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Increased capacity for glutathione synthesis enhances resistance to radiation in *Escherichia coli:* A possible model for mammalian cell protection

(gene transfer/radioprotection/buthionine sulfoximine)

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ABSTRACT **A strain of *Escherichia coli*, enriched in its content of γ-glutamylcysteine synthetase and glutathione synthetase activities by recombinant DNA techniques, is more resistant to the lethal effects of γ-irradiation than is the corresponding wild strain. Although the gene-enriched strain has higher glutathione levels than the wild strain, the observed radioresistance appears to be associated with the increased capacity of the gene-enriched strain to synthesize glutathione when irradiated rather than to the cellular levels of glutathione *per se*. Thus, resistance was abolished in the presence of buthionine sulfoximine, a selective inactivator of γ-glutamylcysteine synthetase that decreases glutathione synthesis but that does not act directly to lower cellular glutathione levels. Conclusions drawn from studies on this *E. coli* model system may have relevance to protection of mammalian cells by glutathione.**

There is much evidence that glutathione functions in the protection of mammalian cells against the effects of radiation, oxidative damage, and certain toxic compounds of endogenous and exogenous origin (1–3). Recent studies have shown that cellular protection may be enhanced by increasing the levels of glutathione, and several methods for increasing cellular glutathione have been developed (3–8). Intracellular synthesis of glutathione requires two enzymes (γ-glutamylcysteine and glutathione synthetases), the amino acid substrates (glutamate, cysteine, and glycine), and a source of energy. It is to be expected that the capacity for glutathione synthesis would be increased by increasing the intracellular content of the synthetases. The possibility of increasing the resistance of cells to oxidative damage, toxic compounds, and radiation by methods involving gene therapy should be considered. This approach would require isolation of the genes for the synthetases and their introduction into cells. Although the mammalian genes for the two synthetases have not yet been isolated, the genes for the synthetases in *Escherichia coli* have been obtained and incorporated into a strain of *E. coli* that exhibits very high capacity for glutathione synthesis (9–12). This strain has been used for the efficient synthesis of isotopically labeled glutathione (13) and of glutathione analogs (14).

In the present work we have used this gene-enriched strain of *E. coli* in a system that may serve in some ways as a model for future approaches involving gene transfer therapy to animals. It has been reported that mutants of *E. coli* deficient in the activities of either of the two synthetases do not exhibit increased sensitivity to radiation as compared to the wild strain (15, 16); thus, the wild strain does not appear to synthesize sufficient glutathione to protect it against radiation. However, we found, as reported here, that a strain of *E. coli* enriched with the genes for the two synthetases is significantly more resistant to radiation than is the corresponding wild strain. We have also found that the enhanced radioresistance exhibited by the gene-enriched strain is associated with increased capacity of such cells to synthesize glutathione rather than solely to their increased cellular levels of glutathione.

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EXPERIMENTAL PROCEDURES

Microorganisms. The strains of *E. coli* studied here are as follows: strain A (increased γ-glutamylcysteine synthetase activity; tetracycline resistant), strain B (increased glutathione synthetase activity; ampicillin resistant), and strain M (increased γ-glutamylcysteine synthetase and glutathione synthetase activities; chloramphenicol resistant). These strains were prepared by Murata and Kimura (9, 10) by transformation of an *E. coli* K-12 parent strain [strain W (C600)]. The details of these studies have been published (9–12).

Materials. 5,5′-Dithiobis(2-nitrobenzoate) (DTNB), glutathione disulfide reductase, NADPH, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma. L-Buthionine-SR-sulfoximine (17–19) and L-γ-glutamyl-L-α-aminobutyric acid (20) were synthesized as described.

