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Expression of γ-Glutamyl Transpeptidase Protects Ramos B Cells from Oxidation-induced Cell Death*

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The ectoenzyme, γ-glutamyl transpeptidase (GGT, EC 2.3.2.2) cleaves glutathione (GSH) to facilitate the recapture of cysteine for synthesis of intracellular GSH. The impact of GGT expression on cell survival during oxidative stress was investigated using the human B cell lymphoblastoid cell line, Ramos. Ramos cells did not express surface GGT and exhibited no GGT enzyme activity. In contrast, Ramos cells stably transfected with the human GGT cDNA expressed high levels of surface GGT and enzymatic activity. GGT-transfected Ramos cells were protected from apoptosis when cultured in cyst(e)ine-deficient medium. The GGT-expressing cells also had lower levels of intracellular reactive oxygen species (ROS). Homocysteic acid and alanine, inhibitors of cystine and cysteine uptake, respectively, caused increased ROS content and diminished viability of GGT expressing cells. Exogenous GSH increased the viability of the GGT-transfected cells more effectively than that of control cells, whereas the products of GSH metabolism prevented death of both the control and GGT-transfected cells comparably. These data indicate that GGT cleavage of GSH and the subsequent recapture of cysteine and cystine allow cells to maintain low levels of cellular ROS and thereby avoid apoptosis induced by oxidative stress.

Glutathione (GSH)[1] is the major intracellular nonprotein thiol defense against free radicals (1). It is a tripeptide consisting of glutamic acid, cysteine, and glycine. The antioxidant effect of GSH is dependent on the reduced sulfhydryl moiety of cysteine. Levels of intracellular GSH are maintained by a series of synthetic enzymes that regulate production of this tripeptide. Because of its relatively high intracellular concentration, GSH is transported out of cells into the extracellular environment. Once in the extracellular environment, there is little or no cellular uptake of intact glutathione. Rather, GSH is metabolized by γ-glutamyl transpeptidase (GGT, EC 2.3.2.2), a plasma membrane enzyme whose catalytic site faces the extracellular environment (reviewed in Ref. 2). GGT removes the γ-glutamyl group from GSH, after which cysteinylglycine can be cleaved by a membrane dipeptidase. The released cysteine can then be transported into the cell and used as a substrate for *de novo* synthesis of GSH. This γ-glutamyl cycle is thought to maintain cellular GSH levels. Despite reports of GGT-related enzymes (3) or direct uptake of intact GSH by cells (4), the γ-glutamyl cycle involving GGT is generally thought to be the major pathway by which cells utilize extracellular GSH for the *de novo* synthesis of intracellular GSH (5).

We have recently shown that primary human memory T cells express higher level of GGT than naive T cells (6). Because these cells are specialized to access extravascular inflammatory sites (7), this observation suggests that increased GGT expression may provide an adaptive advantage in permitting these cells to resist oxidative stress and/or grow more effectively. This possibility is supported by the finding that T cells at sites of inflammation with increased oxidative stress, such as the synovial tissue of rheumatoid arthritis patients, uniformly express high levels of GGT (6).

The role of up-regulated GGT on lymphocytes is not clear. It has been suggested that the expression of GGT on other cell types might be involved in the facilitation of cellular proliferation. GGT expression in mouse fibroblasts and hepatoma cells promotes their growth under conditions of reduced extracellular cysteine and/or depletion of intracellular glutathione (8–10). GGT also has been shown to blunt cell death caused by hydrogen peroxide or cisplatin (11, 12). Whether the role of GGT in protecting cells from oxidative stress or thiol depletion involves resynthesis of intracellular GSH is not known. For example, GGT expression has been correlated with enhanced tumor cell growth, whereas a GGT-related difference in GSH levels was not observed (12).

Up-regulation of GGT expression by B cells has been suggested to account for the ability of nylon-wool adherent peripheral blood mononuclear cells to cleave γ-glutamyl-*p*-nitroanilide (13), although the function of B cell GGT has not been delineated. Because GSH plays a major role in cellular defense against free radicals and GGT exerts an important influence in GSH metabolism, we hypothesized that the expression of GGT might serve to confer a survival advantage to B cells exposed to oxidative stress. To test this hypothesis, we examined the ability of Ramos B cells expressing GGT to survive oxidative stress.

EXPERIMENTAL PROCEDURES

Reagents—All amino acids, peptides, l-buthionine-[*S*,*R*]-sulfoximine (BSO), acivicin, and propidium iodide (PI) were purchased from Sigma. RPMI 1640, Dulbecco's modified Eagle's medium, fetal bovine serum, phosphate-buffered saline, and G418 were obtained from Life Technologies, Inc. Monobromobimane (MBBr) and 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate were purchased from Molecular Probes (Eugene, OR).

Cells—Resting, naïve (IgD^+) human B lymphocytes were isolated from peripheral blood by positive selection using flow cytometry as described (14). They were stained with the monoclonal antibody to human GGT, 3A8 (15), immediately and after a 5-day co-culture with mitomycin-treated, anti-CD3 activated autologous T cells. The trans-

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[1] The abbreviations used are: GSH, glutathione; BSO, l-buthionine-[*S*,*R*]-sulfoximine; GGT, γ-glutamyl transpeptidase; MBBr, monobromobimane; PI, propidium iodide; ROS, reactive oxygen species.

fection of the human B cell lymphoma, Ramos, with cDNA for human GGT has been described (16). Ramos/GGT cells were infected with the retroviral vector, LGGTSN, that expresses the human GGT cDNA regulated by the Moloney murine leukemia virus long terminal repeat. Ramos/X cells were infected with the parental retroviral vector, LXSN. Both cell lines were selected in G418 and maintained in RPMI 1640 containing 0.25 mg/ml of this antibiotic and supplemented with 10% fetal bovine serum.

