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Translocation of glutathione from lymphoid cells that have markedly different γ-glutamyl transpeptidase activities

[cell membrane/transport/L-γ-glutamyl-(*o*-carboxy)phenylhydrazide/6-diazo-5-oxo-L-norleucine/L-serine plus borate]

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ABSTRACT **Translocation of intracellular glutathione to the medium was studied in lymphoid cells (grown in tissue culture) that have very high, very low, or intermediate levels of membrane-bound γ-glutamyl transpeptidase, in the absence and presence of various inhibitors of this enzyme. The data show that glutathione is translocated to the medium by all of the cell lines studied, but that glutathione does not accumulate in the medium unless the cellular transpeptidase activity is either very low or substantially inhibited. Translocation of glutathione does not seem to be directly related to the activity of γ-glutamyl transpeptidase. The present and previous [Griffith, O. W. & Meister, A. (1979) *Proc. Natl. Acad. Sci. USA* 76, 268–272] findings suggest that translocation of intracellular glutathione is a general property of many mammalian cells. Glutathione exported from cells that have membrane-bound transpeptidase may be recovered by the cell in the form of transpeptidation or degradation products. Translocation of glutathione may also reflect operation of a rather general mechanism that protects and maintains the integrity of cell membranes.**

Experiments in which specific inhibitors of γ-glutamyl transpeptidase were administered to mice showed that marked *in vivo* inhibition of the transpeptidase leads to extensive urinary excretion of glutathione and to a substantial increase of the glutathione level in the blood plasma (1). These studies and experiments in which an inhibitor of glutathione synthesis was administered led to the conclusions that (*i*) renal intracellular glutathione is normally translocated to membrane-bound γ-glutamyl transpeptidase as a discrete step in the γ-glutamyl cycle, (*ii*) glutathione is normally translocated from tissues to the blood plasma, and (*iii*) the low but significant steady-state level of glutathione in the blood plasma reflects the intracellular synthesis (and export) of glutathione by certain cells as well as the utilization of glutathione by others. These studies, which were carried out in living animals, thus indicate that glutathione is translocated across the membranes of cells in the kidney, liver, and probably other tissues. It also appears that cells with high levels of membrane-bound γ-glutamyl transpeptidase are able to act upon the glutathione that they synthesize and translocate to the membrane-bound enzyme, whereas cells which have relatively low γ-glutamyl transpeptidase (e.g., liver) translocate glutathione to the plasma.

The previous findings thus show that many mammalian cells have the property of translocating intracellular glutathione out of the cell. It is conceivable, however, that the observed translocation of glutathione is induced by inhibition of γ-glutamyl transpeptidase or possibly that the inhibitor also affects the cell membrane in such a manner as to induce leakage of glutathione. In a further investigation of the translocation of intracellular glutathione, we have examined the consequences of inhibiting the γ-glutamyl transpeptidase activity of several lymphoid cell lines grown in tissue culture. We used three types of cells: (*i*) a cell line with a high γ-glutamyl transpeptidase activity (8000–10,000 units per mg), (*ii*) cell lines with relatively low γ-glutamyl transpeptidase activity (60–230 units per mg), and (*iii*) a cell line with an intermediate level of transpeptidase activity (about 1500 units per mg). Previous studies (2) showed that the γ-glutamyl transpeptidase activity of such lymphoid cells is accessible to externally supplied substrates and to substrates for which the cell is not permeable; this indicates that the enzyme is mainly located on the outer surface of the cell membrane.

The present results indicate that lymphoid cells, like other mammalian cells, normally translocate intracellular glutathione out of the cell and that this process is apparently not directly connected with γ-glutamyl transpeptidase activity. The data suggest, however, that cells that have high levels of transpeptidase utilize the translocated glutathione rapidly whereas, in experiments with cells that have little transpeptidase activity, glutathione accumulates in the medium.