Methods. The bacteria were grown with shaking as described (13) on a medium containing 0.1% glucose, 1% peptone, 1.5% yeast extract, and 0.5% NaCl (modified from ref. 13). Cell growth was determined turbidimetrically at 640 nm. A portion of the culture was removed when the cells reached early logarithmic or stationary phases and irradiated with a γ source; at this time the remainder of the culture was harvested and disrupted by sonication, and the levels of glutathione, total acid-soluble thiol, protein, and γ-glutamylcysteine synthetase and glutathione synthetase were determined. Cells were irradiated in 0.9% NaCl with a ^{60}Co (9600 rads/hr; 1 rad = 0.01 Gy; Memorial Sloan–Kettering Institute) or a ^{137}Cs source (1340 rads/min; Rockefeller University). Cell viability was determined in triplicate by transferring 10-μl portions of the irradiated sample (after a series of 1:10 dilutions) to agar plates impregnated with the appropriate antibiotic (10 μg/ml), incubating overnight at 37°C, and counting colonies (standard deviations were ± 10% or less). (Determination of cells turbidimetrically after irradiation was found to be unreliable.) Protein was determined by the method of Lowry *et al.* (21) using bovine serum albumin as the standard. Glutathione analyses (total glutathione) were performed by the glutathione disulfide reductase/DTNB recycling assay (22) or by reduction with 10 mM dithiothreitol followed by quantitation of glutathione on a Durrum model D-500 amino acid analyzer. Buthionine sulfoximine was determined on the Durrum analyzer using lithium buffers; the retention time was 159 min.

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoate).

Total acid-soluble thiol was determined by treating the samples with DTNB after precipitation of the protein with 5% (final concentration) trichloroacetic acid (23).

γ-Glutamylcysteine and glutathione synthetase activities were measured in cell extracts. Whole cells were collected by centrifugation (10,000 × *g*; 15 min), resuspended in 2 volumes (wt/vol) of Tris·HCl buffer (150 mM; pH 7.4) containing 5 mM $MgCl_2$, and disrupted by sonication (Sonifier cell disruptor, model W 185 D; 80 W; 3 min). The cell extract was separated from particulate material by centrifugation (15,000 × *g*; 15 min) and the clear supernatant was assayed for enzyme activity using a coupled enzyme assay to measure the rate of ADP formation (24). The reaction mixture for γ-glutamylcysteine synthetase assays (final volume, 1.0 ml) contained Tris·HCl buffer (100 mM; pH 8.2), sodium L-glutamate (10 mM), L-α-aminobutyrate (10 mM), $MgCl_2$ (20 mM), Na_2ATP (5 mM), sodium phospho*enol*pyruvate (2 mM), KCl (150 mM), NADH (0.2 mM), pyruvate kinase (5 units), lactate dehydrogenase (10 units), and the enzyme sample. The rate of NADH utilization was followed at 340 nm at 37°C. Glutathione synthetase activity was determined by measuring the rate of ADP formation using the assay described above except that L-γ-glutamyl-L-α-aminobutyrate (5 mM) and glycine (10 mM) were added in place of L-glutamate and L-α-aminobutyrate, respectively; the data were corrected for contaminating ATPase activity—i.e., the rates of ADP formed in the absence of substrates. One unit of enzyme activity is defined as the amount that catalyzes the formation of 1 μmol of product per hour under these conditions. Glutathione disulfide reductase activity was determined as described (25).

RESULTS

The growth curves of strain M and of strain W were the same within experimental variation (±10%). Strain M developed glutathione levels at early stationary phase that were 2- to 3-fold greater than those found in strain W. Strain M was much more resistant to the lethal effects of γ-irradiation than the wild strain (Table 1). Cells of strain M grown to early logarithmic phase (and those of strain W) had much lower levels of glutathione than found after these strains were grown to early stationary phase, but the percent survival after irradiation of strain M grown to early logarithmic phase was significantly greater than that of strain W grown to early logarithmic phase.

It is interesting to note that cells of strain W, whether grown to early logarithmic phase or to early stationary phase, exhibited about the same percent survival after irradiation. Thus, the glutathione level itself is not the sole determinant of radioresistance. The findings suggest that the *capacity* to synthesize glutathione may be an important factor. The survival of strain M after irradiation is greater at a dose of 15,000 rads than at a dose of 25,000 rads (Table 2), suggesting that glutathione synthesis from the available cellular components can compete more effectively with the level of damaging species produced by the lower dose of radiation than that by the higher dose.