GGT expression on primary B cells and Ramos transfectants was determined by flow cytometry using the monoclonal antibody, 3A8 (15). GGT enzymatic activity in whole cell lysates was determined using a commercially available kinetic assay for the conversion of L-γ-glutamyl-3-carboxy-4-nitroanilide to 5-amino-2-nitrobenzoate in the presence of glycylglycine (Sigma). To accomplish this, 10^7 cells were lysed at 10^7/ml in 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl, 1% Triton X-100. 50 μl of cell lysate (150–300 μg of protein) was added to 1.0 ml of the substrate solution provided. The change in the absorbance of the solution at 405 nm was monitored for 3 min. Units of GGT/μg of protein were calculated based on the molar extinction coefficient of the nitrobenzoate product. One unit is defined as the generation of 1 μmol of product/min.

Cell Culture Treatments—To study the role of extracellular cyst(e)ine on the levels of ROS and cell death, Ramos/X and Ramos/GGT cells were first collected from logarithmically growing cultures in standard RPMI 1640 containing 10% fetal bovine serum and washed twice with ice-cold phosphate-buffered saline. 2×10^5 cells were then plated in 24-well plates and incubated in 1 ml of cyst(e)ine-free medium at 37 °C. Cyst(e)ine-free medium was prepared from methionine-free, cyst(e)ine-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with L-glutamine (4 mM), L-methionine (200 μM), and 10% dialyzed fetal bovine serum. In some experiments, inhibitors of cyst(e)ine transport or glutamyl cycle enzymes were added at the time that the cells were shifted to cyst(e)ine-free medium. These include BSO (100 μM), acivicin (200 μM), alanine (10 mM), or homocysteic acid (5 mM).

Determination of Cell Death and Apoptosis—Cell death was measured by flow cytometry using PI staining. After incubation, cells were centrifuged and resuspended in ice-cold phosphate-buffered saline. PI was added at a final concentration of 2 μg/ml, just before analysis using the FACScan (Becton Dickinson, Mountain View, CA) flow cytometer. Total cell death was measured as the percentage of cells stained with PI. The hypotonic PI method was used to measure apoptosis. Cells were centrifuged and resuspended in a hypotonic PI solution (0.1% Triton X-100, 0.1% sodium citrate, and 50 μg/ml PI) and incubated for at least 1 h at 4 °C. Cells containing a hypo-diploid DNA content by flow cytometry were defined as apoptotic cells. Ten thousand cells were counted in each sample, and the data were analyzed with CellQuest™ (Becton Dickinson) software to determine the percentage of dead cells and the percentage of apoptotic cells.

Intracellular GSH Measurement—Cellular GSH levels were analyzed by flow cytometry using MBBr and PI (6). Cells were centrifuged and resuspended in 1 ml of ice-cold phosphate-buffered saline, and 20 μl of a 4 mM stock solution of MBBr in 100% ethanol was added. The sample was then incubated for 10 min at room temperature in the dark. PI (1 μg/ml) was added just before measurement to gate out dead cells. The mean fluorescence intensity for the live cells was recorded using the FACStar Plus flow cytometer (Becton Dickinson). Ten thousand cells were measured in each sample. Fluorosbrite™ carboxy BB 6-μm microspheres (Polysciences, Warrington, PA) were analyzed before each experiment to normalize the fluorescence intensity measurement conditions. To determine the level of background staining, *N*-ethylmaleimide (100 μM) was added to a parallel sample immediately before MBBr staining. *N*-Ethylmaleimide is a sulfhydryl-reactive agent that rapidly depletes intracellular thiols. The mean fluorescence intensity of the *N*-ethylmaleimide-treated sample was subtracted from the mean fluorescence intensity of the experimental sample, and the difference was defined as the cellular GSH level of the live cells. The GSH level of fresh cells was also determined using a Glutathione Assay Kit (Calbiochem, San Diego, CA), which is based on the nonenzymatic reaction of thiols with a quinolinium chromogen (17). Briefly, 1.6×10^7 cells were lysed in 400 μl of 5% metaphosphoric acid, and the insoluble material was removed by centrifugation. A 40-μl aliquot was diluted with 100 μl of a buffer comprised of 200 mM potassium phosphate, pH 7.8, 0.2 mM diethylene triamine pentaacetic acid, and 0.025% Lubrol. 10 μl of a 12 mM solution of 4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate in 0.2 N HCl was added with mixing, followed by 50 μl of 30% NaOH. The reaction was allowed to proceed for 10 min at room temperature in the dark. The absorbance at 400 nm was measured and compared with a standard curve of freshly prepared GSH in metaphosphoric acid. All assays were performed in triplicate, and the GSH content was expressed as nmol/10^6 cells.

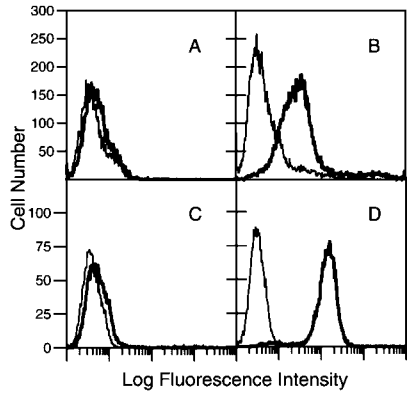

FIG. 1. **Surface expression of GGT by peripheral blood B cells and B cell lines.** Resting $CD19^+$ IgD^+ peripheral blood B cells were analyzed immediately after purification (*A*) and after a 5 day co-culture with anti-CD3-activated autologous T cells (*B*). Ramos B lymphoma cells were transfected with an empty retroviral vector (*C*) or with a vector expressing the cDNA for human GGT (*D*). The cells were stained with the monoclonal antibody, 3A8, recognizing human GGT (*thick lines*) or an isotype-matched control monoclonal antibody (*thin lines*).