MATERIALS AND METHODS

Cell Lines. MOLT, a human T-cell line, and RPMI 6237, a human B-cell line, were obtained from Associated Biomedic Systems, Inc. (Buffalo, NY). 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). 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. The cell cultures were kept in exponential growth phase by addition of fresh culture medium on alternate days. The viability of cells used for the experiments was >90%, as assessed by the trypan blue exclusion assay.

Experimental Procedure. The cultured cells were washed twice with pH 7.2 phosphate-buffered saline (P_i/NaCl) and suspended ($1.6–2.0 \times 10^7$/ml) in P_i/NaCl containing various compounds as specified below. The suspensions were incubated at 37°C for 60 min with shaking. After centrifugation at 1000 × *g* for 10 min, the supernatant was removed (see below) and the cell pellet was suspended in P_i/NaCl. After addition of 0.1 vol of 0.1 M HCl, the cells were disrupted by freezing and thawing (three times), and the glutathione content was determined (3). The content of glutathione in the cells was determined before and after the experimental period and was found to be essentially unchanged. The clear supernatant solutions obtained after centrifugation of the cells were mixed with 0.1 vol of 0.1 M HCl and then assayed for glutathione (3). The glutathione present in the supernatant solution is expressed as nmol/mg of cellular protein in the corresponding pellet. In several experiments it was found that (*i*) the amount of gluta-

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Abbreviation: P_i/NaCl, phosphate-buffered saline, pH 7.2.

thione found in the medium was essentially linear with time over a period of 30–120 min and (*ii*) that more than 90% of the glutathione (GSH + GSSG) was in the form of GSH. Cell viability was found to be at least 90% at the end of the experiments.

Determination of γ-Glutamyl Transpeptidase Activity. Transpeptidase activity was determined with L-γ-glutamyl-*p*-nitroanilide and glycylglycine (2). The cultured cells were washed twice with P_i/NaCl and suspended (10^6 cells per ml) in P_i/NaCl. The assay solution (final volume, 1 ml) contained 0.1 ml of cell suspension, 2.5 mM of L-γ-glutamyl-*p*-nitroanilide, 30 mM glycylglycine (adjusted to pH 8.0 with NaOH), and 50 mM Tris·HCl (pH 8.0). The various inhibitors were added at the concentrations specified. After incubation at 37°C for 10–30 min with shaking, the cells were removed by centrifugation and the absorbance of the supernatant solution was measured at 410 nm. Activity is expressed as nmol/mg of protein (4) per hr.

RESULTS

Table 1 describes experiments in which suspensions of lymphoid cells that exhibit moderate levels of γ-glutamyl transpeptidase activity were suspended in a solution containing various concentrations of the γ-glutamyl transpeptidase inhibitor L-γ-glutamyl-(*o*-carboxy)phenylhydrazide (1). After 60 min, the cells were sedimented by centrifugation and the glutathione content of the supernatant solution was determined. The γ-glutamyl transpeptidase activity of the cells and their content of glutathione were also determined. In Exp. 1, an insignificant amount of glutathione was found in the medium in the control, but increasing amounts of glutathione were found in the medium as the concentration of L-γ-glutamyl-(*o*-carboxy)-phenylhydrazide was increased. As expected, the transpeptidase activity of the cells was substantially inhibited under these conditions. In Exp. 2, the effects of several other hydrazides were studied; these have little or no effect on transpeptidase. Most of these agents did not increase the glutathione content of the medium appreciably. On the other hand, in the presence of L-serine plus sodium borate and of 1-(L-γ-glutamyl-)-2-(DL-1-carboxymethyl)hydrazide, which inhibit transpeptidase effectively, a substantial amount of glutathione was found in the medium. As shown by the results of Exp. 3, the medium of cells incubated with L-serine or sodium borate separately did not contain amounts of glutathione significantly greater than in the control study. When 6-diazo-5-oxo-L-norleucine was added to the medium (Exp. 4), transpeptidase was inhibited as expected (5), and a significant amount of glutathione appeared in the medium.

