Glutathione Regulates Interleukin-2 Activity on Cytotoxic T-cells*

Chi-Ming Liang‡, Nancy Lee, Debra Cattell, and Shu-Mei Liang

From the Division of Blood and Blood Products and the Division of Cytokine Biology, Food and Drug Administration, Bethesda, Maryland 20892

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In this study, we examined whether and how the cellular activity of interleukin-2 (IL-2) is affected by glutathione (GSH), an important tripeptide existing in most cells. Cell culture and thymidine incorporation assay showed that addition of GSH enhanced the effect of IL-2 on the proliferation and thymidine incorporation of IL-2-dependent cytotoxic T-cells such as CTLL-2 and CT-4R. Treatment of the cells with GSH resulted in a 2-fold increase in the amount of IL-2 bound to the cells and a rapid internalization of the bound IL-2. In addition, the degradation of IL-2 in the cells was enhanced by GSH treatment. These effects of GSH were accompanied by an increase in the intracellular GSH level. L-Buthionine-(S,R)-sulfoximine, an inhibitor of de novo GSH synthesis, blunted the increase of intracellular GSH level and modulated the effect of GSH on IL-2 activity. These results suggest that GSH regulates the binding, internalization, degradation, and T-cell proliferative activity of IL-2; alterations of cellular GSH concentration may thus affect the growth and replication of IL-2-sensitive cytotoxic T-cells.

Interleukin-2 (IL-2), an important lymphokine, promotes the long term proliferation of activated T-cells (1, 2). In addition, it has an immunomodulatory effect on other antigen-specific cytotoxic T-lymphocytes (3, 4), natural killer cells (5), and lymphokine-activated killer cells (6).

The activities of IL-2 can be monitored by assaying its proliferative effect on IL-2-dependent cell lines such as CTLL-2 and TN-9 in vitro (7–13). It is generally believed that these effects are initiated after IL-2 binds to its receptors (14, 15). Three types of receptor proteins, i.e. low affinity (Kd = 10 nM; IL-2Rα), intermediate affinity (Kd = 1–2 nM; IL-2Rβ), and high affinity (Kd = 10 pm) have been reported (16–23). The IL-2Rα protein together with IL-2Rβ protein may form the high affinity IL-2 receptor (21–23). Binding of IL-2 to high or intermediate affinity but not low affinity receptors appears to be responsible for the biological activity of IL-2 (19, 20, 24). Structure-activity study indicates that the active region of IL-2 may involve N-terminal residues 8–27 and internal residues 33–54 (25–29). Using site-directed mutagenesis and partial sequence deletion, we have shown that the C-terminal residues in the vicinity of cysteine 125 are also essential (8). Recently, we have further demonstrated that the terminal residues in the vicinity of cysteine 125 play important roles in the binding of IL-2 to the intermediate affinity receptor protein (30).

The binding of IL-2 to receptors causes the biological effects. To understand the mechanism of IL-2 action, it is important to examine whether factor(s) other than the binding of IL-2 to receptors may affect the biological activity of IL-2.

Glutathione (GSH), a tripeptide thiol found in virtually all cells, activates thiol-requiring enzymes (31), protects the cells from oxidative damage (31, 33), and regulates microtubule formation (34). In addition, it affects the initiation and progression of lymphocyte activation (35, 36). The mechanism of GSH action on lymphocytes is, however, not well established.

Since both GSH and IL-2 are involved in the growth and replication of activated lymphocytes, in this paper we examined the functional relationship between these two biologically active molecules. Moreover, we studied whether GSH affects the binding and internalization of IL-2 to evaluate its mode of action.

EXPERIMENTAL PROCEDURES

Materials

Recombinant Escherichia coli-derived IL-2 (rIL-2; specific activity, 2 × 10^6 units/mg) was a generous gift from Glaxo (Geneva, Switzerland). CT-4R, an IL-2- and IL-4-dependent murine cytotoxic T-cell line, was a generous gift from Dr. W. E. Paul of the National Institutes of Health (Bethesda, MD). [3H]-Labeled thymidine (78 Ci/mmol) and 3H-labeled IL-2 (30–50 Ci/μg) were purchased from Du Pont-New England Nuclear. CTLL-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), minimum Eagle's medium nonessential amino acids solution (100× concentrate), glutamine, and sodium pyruvate were from GIBCO. GSH, NADPH, GSH reductase, 5,5'-dithiobis(2-nitrobenzoic acid) (Eltman's reagent) and L-buthionine-(S,R)-sulfoximine (BSO) were obtained from Sigma. 2-Vinylpyridine was purchased from Aldrich and stored at −20 °C.