Determination of Cellular ROS—The relative level of intracellular ROS was analyzed by flow cytometry using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate and PI. 10 μM (final) of 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate or vehicle alone was added to each sample 30 min before the end of the incubation with different stressors. Just before analysis, PI was added to each sample as described above, and the samples were analyzed using the FACScan flow cytometer. Ten thousand cells were analyzed in each sample, and dead cells were gated out by PI staining. The mean fluorescence intensity for the cells that were not stained with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate was subtracted from that of cells stained with 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, and the difference was defined as the relative level of ROS for the live cells.

RESULTS

GGT-transfected Ramos Cells as a Model of Activated B Cells—Using a recently developed monoclonal antibody (3A8) to human GGT (15) and flow cytometry, expression of GGT by human T lymphocytes was previously found to increase with cellular activation (6). The same approach was used to assess the level of GGT expression on human B lymphocytes. Resting B cells express little or no surface GGT by 3A8 staining (Fig. 1). However, with activation, there was marked up-regulation of GGT. Similarly, the Ramos B cell line failed to express GGT. The Ramos cell line was chosen as a model for mature B cells not only because it does not express surface GGT, but also it had been extensively used to study B cell signaling and apoptosis (18–20). To study the impact of GGT on B cell function, this transformed B cell line was transfected with the cDNA for human GGT under the control of a murine retroviral promoter (16). The transfected Ramos/GGT cells express high levels of GGT on their surface, whereas Ramos/X (vector control transfectant) expresses little or no surface GGT. The level of GGT staining on the Ramos/GGT cells was approximately five times the level on the activated peripheral blood B cells. This apparent difference in GGT expression per cell is offset by the larger size of the Ramos lymphoblastoid cells with respect to peripheral blood B cells (approximately twice the diameter; thus four times the surface area). Finally, the transfection resulted in a high level of GGT enzymatic activity in the Ramos/GGT cells (97.1 ± 1.7 milliunits of enzyme activity/mg cellular protein), whereas no GGT enzymatic activity could be detected in the parental Ramos or Ramos/X cells, confirming previous observations (16).

The Effect of GGT Expression on Intracellular GSH and ROS

TABLE I

Intracellular GSH levels, reactive oxygen species and cell death in log phase cultures of Ramos/X and Ramos/GGT

Ramos/X and Ramos/GGT transfectants were harvested from log phase growth in RPMI 1640/10% fetal bovine serum. The intracellular GSH level was measured both colorimetrically and by reaction with MBBr. Reactive oxygen species were quantified by flow cytometry using dichlorofluorescein (DCF); the percentage of dead cells was determined by exclusion of PI. Data shown are the means ± S.E. from four separate experiments for colorimetric GSH levels and 15 experiments for the others. The Mann-Whitney test was used for statistical analysis.

	Cell Line	
	Ramos/X	Ramos/GGT
GSH (nmol/10^6 cells, colorimetric assay)	1.97 ± 0.24 (n = 6)	1.21 ± 0.20 (n = 6)[a]
GSH (mean fluorescent intensity of MBBr staining)	144.9 ± 11.5 (n = 15)	88.6 ± 5.7 (n = 15)[b]
Reactive oxygen species (mean fluorescent intensity of DCF staining)	46.3 ± 5.0 (n = 15)	33.7 ± 4.2 (n = 15)[a]
Dead cells (percentage propidium iodide positive)	17.4 ± 1.1 (n = 15)	13.0 ± 0.8 (n = 15)[c]

[a] $p < 0.05$.
[b] $p < 0.001$ for comparison of Ramos/GGT with Ramos/X.
[c] $p < 0.01$.

Levels—GSH enters cells as the intact tripeptide poorly, if at all, under physiological conditions (3–5). The function of GGT has been presumed to be to facilitate the recapture of extracellular GSH. Flow cytometric and biochemical assays were therefore carried out to quantify the intracellular levels of GSH in the Ramos/X and Ramos/GGT cells during log phase growth. First, MBBr fluorescence of living cells was quantified. MBBr is capable of binding to all intracellular thiols. The majority of the signal, however, will reflect GSH, the dominant intracellular thiol. This was confirmed by incubating cells with BSO, to block *de novo* GSH biosynthesis. The MBBr signal was reduced 60–70% by BSO treatment (data not shown) in agreement with other studies (21). In addition, the MBBr concentration and time of incubation were kept to a minimum in these experiments to produce a signal that is proportional to the intracellular GSH concentration (22). Contrary to the expected result, Ramos/X had significantly higher levels of GSH in comparison with Ramos/GGT (Table I). This finding was corroborated by a second assay performed on cell lysates prepared from cells taken directly from culture. This chromogenic assay employs a quinolinium reagent that reacts with either cysteine or GSH to form products with similar absorption coefficients (17). Under normal circumstances, the concentration of intracellular GSH is 40–80 times that of cysteine (23, 24). Thus, it is formally possible but highly unlikely that these results reflect a much greater intracellular cysteine level in Ramos/X cells compared with Ramos/GGT.