In similar studies on a cell line that exhibits very high transpeptidase activity (RPMI 8226) (2), we found no glutathione in the medium of the controls or in the presence of 5, 20, 50, or 100 μM L-γ-glutamyl-(*o*-carboxy)phenyl-hydrazide (Table 2; Exp. 1). When the concentration of the hydrazide was 500 or 1000 μM, a substantial amount of glutathione was found in the medium. With this cell line, a moderate amount of glutathione was found in the medium when the cells were suspended in solutions containing L-serine plus sodium borate or containing 6-diazo-5-oxo-L-norleucine.

In contrast to the results described above on cells that have appreciable transpeptidase activity, we found that glutathione accumulated in the medium of cell lines with very low transpeptidase activity (Table 3; controls). In a cell line (CEM) that has a very low level of transpeptidase (60 nmol/mg of protein per hr), addition of 1000 μM L-γ-glutamyl-(*o*-carboxy)-phenylhydrazide to the medium did not increase the amount of glutathione found. In another cell line (MOLT), which had a somewhat higher transpeptidase activity (232 nmol/mg of protein per hr), addition of the inhibitor led to some increase in the amount of glutathione in the medium.

Table 1. Effect of inhibition of γ-glutamyl transpeptidase of a human lymphoid cell line (RPMI 6237) on glutathione content of the culture medium

Inhibitor	Inhib. conc., μM	γ-Glutamyl transpeptidase, nmol/mg/hr	Glutathione, nmol/mg
	Exp. 1		
None (control)	—	1750	0.10*
L-γ-Glu-(*o*-carboxy)phenyl-hydrazide	10	1490	0.48
	50	1130	1.2
	500	276	2.9
	1000	184	3.4
	Exp. 2		
None (control)	—	1650	0.10*
L-γ-Glu-(*o*-carboxy)phenyl-hydrazide	50	607	1.0
	1000	202	1.6
L-γ-Glu-(*p*-carboxy)phenyl-hydrazide	50	1540	0.12
	1000	1410	0.22
L-γ-Glu-phenylhydrazide	50	1550	0.29
	1000	1520	0.29
1-(L-β-Asp)-2-(DL-1-carboxy-methyl)hydrazide	50	1680	0.26
	1000	1680	0.18
1-(L-γ-Glu)-2-(DL-1-carboxy-methyl)hydrazide	50	1590	0.37
	1000	1120	0.60
L-Serine + Na borate	2000/2000	636	0.76
	Exp. 3		
None (control)	—	1300	0.03*
L-Serine + Na borate	1000/1000	805	0.08
	2000/2000	411	0.78
	4000/4000	142	1.5
L-Serine	2000	1130	0.07
Na borate	2000	1290	0.05
L-γ-Glu-(*o*-carboxy)phenyl-hydrazide	50	720	0.69
	1000	142	1.23
	Exp. 4		
None (control)	—	2030	0.07*
L-γ-Glu-(*o*-carboxy)phenyl-hydrazide	50	1000	1.1
	100	684	1.5
6-Diazo-5-oxo-L-norleucine	500	1440	0.05
	1000	1220	0.24
	2000	837	0.34

The intracellular glutathione content was 8.7, 6.5, and 8.5 nmol/mg of protein in Exps. 1, 2, and 4, respectively.

* Under the conditions of the assay, these values are not significantly different from zero.

The data in the tables indicate that there is not a direct correlation between the level of transpeptidase and the amount of glutathione found in the medium. The data obtained in four separate experiments on cells of line RPMI 6237 are plotted in Fig. 1. Within each experiment, the amount of glutathione found in the medium was related to the transpeptidase value; however, the relationship between transpeptidase and glutathione found in the medium was different in the different experiments. In a given experiment, the correlation between amount of glutathione in the medium and the transpeptidase activity seemed to be independent of the inhibitor used (Fig. 2). It is notable that the different slopes evident in Fig. 1 apparently are not due to variations in the initial intracellular glutathione concentration or to variations in the cell density

