factors, some perhaps closely associated with differences in cell type, are involved. We found here that exogenously supplied GSH does not protect cells under conditions where GSH was not a substrate of membrane-bound γ-glutamyl transpeptidase or where intracellular synthesis of GSH was markedly inhibited; this suggests that radioprotection requires intracellular GSH and that extracellular GSH, of itself, offers little or no protection.

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