Spontaneous cell death in both transfected cell lines was low, although there was a small but significant increase in the number of dead cells in Ramos/X compared with Ramos/GGT (17.4% *versus* 13.0%, $p < 0.01$, Mann-Whitney test). Intracellular ROS, measured by dichlorofluorescein fluorescence, was also significantly lower in Ramos/GGT compared with Ramos/X (mean fluorescence intensity of 33.7 *versus* 46.3; $p < 0.05$). Thus, expression of GGT in Ramos cells appeared to lower their relative level of ROS and protect them modestly from cell death but unexpectedly also appeared to result in a decrease in intracellular GSH levels.

Standard tissue culture medium contains high concentrations of cystine. For example, both Dulbecco's modified Eagle's medium and RPMI 1640 have ~200 μM cystine, compared with the 40 μM found in human serum. Therefore, GGT expression may not provide a particular advantage in this situation. However, it may be beneficial when cyst(e)ine is limiting. To examine this, Ramos/X and Ramos/GGT cells were incubated in medium lacking cyst(e)ine. Under this form of oxidative stress (extracelular thiol depletion), significantly more Ramos/X cell died than did Ramos/GGT cells. The increase in the percentage of dead Ramos/X cells was noted within 3 h in cyst(e)ine-free medium and continued to increase thereafter (Fig. 2*A*). Approximately one-half of the spontaneous death of Ramos cells in cyst(e)ine-free medium was apoptotic as measured by the appearance of hypodiploid nuclei, and the remainder was a result of necrosis. Despite the marked differences in the spontaneous death of Ramos/X and Ramos/GGT cells, there was a comparable percentage decline in intracellular GSH levels when Ramos/X and Ramos/GGT cells were cultured in cysteine-free medium (Fig. 2*B*). At all time points examined, Ramos/X manifested both a greater percentage of dead cells and a higher level of cellular GSH in the residual live cells. However, intracellular ROS were significantly greater in the live Ramos/X cells relative to the Ramos/GGT (Fig. 2*C*). The rapid rise in relative levels of ROS in the live cells appeared to precede the marked increase in the percentage of dead cells that appeared in both Ramos/X and Ramos/GGT with longer incubations. These results suggest that relative levels of ROS and cell death may be controlled by the expression of GGT in Ramos cells, whereas cellular levels of GSH are not.

Effect of γ-Glutamyl Cycle Inhibitors Cell Death—Acivicin, an irreversible inhibitor of GGT activity was used to confirm that expression of GGT determined the differences in cell death. This compound also inhibits other enzymes that utilize glutamine as a substrate, such as those involved in nucleoside biosynthesis (25). Thus, it can contribute to the background level of cell death (26). As seen in Table II, the addition of 50 μM GSH to Ramos/X and Ramos/GGT cells cultured in cyst(e)ine-free, GSH-free medium was able to prevent cell death in 20–40% of the Ramos/X cells and 50–70% of the Ramos/GGT cells. The ability of GSH to prevent death of Ramos/GGT cells was inhibited by acivicin. The effect of acivicin on Ramos/X cells was variable, and in some experiments, it caused a small increase in cell death in the Ramos/X culture, consistent with its pleiotropic effects. Therefore, the effects of acivicin confirm that the expression of GGT seen in the Ramos/GGT cells helps to protect them from oxidation-induced cell death by the metabolism of GSH.

Extracellular GSH is cleaved by GGT producing glutamate and the dipeptide, cysteinylglycine, which is subsequently hydrolyzed by an extracellular membrane-bound dipeptidase (27). The cysteine that is produced can be taken up by cells in either its reduced or oxidized form (2). To confirm that this pathway was involved in the GGT-dependent protection of cells from oxidative stress, Ramos/GGT cells were cultured in cyst(e)ine-free medium containing homocysteic acid, an x_c^- system competitor, and alanine, an ASC system competitor (23), to inhibit uptake of cystine and cysteine, respectively (Fig. 3). The

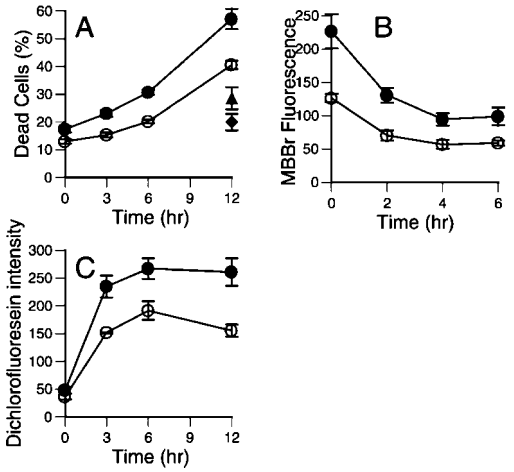

FIG. 2. **Cell death, intracellular GSH levels, and relative content of reactive oxygen species in Ramos/X and Ramos/GGT.** Cells were removed from RPMI 1640 during log phase growth and incubated for various time periods in medium lacking cyst(e)ine and GSH. Cell death (*A*) was quantified by exclusion of propidium iodide, intracellular GSH was determined by monobromobimane staining (*B*), and the level of intracellular reactive oxygen species (*C*) was determined by dichlorofluorescein staining. , Ramos/X; ○, Ramos/GGT. Data are the means ± S.E. for 15 determinations. In *A*, the percentage of cells with hypodiploid nuclei indicative of apoptosis are shown for Ramos/X (▲) and Ramos/GGT (♦).

TABLE II
The effect of the GGT inhibitor acivicin on oxidation-induced cell death

Ramos/X and Ramos/GGT cells were incubated for 12 h at 37 °C in cyst(e)ine-free, GSH-free medium alone or with 50 μM GSH, 10 μM acivicin, or both. Each experiment represents the analysis of a single culture for each condition. The values represent the percentages of dead cells in each culture as determined by uptake of propidium iodide.