Table 2. Effect of inhibition of γ-glutamyl transpeptidase of a human lymphoid cell line (RPMI 8226) on glutathione content of the culture medium

Inhibitor	Inhib. conc., μM	γ-Glutamyl transpeptidase, nmol/mg/hr	Glutathione, nmol/mg
	Exp. 1		
None (control)	—	8700	0
L-γ-Glu-(*o*-carboxy)-phenyl hydrazide	5	8060	0
	20	6540	0.10*
	50	4840	0.10*
	100	3110	0.10*
	500	899	0.60
	1000	555	0.92
	Exp. 2		
None (control)	—	8750	0.09*
L-γ-Glu-(*o*-carboxy)-phenyl hydrazide	50	4520	0.55
	1000	577	0.81
L-Serine + Na borate	2000/2000	2480	0.27
	2000/4000	1220	0.81
6-Diazo-5-oxo-L-norleucine	2000	3730	0.27

The intracellular glutathione content was 11.8 and 15.6 nmol/mg of protein in Exps. 1 and 2, respectively.
* Under the conditions of the assay, these values are not significantly different from zero.

used in individual experiments (which was constant within ±16%).

DISCUSSION

These findings show that glutathione is translocated to the medium by the lymphoid cell lines studied. A large fraction (>90%) of the total glutathione (GSH and GSSG) in the medium was found to be in the reduced form, suggesting that this form is translocated; oxidation to GSSG may take place nonenzymatically under these conditions. There seems to be no obvious relationship between the amount of glutathione translocated and the initial transpeptidase activity of the cell lines. The data in Fig. 2 indicate that, in a given experiment, the amount of glutathione that accumulates in the medium correlates with the extent of transpeptidase inhibition and that it is independent of the nature of the inhibitor. However, in individual experiments with RPMI 6237, the amount of glutathione that accumulated was not a direct function of transpeptidase activity (Fig. 1). In the studies with RPMI 8226, which has very high transpeptidase activity (Table 2), no translocation of glutathione could be detected unless high levels of the inhibitor were added. In contrast, translocation of glutathione from cells with very low transpeptidase activity (Table 3) was detectable even without addition of inhibitor. The findings indicate that all of these cells translocate glutathione but that glutathione does not accumulate in the medium unless the transpeptidase activity is low or is substantially inhibited.

Table 3. Effect of inhibition of γ-glutamyl transpeptidase of human lymphoid cell lines (CEM, MOLT) on glutathione content of the culture medium

Cell line	Inhibitor, μM	γ-Glutamyl transpeptidase, nmol/mg/hr	Glutathione, nmol/mg
CEM	None (control)	60	1.2
	1000	16	1.3
MOLT	None (control)	232	0.61
	1000	20	1.4

The inhibitor was L-γ-Glu-(*o*-carboxy)phenylhydrazide in both experiments. The content of intracellular glutathione in Exps. 1 and 2 was 9.8 and 11.8 nmol per mg of protein, respectively.

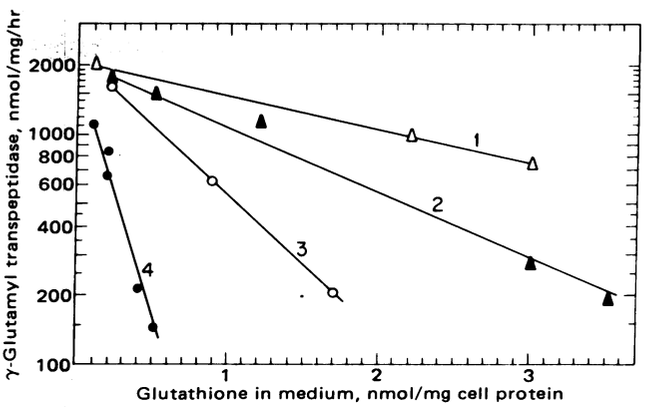

FIG. 1. Relationship between transpeptidase activity and content of glutathione in the medium in several experiments (curves 1–4) with L-γ-glutamyl-(*o*-carboxy)phenylhydrazide and cells of line RPMI 6237. The intracellular content of glutathione was 8.5, 8.7, and 6.5 nmol/mg of protein in experiments 1, 2, and 3, respectively.