Methods

Cell Culture—CTLL-2 and CT-4R cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 0.5 mM sodium pyruvate, 1 mM arginine, 3–30 units/ml rIL-2, and 3 × 10^−7 M 2-mercaptoethanol. All cells were incubated in a humidified atmosphere of 10% CO2 in air at 37 °C until they reached a density of 2 × 10^5–2 × 10^6 cells/ml. Some of the cells were subcultured, while the others were used in IL-2 or GSH assay. Because the culture medium contains not only IL-2 but also 2-mercaptoethanol, which increased the intracellular GSH level (data not shown), the cells were thoroughly washed with 0.5% FCS, DMEM or phosphate-buffered saline before they were used for assays.

Thymidine Incorporation Assay—The cells were centrifuged at 500 × g for 5 min and washed with 0.5% FCS, DMEM three times. The cells were resuspended in 10% FCS, DMEM, and serial dilutions of IL-2 and GSH were added as needed. The assay was determined as

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†To whom correspondence should be addressed.

‡The abbreviations used are: IL-2, interleukin-2; rIL-2, recombinant E. coli-derived IL-2; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; BSO, L-buthionine-(S,R)-sulfoximine.
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described previously (11). One unit of biological activity was defined as the amount of IL-2 needed to yield 50% maximal incorporation of tritiated thymidine under the assay conditions.

Assay for GSH—GSH assays were performed according to Tietze (37) and Griffith (38) with some modifications. Briefly, solutions containing serial dilutions (6.5 x 10^-5, 1.3 x 10^-4, 2.6 x 10^-4, 5.2 x 10^-4, 1 x 10^-3, 2 x 10^-3 M) of GSH in 0.1 M sodium phosphate, 5 mM EDTA buffer, pH 7.5, were distributed to a 96-well microtiter plate (50 ll/well). Fifty ll of Eillman's reagent (2.4 mM in 0.1 M sodium phosphate, 5 mM EDTA buffer, pH 7.5) was then added to each well, and the mixture was incubated at room temperature for 10 min. Since Eillman’s reagent reacts directly with high concentrations (>2 x 10^-3 M) of 2-mercaptoethanol, dithiothreitol, and probably other reducing agents to yield chromophores that absorb at 405 nm, the mixture was scanned with a Microplate Auto-reader (Bio-Tek Instruments, Inc.) for its absorption at 405 nm to exclude the possibility of GSH-unrelated reaction. GSH reductase (40 pg/ml; 50 ll/well) and NADPH (0.667 mg/ml; 50 ll/well) were subsequently added, and the GSH-specific reaction was carried out at room temperature for 6 min. The chromophores resulting from this reaction were then detected by the absorption at 405 nm. To distinguish the reaction of free GSH from that of glutathione disulfide, the samples were treated with 2-vinylpyridine according to the method of Griffith (38).

Receptor-binding Assays—High affinity IL-2 receptor-binding assays were performed by determining the binding of 125I-labeled IL-2 to CTLL-2 cells as described previously (30). Each binding assay was carried out in triplicate. Nonspecific binding was determined by inclusion of an excessive amount of unlabeled human rIL-2 (100 nM) in the assay. The specific binding was defined as the difference between the total and nonspecific binding.