	Ramos/X	Ramos/GGT
	%	%
Experiment 1:		
Medium alone	98	98
+ 50 μM GSH	78	48
+ 10 μM acivicin	98	98
+ 50 μM GSH and 10 μM acivicin	73	61
Experiment 2:		
Medium alone	98	99
+ 50 μM GSH	64	27
+ 10 μM acivicin	98	98
+ 50 μM GSH and 10 μM acivicin	74	45

survival advantage manifested by Ramos/GGT was blocked by the combination of both compounds. Under these experimental conditions, the blockade of cyst(e)ine uptake causes an increase in the intracellular ROS of both Ramos/X and Ramos/GGT cells ($p < 0.001$, Student's *t* test). This suggests that the activity of GGT as well as the capacity to take up cyst(e)ine were critical determinants in the survival advantage of Ramos/GGT cells. These results complement similar studies documenting a GGT-dependent accumulation of [^{35}S]cysteine derived from radiolabeled extracellular GSH in mouse hepatoma cells (9).

The Effect of GSH and Its Components on Cell Survival—Survival of the two Ramos transfectants in cyst(e)ine-free, GSH-free medium was dependent on GSH released from the cells and metabolized by GGT. To analyze the metabolic steps involved in cell survival, different sulfhydryl-containing compounds were tested. Death of both Ramos/X and Ramos/GGT cells was nearly 100% after culture in cyst(e)ine-free GSH-free medium for 24 h (Fig. 4). The addition of 10 μM GSH protected Ramos/GGT cells from death but did not affect the survival of

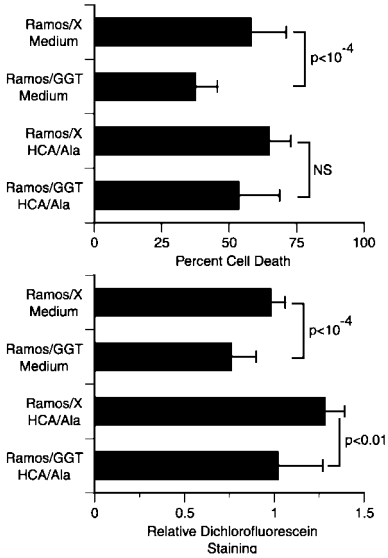

FIG. 3. **Inhibition of cysteine uptake nullifies the protective effect of GGT expression.** Ramos/X and Ramos/GGT cells were incubated for 12 h in cyst(e)ine-free, GSH-free medium with or without both homocysteic acid and alanine (5 and 10 mM, respectively). The percentage of dead cells and relative levels of ROS were measured by flow cytometry as described. Data shown are the means ± S.E. of 14 experiments. The difference in ROS levels seen in untreated *versus* homocysteic acid/alanine-treated cultures is statistically significant ($p < 0.001$, Student's *t* test) for both cell types. Other statistically significant differences are indicated on the figure. *NS*, not significant.

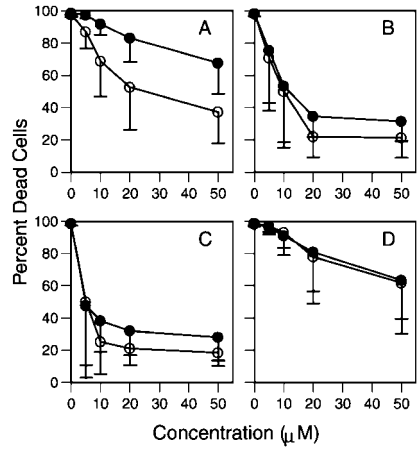

FIG. 4. **Effect of exogenous thiols on cell death.** Ramos/X and Ramos/GGT cells were incubated for 24 h in cyst(e)ine-free, GSH-free medium supplemented with the indicated concentrations of GSH (*A*), cysteine (*B*), cystine (*C*), or cysteinylglycine (*D*). The percentage of dead cells was measured by PI staining. , Ramos/X; ○, Ramos/GGT. Data shown are the means and S.E. of two independent experiments.

Ramos/X cells. Higher concentrations of GSH protected both cell lines, although the degree of survival of Ramos/GGT was greater than Ramos/X at all concentrations of GSH tested. The mechanism by which Ramos/X is protected from cell death by GSH is not known. The GGT assay can detect activity down to 10 milliunits/ml, and no activity was seen when either the Ramos/X cells or the fetal calf serum used to culture them were tested. One possible explanation is the possible presence of the enzyme, GGT-rel (3). This enzyme is capable of degrading GSH but is not detected by the chromogenic substrate used here. The expression of GGT-rel on lymphoid cell lines is under investigation.

Whereas 10–20 μM of GSH resulted in survival of 50% of Ramos/GGT cells, no concentration of GSH was found that could rescue Ramos/X cells to this degree. In contrast, the

TABLE III
The effect of GSH and its components on cellular GSH levels

Ramos/X and Ramos/GGT cells were incubated for 6 h at 37 °C in cyst(e)ine-free medium with or without GSH, cysteine, cystine, or cysteinylglycine. Intracellular GSH levels were quantified by monobromobimane staining. The mean fluorescence intensity of the MBB peak corrected for nonthiol staining is indicated (mean ± S.E. of three to six independent experiments).