Table 1. Cell survival after γ-irradiation

Cell type	GSH,* μmol/g	Total thiol, μmol/g	% survival†
Strain W‡	5.0 (6.0)	5.1	14
Strain M‡	14.0 (11.0)	13.0	72§
Strain W¶	0.5	0.5	13
Strain M¶	0.7	0.7	35

*Determined enzymatically (22); values in parentheses were determined on the amino acid analyzer; expressed as μmol/g of wet weight of packed cells.
†After a 15,000-rad dose (^{137}Cs source).
‡Grown to early stationary phase; the activities of strain M of γ-glutamylcysteine and glutathione synthetases were 2.1 and 2.8 units/mg of protein, respectively. The corresponding values for strain W were 0.79 and 0.69 unit/mg of protein, respectively.
§Cells of strains A and B (enriched in activities of γ-glutamylcysteine and glutathione synthetases, respectively) exhibited cell survival values of 12% and 42%, respectively.
¶Grown to early logarithmic phase.

Table 2. Cell survival at two doses of radiation

Cell type	% survival	
	15,000 rads	25,000 rads
Strain W*	14	1.5
Strain M*	75	28
Strain W†	5	5
Strain M†	84	37

Cells were grown to early stationary phase and the cultures were serially diluted into 0.9% NaCl; cell densities were 10^8–10^9 cells per ml. After irradiation cell viability was determined.
*^{137}Cs source (dose rate, 1340 rads/min).
†^{60}Co source (dose rate, 9600 rads/hr).

To pursue the idea that the capacity for glutathione synthesis is a key factor in radioresistance of strain M, buthionine sulfoximine, a selective inhibitor of γ-glutamylcysteine synthetase (17–19), was added to suspensions of strain M 10 min prior to irradiation. Cells of strain M, treated in this way, showed virtually no survival after irradiation (Table 3, Exp. 3). Strain M cells treated with buthionine sulfoximine but not irradiated (Exp. 4) exhibited high survival as did cells of this strain that were treated with buthionine sulfoximine that was previously irradiated (Exp. 5). The results of Exp. 5 exclude the possibility that the effect of buthionine sulfoximine in Exp. 3 is mediated through a radiation-induced breakdown product of buthionine sulfoximine. Addition of 10 mM glutathione to the cell suspension did not affect survival, consistent with the view that the protective effect of glutathione is exerted intracellularly.

These studies strongly suggest that the effect of buthionine sulfoximine in decreasing cell survival (Table 3, Exp. 3) is due to inhibition of glutathione synthesis. In experiments carried out under these conditions, buthionine sulfoximine was found to enter the cells and to inhibit the γ-glutamylcysteine synthetase activity of strain M. In these studies, cells of strain M were incubated as described in Table 3 in 0.9% NaCl containing 5 mM buthionine sulfoximine for 10 min; the cells were then separated by centrifugation and washed twice with cold 0.9% NaCl. The cells were suspended in 5 volumes of 0.15 M Tris·HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and disrupted by sonication at 0°C. After centrifugation at 15,000 × *g* for 15 min at 4°C, the clear supernatant solution was assayed for γ-glutamylcysteine synthetase activity and was also analyzed for buthionine sulfoximine and glutathione. The synthetase activity was 0.72 unit/mg of protein, as compared to 2.4 units/mg of protein for control cells not treated with buthionine sulfoximine. Analysis indicated 60–

Table 3. Cell survival after irradiation of strain M cells treated with buthionine sulfoximine

Exp.	BSO	Radiation	% survival
1	0	0	100
2	0	+	70
3	+	+	0
4	+	0	100
5	+*	0	100

Cells of strain M (2.2×10^7 per ml) were suspended in 0.9% NaCl containing 5 mM buthionine sulfoximine (BSO); after 10 min, irradiation was carried out (15,000 rads from ^{137}Cs source) as stated.
*The cells were suspended for 10 min in a solution containing 5 mM buthionine sulfoximine previously irradiated with 15,000 rads.