Determination of Internalized IL-2—The internalization procedures were performed according to Robb and Greene (24). Briefly, CTLL-2 cells (1 x 10^6 cells/ml) were incubated with 100 pm 125I-labeled IL-2 in medium containing RPMI 1640 and 1% bovine serum albumin at 4°C for 90 min. After incubation, the cells were washed three times with ice-cold RPMI-bovine serum albumin to remove unbound IL-2 and resuspended in RPMI-bovine serum albumin containing 200 uM chloroquine. The cells were incubated at 37°C in a water bath. At selected times, 100-ll aliquots of the cell suspension in triplicate were removed and diluted with 1 ml of ice-cold RPMI-bovine serum albumin. The cells were pelleted, and the radioactivity in the supernatant was measured to determine the level of 125I-labeled IL-2 that had dissociated from the receptor sites. The cell pellets were then treated with 100 ll of chilled 0.5 M NaCl, 0.5 M acetic acid, pH 2.6, at 4°C for 10 min. The cell suspensions were layered onto a cushion of 80% dibutyl phthalate, 20% olive oil and spun for 4 min in a Beckman Microfuge. The radioactivity in the cell pellet and in the supernatant was measured to determine the level of acid-resistant internalized IL-2 and the level of acid-sensitive cell surface-bound IL-2, respectively.

RESULTS

Although GSH has been implicated in playing an important role in the initiation and progression of cellular activation of lymphocytes (35, 36), the mechanism of action is unclear. To examine whether GSH affects lymphocytes through its effect on the activity of cytokines, the influence of GSH on the T-cell proliferative activity of IL-2 was evaluated. Cell culture studies of CTLL-2 cells showed that GSH (0.5-16 mM) stimulated the replication of CTLL-2 (from 1 x 10^5 to 1 x 10^6 cells/ml) in the medium containing 10% FCS and 3-30 units/ml rIL-2. The GSH-treated cells exhibited a doubling time of 18-20 h, whereas the cells in the medium without GSH showed little, if any, replication. The cell proliferative activity of IL-2 was accompanied by an increase in thymidine incorporation, and this effect of GSH was concentration dependent; it reached a plateau at 3-10 mM GSH. In addition, the effect of GSH was time dependent. As shown in Fig. 1 rIL-2 increased the thymidine incorporation of CTLL-2; pretreatment of the cells with GSH (0.65 mM) for 6 h enhanced the thymidine incorporation to a level 20-fold higher.

Because the T-cell proliferative activity of IL-2 is initiated

![Graph](https://example.com/graph.png)

**FIG. 1.** Effect of GSH on the response of CTLL-2 to IL-2. The cells in the presence of serial dilutions of IL-2 were incubated at 37°C, 10% CO2 in 15% FCS, DMEM for 18 h (□); or in medium for 16 h followed by medium supplemented with GSH (0.65 mM) for 2 h (▲), or in medium supplemented with GSH (0.65 mM) for 18 h (◆). The cells were then pulsed with 3H-labeled thymidine (3H-TdR; 0.25 µCi/well) for 4 h. The radiolabeled cells were harvested with a cell harvester at the end of the incubation. The values are the means ± S.E. of three experiments.

![Graph](https://example.com/graph.png)

**FIG. 2.** Effect of pretreatment with GSH on the binding of 125I-labeled IL-2 to CTLL-2 cells. Cells were incubated at 37°C for 18 h in the presence (∆) or absence (○) of 8 mM GSH. After incubation, the cells were treated with 10 mM sodium citrate, 0.14 M NaCl, pH 4.0, for 12 s to remove surface-bound ligands and then washed, followed by incubation with various concentrations of 125I-labeled IL-2 at 4°C for 90 min as indicated. The cultures were performed in triplicate. Cell-bound radioactivity was separated from unbound radioactivity as described under “Methods.”