Addition	Ramos/X	Ramos/GGT
None	60 ± 4 (n = 3)	41 ± 3 (n = 3)
10 μM GSH	140 ± 8 (n = 3)[a]	89 ± 2 (n = 3)[a]
50 μM GSH	178 ± 8 (n = 6)[a]	120 ± 10 (n = 5)[a]
10 μM cysteine	160 ± 10 (n = 4)[a]	111 ± 10 (n = 3)[a]
10 μM cystine	178 ± 16 (n = 4)[a]	122 ± 14 (n = 3)[a]
50 μM cysteinylglycine	145 ± 5 (n = 4)[a]	98 ± 7 (n = 3)[a]

[a] $p < 0.005$ for comparison with control (no addition) within cell lines.

metabolic products of GSH, cysteinylglycine, and either cysteine or cystine prevented cell death in both Ramos/GGT and Ramos/X cells. Despite the marked differences in the survival of Ramos/GGT and Ramos/X caused by the addition of GSH, the changes in intracellular GSH levels in the two cell lines were comparable (Table III). These results are consistent with the conclusion that the major difference between the capacity of Ramos/GGT and Ramos/X cells to resist oxidative damage relates to the activity of GGT. Following metabolism of GSH by GGT, both cell types are comparable in their capacity to utilize sulfhydryl-containing compounds to protect themselves from cell death.

Finally, the effect of cyst(e)ine uptake inhibitors on the survival of Ramos/GGT was tested in the presence of physiologic concentrations of the thiols GSH (10 μM), cysteine (10 μM), and cystine (50 μM). This is in contrast to the experiments shown in Fig. 3, which were carried out in thiol-free medium. In the absence of added thiol, cell death was 95% (data not shown). As above, there was partial protection from cell death by a low concentration of GSH (Fig. 5) in the absence of any transport inhibitor. The higher concentration of GSH, as well as physiological concentrations of cysteine or cystine prevented cell death in 75% of the cells. Homocysteic acid and alanine each appeared to have partial effects in the presence of 10 μM GSH, which were additive when the two compounds were used together. This effect was blunted in the presence of 50 μM GSH, suggesting competition between the inhibitors and the thiols produced from GSH. Overall, the effect of GSH appears to involve the uptake of both cysteine and cystine, because each compound showed partial inhibition. Homocysteic acid, alone or in combination with alanine, decreased the survival of cells treated with cysteine or cystine to only 25%, whereas alanine had little effect. This suggests that little cysteine was being taken up by the ASC system. This suggests that the cysteine added to tissue culture medium is likely to be oxidized to cystine under aerobic culture conditions. Interpretation of the results seen with the higher concentration of GSH are complicated by the fact that GSH can reduce cystine to cysteine in the extracellular medium (28). Alternatively, cysteinylglycine itself is being transported into the cell under these conditions by a mechanism not inhibited by homocysteic acid.

Effect of γ-Glutamyl Cycle Inhibitors on Cell Survival—Finally, the mechanism by which exogenous GSH protects the Ramos transfectants from death in cyst(e)ine-free medium was investigated. GSH, cysteine, and cystine all prevented death of Ramos/GGT cells from death (Fig. 6). In the absence of any thiol, cell death was 100% (data not shown). The GGT inhibitor acivicin partially inhibited the effect of 50 μM GSH, although this was not statistically significant. Acivicin also blunted the ability of physiologic concentrations of cysteine and cystine to improve cell survival. BSO, an inhibitor of γ-glutamylcysteine

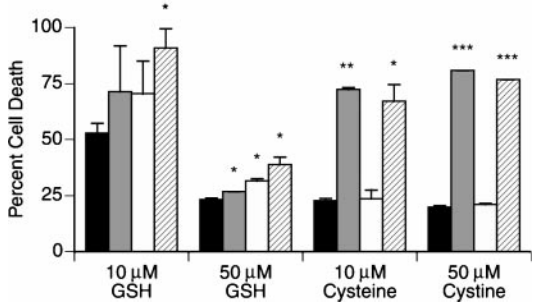

FIG. 5. **The effect of cyst(e)ine uptake inhibitors on cell survival in the presence of various thiols.** Ramos/GGT cells were incubated for 6 h in cyst(e)ine-free, GSH-free medium supplemented with the indicated thiols in the absence of inhibitors (*black columns*), with 5 mM homocysteic acid (*shaded columns*), with 10 mM alanine (*open columns*), or with homocysteic acid and alanine together (*hatched columns*). Exogenous GSH, cysteine, or cystine was added at the indicated concentration. The percentage of dead cells was measured by PI staining. Data shown are the means and S.E. of three independent experiments. Some *error bars* are too small to be visible. *, $p < 0.05$; **, $p < 0.05$; ***, $p < 0.005$ (Student's t test compared with untreated cells for each thiol).

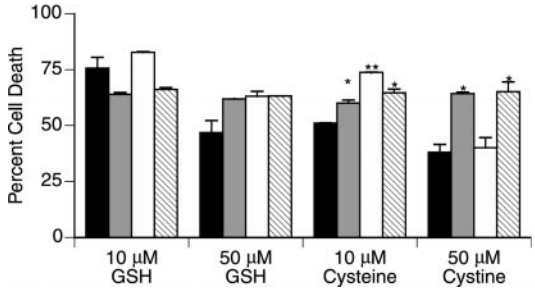

FIG. 6. **The effect of γ-glutamyl cycle inhibitors on cell survival.** Ramos/GGT cells were incubated for 6 h in cyst(e)ine-free, GSH-free medium supplemented with the indicated thiols in the absence of inhibitors (*black columns*), with 200 μM acivicin (*shaded columns*), with 100 μM BSO (*open columns*), or with acivicin and BSO together (*hatched columns*). Exogenous GSH, cysteine, or cystine was added at the indicated concentration. The percentage of dead cells was measured by PI staining. Data shown are the means and S.E. of three independent experiments. *, $p < 0.005$; **, $p < 0.001$ (Student's t test compared with untreated cells for each thiol).

synthetase, was used to examine the requirement for *de novo* GSH synthesis in cell survival. BSO blunted the protective effect of low concentrations of cysteine, suggesting that the synthesis of intracellular GSH is responsible, in part, for the rescue of these cells from the oxidative stress of culture in cyst(e)ine-free medium. A high (50 μM) concentration of cystine, however, prevented cell death in the presence of BSO. When acivicin and BSO were applied together, the effect of the cystine was inhibited. Taken together, these data support the hypothesis that GGT can form γ-glutamylcystine directly in the extracellular environment, thus bypassing the block imposed by BSO.