100 μM buthionine sulfoximine. The levels of glutathione were 14.0 and 13.0 μmol/g in the control cells and the buthionine sulfoximine-treated cells, respectively; this difference is probably not significant. The enzyme activity data and the analytical data show that buthionine sulfoximine enters the cells under these conditions.

Growth of strain W and of strain M was not significantly affected by addition of 10 mM buthionine sulfoximine to the growth medium. Cells examined at early and midlogarithmic phase and in the stationary phase were found to contain 75–240 μM buthionine sulfoximine. The glutathione levels of the cells were not significantly affected by the presence of buthionine sulfoximine in the medium.

The glutathione reductase activities of strain M and strain W were essentially the same—i.e., 2.2 and 2.8 units/g of wet weight of cells, respectively.

DISCUSSION

The findings indicate that the observed radioresistance of strain M as compared to strain W is due to the high capacity for glutathione synthesis exhibited by strain M cells. Studies on *E. coli* mutants deficient in either of the synthetases (15, 16) did not lead to increase of radiosensitivity, indicating that the level of glutathione found in wild strain cells (and its associated relatively low capacity for glutathione synthesis) does not afford protection against radiation. [However, there is evidence that glutathione protects *E. coli* against chemical toxicity (15, 26, 27).] The present studies do not reveal correlation between glutathione levels and protection. Presumably a signal, perhaps a markedly decreased level of cellular glutathione [with consequent release from feedback inhibition (10, 28)], turns on glutathione synthesis during irradiation. Rapid glutathione synthesis is possible in strain M (which has high enzyme levels), but this is not possible in strain W. This response is selectively prevented in strain M by addition of buthionine sulfoximine.

The interaction of species induced by irradiation with glutathione probably leads initially to formation of glutathione disulfide [nonenzymatically or enzyme-catalyzed (29)], which may be (*i*) enzymatically reduced to glutathione, (*ii*) exported, or (*iii*) further oxidized to products such as glutathione sulfonic acid. Regardless of the pathways involved, the availability of glutathione would ultimately be limited by the amounts of amino acid substrates, the supply of energy (for peptide bond synthesis and reduction), or the stability of NADPH and the enzymes involved.

Buthionine sulfoximine has no effect on the growth of strains W and M, findings consistent with observations that indicate that mutants of *E. coli* that lack γ-glutamylcysteine synthetase or glutathione synthetase grow at normal rates (15, 16, 30). The glutathione levels of strains W and M were not affected by adding buthionine sulfoximine to the medium; it was found that buthionine sulfoximine is transported into the cells. It thus appears that sufficient synthesis of γ-glutamylcysteine synthetase occurs so as to maintain cellular glutathione levels, and it also seems probable that there is, under usual growth conditions, relatively little utilization or transport of glutathione. However, a marked increase in the rate of glutathione synthesis must occur when the cells are exposed to radiation.

The findings made on the model system studied here may have relevance to the protection of animal cells by glutathione. Thus, the capacity of mammalian cells to synthesize glutathione in the presence of a challenge may be of major importance, perhaps in some cases more important than the level of glutathione within the cell at the time of challenge. In recent studies on the role of glutathione in mediating multidrug and radiation resistance, it has generally been assumed that the cellular level of glutathione is the factor of major importance. The cellular levels of glutathione are typically increased (often 1.5- to 2-fold) in drug- and radiation-resistant tumor cells (3); such increase may reflect enhanced capacity for glutathione synthesis, but the capacity itself may be the major determinant of resistance. The relative importance in cellular protection of the levels of glutathione as compared to the capacity for glutathione synthesis may depend upon the intensity and type of challenge. These considerations suggest the importance, in studies on cellular glutathione protection, of determining the levels of glutathione and the cellular capacity for glutathione synthesis.

It is of interest that *E. coli*, an organism that does not require glutathione for growth or use it for protection (of the wild strain) against radiation, can nevertheless be afforded protection by enrichment with genes that code for glutathione synthesis. The present studies suggest the possibility that transfer of the genes responsible for glutathione synthesis into mammalian cells might, as in the case of *E. coli*, increase their capacity for protection against radiation and also against toxic compounds.

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