The mechanism by which glutathione is translocated and the factors that regulate glutathione translocation need to be determined. The differences in glutathione accumulation found in different experiments with cells of the RPMI 6237 line are evidently due to different rates of translocation, which are probably not directly related to the intracellular level of glutathione. There was some variability observed in the concentration of intracellular glutathione even with the same cell line. The reason or reasons for such variability are not yet fully understood but may be related to the metabolic status of the cells or possibly to an influence associated with the stage of the cell cycle. The mechanism of translocation and the manner in which glutathione levels and translocation are controlled require further study; experiments in which glutathione synthesis is blocked by specific inhibitors (6) may help to elucidate these phenomena.

The present results are in accord with the conclusion (1) that translocation of intracellular glutathione to membrane-bound γ-glutamyl transpeptidase is a discrete step in the γ-glutamyl cycle. The lymphoid cell lines used here seem, at least in one respect, to be similar to other mammalian cells such as those of

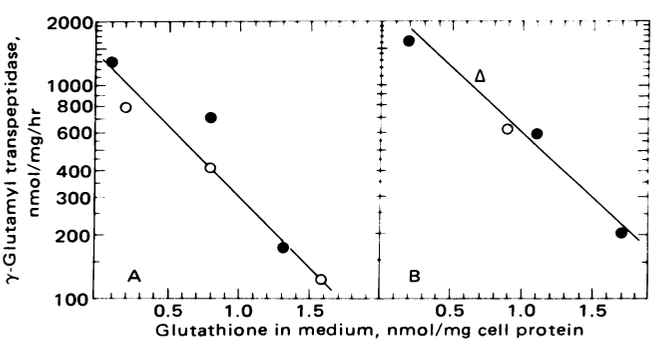

FIG. 2. Relationship between transpeptidase activity and content of glutathione in the medium in experiments with cell line RPMI 6237 and various inhibitors. The intracellular content of glutathione was 6.7 and 6.5 nmol/mg of protein in *A* and *B*, respectively. ●, L-γ-Glutamyl-(*o*-carboxy)phenylhydrazide; ○, L-serine + Na borate; △, L-γ-glutamyl-2-(DL-1-carboxymethyl)hydrazide.

liver and kidney which contain, respectively, very low and substantial levels of γ-glutamyl transpeptidase. Thus, evidence previously reported (1) led to the conclusion that glutathione is translocated out of renal cells into the tubular lumen where it is efficiently utilized by γ-glutamyl transpeptidase. In contrast, cells of the liver, and probably other organs that do not have substantial amounts of transpeptidase activity, translocate glutathione into the blood plasma from which it is removed efficiently by transpeptidase located in the kidney and other organs.

The data reported here render unlikely the possibility that translocation of glutathione is directly related to the activity of membrane-bound γ-glutamyl transpeptidase. However, the findings do not exclude the possibility that there is some type of indirect connection between translocation and transpeptidase. As noted previously (7), the enzyme may also exist within the cell membrane as well as intracellularly.

The present and previous findings suggest that translocation of intracellular glutathione may be a property of many cells. Glutathione translocated from a cell equipped with transpeptidase may be recovered by the cell in the form of its transpeptidation or degradation products as discussed (1, 7). Thus, one function of the translocation process may be connected with amino acid and peptide transport (8). In addition, the translocated glutathione may function to protect the cell membrane itself against oxidation, or it may be used extracellularly in the vicinity of the cell in a mechanism associated with transport. It is generally believed that glutathione functions in the protection of the erythrocyte membrane, and it has been reported that glutathione is required to permit or preserve assembly of microtubules (9). The finding that glutathione is translocated across the cell membranes of many mammalian cells may reflect the operation of a rather general mechanism that protects and maintains the integrity of cell membranes.

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