DISCUSSION

The current studies clearly document the capacity of membrane GGT to protect cells from cell death resulting from oxidative stress. The use of a B cell line transfected with the cDNA for human GGT in combination with chemical inhibitor studies underscore this observation. The mechanism relates to the capacity of GGT to metabolize GSH, resulting in the liberation of cysteine that can be taken up by the cells in either the reduced or oxidized state (Fig. 7).

GSH is present intracellularly in mM concentrations, whereas extracellular concentrations in human serum are estimated to be 5–11 μM. Intact GSH is not transported across

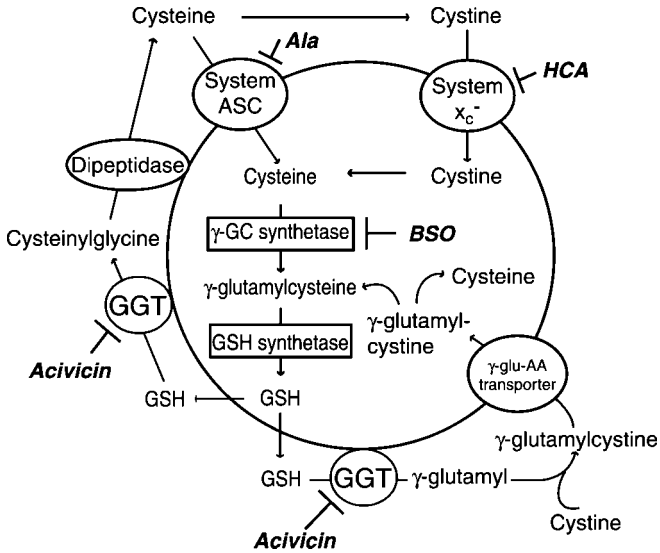

FIG. 7. **The γ-glutamyl cycle and the impact of specific inhibitors.** The roles of GGT and the cysteine and cystine transporters in maintaining intracellular thiol balance are shown. *γ-GCS*, γ-glutamylcysteine synthetase; *HCA*, homocysteic acid. See "Discussion" for details.

this concentration gradient in most cells, and, therefore, intact extracellular GSH cannot directly replenish intracellular levels. The "glutamyl cycle" has long been proposed as the mechanism for the recapture of GSH. By removing the γ-glutamyl group from GSH and thus permitting the uptake of cyst(e)ine by the cell, GGT is the critical first step in this cycle. Although its role in maintaining GSH levels suggests that GGT would be important as part of cellular defense against oxidative stress, its exact role in maintaining intracellular ROS levels has not been completely delineated. This is particularly relevant in lymphocytes, whose migratory patterns expose them to varying degrees of oxidative stress. The current data support the general conclusion that membrane GGT plays a central role in maintaining cellular viability in low cyst(e)ine environments but expands the mechanisms involved to include not only the provision of the substrates for intracellular GSH synthesis but also the protection of cells from cell death due to oxidative stress.

An unexpected observation was the finding that the level of intracellular GSH seen in the Ramos/GGT cells is lower compared with Ramos/X cells when each is cultured in complete medium containing both GSH and cystine. This finding was noted using both MBBr staining for flow cytometry as well as colorometric assays using a quinolinium compound (Table I) and the Tietze assay using Ellman's reagent in combination with glutathione reductase (data not shown). Similar data have been observed with other transfected lymphoma cell lines (data not shown) and V79 cells (29). The mechanism for this is unknown. It has recently been suggested that the high levels of GGT expressed by some cell lines generates amounts of cysteinylglycine that are pro-oxidant when the cells are exposed to artificially high levels of substrate (GSH) (30). It is not known whether this effect is observed with all cells nor whether this can be translated to a lower intracellular GSH level. It is also possible that the high turnover of GSH in complete tissue culture medium generates sufficient glutamate to block cystine uptake. Both the Ramos/X and Ramos/GGT cells were processed simultaneously in these experiments, and GSH determinations were made within 1 h of culture. It is therefore unlikely that the observed differences reflect differential loss of GSH during analysis.

Intracellular GSH has a number of functions. It maintains the reduced state of protein and low molecular weight thiols. It is oxidized by glutathione peroxidase in a reaction that detoxifies intracellular peroxides. In some tissues such as the liver, GSH is conjugated to a number of xenobiotics by glutathione *S*-transferse, promoting their excretion. Glutathione also serves as a reservoir of cysteine. Cysteine and cystine are nonessential amino acids synthesized from methionine by trans-sulfuration in hepatocytes and certain other cell types (23). Many cells lack sufficient activity in the trans-sulfuration pathway and thus require exogenous cyst(e)ine, either as the isolated amino acid or as GSH. As shown here, for example, Ramos cells die rapidly when deprived of cyst(e)ine even when cultured in adequate levels of methionine. Moreover, the viability of Ramos cells can be maintained by either exogenous cysteine or *de novo* synthesized cyst(e)ine derived from extracellular GSH.