after the binding and internalization of IL-2 (14, 15, 39, 40), the influence of GSH on the incorporation of IL-2 into the cells was evaluated. Fig. 2 shows that preincubation of CTLL-2 cells with GSH enhances the binding of IL-2 to its high affinity receptors. Scatchard plot analysis showed that the Kd decreased from 75 ± 19 to 29 ± 6 pm (n = 5; p < 0.05), whereas the receptor number was not significantly affected (from 2853 ± 422 to 3036 ± 382 per cell; p > 0.2) by GSH treatment. The internalization study showed that in the GSH-pretreated CTLL-2 cells, IL-2 was rapidly internalized with a maximum of intracellular IL-2 found 40 min later (Fig. 3A). At this time, most of the membrane-bound radioactivity disappeared from the cell surface. Meanwhile, the degraded IL-
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**FIG. 3.** Effect of preincubation of CTLL-2 cells with GSH on the internalization of IL-2. (A) CTLL-2 cells were preincubated with 8 mM GSH for 18 h in the presence of 3 units/ml (6.7 × 10^{-11} M) rIL-2. After incubation, cells were treated with 10 mM sodium citrate, 0.14 M NaCl, pH 4.0, for 12 s to remove surface-bound ligands and then washed, followed by incubation with 100 pm 3H-labeled IL-2 at 4 °C for 90 min. The cells were washed and incubated at 37 °C for various durations as indicated. Aliquots of the cell suspension were removed and treated as described under “Experimental Procedures.” The radioactivities in the culture supernatants (○), on the cell surface (△), inside the cells (▲), and the sum of the counts of all three fractions (■) are shown. (B) CTLL-2 cells were preincubated in the presence of 3 units/ml rIL-2 for 18 h without addition of GSH or other thiol compounds.

2 (soluble in 10% trichloroacetic acid) started to appear in the culture medium, and its amount increased rapidly during the next 140 min even in the presence of 200 μM chloroquine, a lysosomotropic agent (41); whereas in GSH-unincubated control CTLL-2, the intracellular IL-2 did not reach a plateau until 160 min, and the total amount of IL-2 internalized was only half of the GSH-pretreated cells. Moreover, there was little, if any, degraded IL-2 in the medium even after 160 min (Fig. 3B). These results suggest that GSH plays an important role in the internalization of IL-2.

**FIG. 4.** Effect of GSH on the thymidine incorporation of CT-4R cells. CT-4R cells in the presence of serial dilutions of rIL-2 were incubated in 10% FCS, DMEM for 18 h (■); or in medium for 16 h followed by medium supplemented with GSH (3.25 mM) for 2 h (△); or in medium supplemented with GSH (3.25 mM) for 18 h (●). The cells were then pulsed with 3H-labeled thymidine (3H-TdR:0.25 μCi/well) for 4 h. The radiolabeled cells were harvested at the end of the incubation. The values are the means ± S.E. of three experiments.

**FIG. 5.** Effect of preincubation of CT-4R cells with GSH on the internalization of IL-2. (A) CT-4R cells were preincubated with 1.6 mM GSH for 18 h in the presence of 3 units/ml (6.7 × 10^{-11} M) rIL-2. (B) CT-4R cells were preincubated in the presence of 3 units/ml rIL-2 for 18 h without addition of GSH or other thiol compounds. The procedure of IL-2 internalization study was the same as described in the legend of Fig. 3.

| Table I: The cellular content of GSH in various cell lines |
|----------------------------------------------------------|
| Cell type* | Intracellular GSH | ng/10^6 cells |
|------------|--------------------|----------------|
| CTLL-2     | <50                |                |
| CT-4R      | <50                |                |
| MLA-144    | 1283 ± 30          |                |
| Hut-78     | 1165 ± 180         |                |
| Molt-3     | 3900 ± 100         |                |
| Molt-4     | 3700 ± 200         |                |

*CT-4R and CTLL-2 are IL-2 dependent. MLA-144, Hut-78, Molt-3, and Molt-4 are IL-2 independent.

GSH was measured by its selective reaction with Ellman’s reagent in the presence of GSH reductase and NADPH. The existence of GSH disulfide was excluded by using 2-vinylpyridine according to the method of Griffith (38). The values represent means ± S.E. of at least three determinations.
and degradation of IL-2 were enhanced when the CT-4R was pretreated with GSH (Fig. 5). The values represent the means of at least three determinations.

$^a$Cells were cultured in 10% FCS, DMEM with $0.7 \times 10^{-4}$ M rIL-2 at 37°C, 10% CO$_2$ for 22 h. GSH (final concentration, 0.65 mM) and BSO (final concentration, 1 mM) were added as indicated. After incubation, the cells were centrifuged and washed with 10 ml of phosphate-buffered saline three times, and the number of the viable cells was counted. The cells were broken by freezing and thawing three times.

GSH was determined by its selective reaction with Ellman’s reagent in the presence of GSH reductase and NADPH. BSO (1 mM) did not interfere with the reaction. The values represent the means of two experiments.