The intracellular concentration of reduced GSH is determined by a balance between the import of intact GSH, the uptake of constituent amino acids, intracellular synthesis and oxidation of GSH, and the export of GSH from the cell. Uptake of intact GSH by an active, sodium-dependent transporter against the intracellular concentration gradient has been demonstrated for only a few cell types including renal tubular epithelial cells, type II pneumocytes, and small intestinal epithelial cells (4). It has not been documented under normal conditions for other cells. However, there is ample evidence for efflux of intact GSH from cells. The activities of both the GSH transporter and GGT itself depend on the level of extracellular thiols. A more reduced extracellular state (*e.g.* higher extracellular concentration of GSH) favors the efflux of GSH and decreases GGT activity (31), whereas an oxidized extracellular state (*e.g.* high cystine concentration) inhibits the loss of GSH by the cell and activates GGT. This may contribute to the increased intracellular GSH levels exhibited by Ramos/X cells cultured in standard tissue culture medium containing high concentrations of both cystine and GSH. An alternative and more plausible hypothesis derives from the fact that the activity of the rate-limiting enzyme in GSH synthesis, γ-glutamylcysteine synthetase, is increased by oxidative stress (32). Both transcriptional and post-transcriptional mechanisms have been proposed. Thus, the higher levels of ROS observed in the GGT-deficient Ramos cells would not only tend to deplete GSH but also increase the activity of the GSH biosynthetic pathway. In the face of a high concentration of cystine in the medium, the increased synthetase activity could result in a higher GSH level. This model would also suggest that the higher level of cyst(e)ine that would be produced in the Ramos cells expressing GGT could directly decrease ROS, without a concomitant increase in steady-state GSH levels. Whether the observation that GGT expression correlates with a slightly lower level of intracellular GSH is a feature of transformed cell lines with abnormally high enzyme activity or is seen in primary B cells activated under physiological conditions is not known.

The expression of GGT by the transfected Ramos cells protected them from cell death caused by cyst(e)ine depletion and blunted their accumulation of ROS. In a variety of cells, the detoxification of ROS involves the oxidation of GSH by glutathione peroxidase. The intracellular synthesis of GSH depends primarily on the availability of cysteine and the activity of γ-glutamylcysteine synthetase. The activity of this enzyme is negatively regulated by GSH and positively by ROS, as mentioned. The primary metabolic effect of GGT expression on lymphocytes is likely to be the increased uptake of cyst(e)ine derived from GSH, as has been shown for transfected mouse fibroblasts and hepatoma cells (9, 10). The GSH used by the enzyme may be derived either from the environment or ex-

truded from the cell. In contrast to the situation observed in complete tissue culture medium, GGT expression confers a distinct advantage to Ramos cells cultured at more physiological thiol concentrations (Table III). This is similar to the effect seen in mouse fibroblasts (8). Therefore, the GGT-facilitated increase in intracellular cysteine counteracts the effect of cyst(e)ine depletion in the medium. Both activity of GGT and transport of cyst(e)ine are necessary, because inhibition of either part of the glutamyl cycle nullifies the effect of GGT expression.

In these experiments, at least 50% of the observed Ramos cell death was caused by apoptosis. Although oxidative stress and the generation of ROS can cause nonspecific damage to cellular macromolecules, resulting in necrotic cell death, there is clear evidence that ROS regulate apoptosis as well (33–35). ROS are generated endogenously, as a result of mitochondrial respiration as well as cellular activation with tumor necrosis factor-α, phorbol esters, and cytokines. In some cells, including T lymphocytes, apoptosis is more pronounced in cells depleted of cyst(e)ine itself, rather than glutathione depletion (36, 37). The potential role for cyst(e)ine in protecting B cells from apoptosis is clearly demonstrated by these studies. Either inhibition of GGT or inhibition of both cysteine and cystine transport negates the protective effect of GGT expression on both apoptosis and endogenous ROS generation. Inhibition of either cysteine or cystine transport alone resulted in a partial loss of the GGT effect. Thus, GGT metabolism of GSH generates several thiol species that may interconvert in cell culture, each of which is capable of protecting B cells from programmed cell death. However, it is clear that at low concentrations, transport of cysteine is not sufficient to prevent cell death in this system. GSH synthesis is required, because treatment with BSO reverses the effect of both extracellular GSH and cysteine in preventing cell death (Fig. 6). Only at larger concentrations of GSH can cyst(e)ine directly protect B cells from apoptosis. Inhibition of GGT with acivicin also reverses the protection seen with GSH and low concentrations of cysteine. The effect on GSH rescue from cell death is expected because acivicin inhibits GSH cleavage by GGT. The fact that inhibition of GGT abolishes the effect of cysteine is surprising. One possible mechanism lies in the fact that cysteine and cystine are acceptors for the glutamyl group transferred from GSH by GGT (38). In this case, the GSH involved is likely to have been transported from inside the cell. The γ-glutamylcyst(e)ine that is formed can be transported into the cell (39) and serve as a substrate for GSH synthesis that bypasses the step catalyzed by γ-glutamylcysteine synthetase (40). When higher concentrations of cyst(e)ine are present in the extracellular medium, there is less of an advantage derived from the formation of γ-glutamylcysteine by GGT.

In summary, these data show that the expression of GGT in the B lymphocyte cell line, Ramos, protected those cells from death caused by oxidative stress. As expected, this protection was mediated by the increased uptake of cystine and cysteine by cells expressing GGT. The effect of GGT expression was not observed under tissue culture conditions where the concentration of cyst(e)ine was artificially high. When the cells were placed under the oxidative stress of relative cyst(e)ine depletion, the expression of GGT led to increased GSH levels, lowered intracellular ROS levels, and prevented cell death. These findings point to the important role for this ectoenzyme in maintaining the redox balance in B cells.

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