### Table II

| Treatment | Intracellular GSH level$^a$ |
|-----------|----------------------------|
| No GSH treatment | <50 | <50 |
| GSH (for 22 h) | 1289 | 1815 |
| GSH and BSO (for 22 h) | <50 | 100 |

$^a$Cells were cultured in 10% FCS, DMEM with 6.7 x 10^-4 M rIL-2 at 37°C, 10% CO$_2$ for 22 h. GSH (final concentration, 0.65 mM) and BSO (final concentration, 1 mM) were added as indicated. After incubation, the cells were centrifuged and washed with 10 ml of phosphate-buffered saline three times, and the number of the viable cells was counted. The cells were broken by freezing and thawing three times.

GSH was determined by its selective reaction with Ellman’s reagent in the presence of GSH reductase and NADPH. BSO (1 mM) did not interfere with the reaction. The values represent the means of two experiments.

### Discussion

A variety of mitogens such as plant lectins, phytohemagglutinin, and Con A (47) is capable of inducing lymphocyte activation. This activation of lymphocytes and replication of the activated lymphocytes can be regulated by GSH, an abundant thiol compound in most cells (45, 48). It is not clear, however, how GSH can possess such activities. In this study, we found that GSH enhanced the T-cell proliferative activity of IL-2 (Fig. 1). Since mitogens enhance the production and secretion of IL-2, which may in turn play an important role in the replication and differentiation of T-lymphocytes (1-6), our results suggest that the effect of GSH on the replication of activated T-lymphocytes is partially, if not mainly, due to its influence on the activity of IL-2.

The cysteine residues of IL-2 at positions 58 and 105 form an intramolecular disulfide bond (8, 25-27). GSH, on the other hand, has a free thiol group on its cysteine residue (49). A literal interpretation for the influence of GSH on IL-2 activity would be that GSH interacts with IL-2 directly by reducing the disulfide bond of IL-2. We found, however, that the reduced form of IL-2 is only one-tenth as potent as nonreduced IL-2 (8). It is thus unlikely that the potentiating effect of GSH on IL-2 activity is due to its reduction-oxidation reaction with the IL-2 molecule.

An alternative explanation is that GSH affects the IL-2 activity indirectly through its effect(s) on the target cells. Although GSH is not effectively transported into certain cell lines such as HSB, Molt, CEM, or normal human skin fibroblasts (50), partial repletion of intracellular GSH by adding GSH to the medium has been reported (51). In this study, we found that pretreatment of CTLL-2 and CT-4R cells with GSH increased their intracellular GSH concentration (Table II). Moreover, we found that GSH enhanced the binding, internalization, and degradation of IL-2. Whether some or all of these GSH effects are involved in the potentiating of IL-2 activity is currently under study.

Most circulating T-cells are in a resting state; only after reacting with antigen or mitogen do specific clones of cells proliferate and differentiate into effector cells such as cytolytic T-cells and helper T-cells (7). Because resting T-cells do not produce IL-2, nor are they responding to IL-2 (39), it is likely that production of IL-2 and IL-2 receptors determines the response of T-cells to antigen activation. By examining the response of activated T-cells to IL-2 and the distribution of IL-2 receptor density, Cantrell and Smith (39) proposed that the effects of IL-2 on lymphocytes are mainly determined by the IL-2 concentration, the IL-2 receptor density, and the duration of interaction between IL-2 and the IL-2 receptors. In view of our finding that GSH in physiological concentrations (0.1-10 mM; Ref. 49) affects the response of IL-2-dependent cells to IL-2, we suggest that the effect of IL-2 may...
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also be regulated by changing the tissue GSH level.

In summary, we have shown that GSH enhances the effect of IL-2 on replication and thymidine incorporation of IL-2-dependent cells in vitro. This potentiating effect of GSH is accompanied by an increase in the intracellular GSH level and enhancement in the binding, internalization, and degradation of IL-2. It will be interesting to examine whether de novo biosynthesis and secretion of GSH may regulate the response of activated lymphocytes to IL-2 and thus play an important role in the initiation and progression of immune responses in vivo.

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