Determination of glutathione in lymphocytes and possible association of redox state and proliferative capacity of lymphocytes

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The glutathione (GSH) content of mouse T- and B-cells was determined and compared with the GSH content of human peripheral blood lymphocytes and human erythrocytes. Owing to the difficulty of obtaining large numbers of purified lymphocytes, a technique was developed to measure picomolar quantities of GSH. By this technique, mouse T- and B-cells, as well as mouse peripheral-blood lymphocytes, were found to contain approx. 30% of the GSH found in human peripheral-blood lymphocytes. The concanavalin A response of human peripheral-blood lymphocytes and human spleen cells was insensitive to 2-mercaptoethanol as well as to culture in 17% O₂, whereas mouse lymphocyte responses were altered by 2-mercaptoethanol and inhibited by 17% O₂. The capacity of human peripheral-blood lymphocytes, human erythrocytes, mouse T-cells and mouse B-cells to regenerate GSH stores after chemical oxidation by diamide was tested, and it was found that mouse cells were less capable of regenerating GSH than human erythrocytes or human peripheral-blood lymphocytes. In addition, the latter lymphocytes were less sensitive to oxidation of GSH and to inhibition of proliferation by diamide.

The proliferative and functional activities of mouse T- and B-lymphocytes are sensitive to thiol compounds *in vitro* (Noelle & Lawrence, 1980a), and O₂ at concentrations of only 7% is known to be optimal for mouse lymphocyte responses *in vitro* (Mishell & Dutton, 1967). In addition, oxidation of lymphocyte cell-surface thiol groups can ablate the concanavalin A-induced response, and the oxidative inhibition by O₂ or by the impermeant oxidant copper–phenanthroline could be completely reversed by 2-mercaptoethanol (Noelle & Lawrence, 1981). The inhibition of concanavalin A-induced lymphocyte proliferation by oxidants has been suggested to involve GSH-metabolic pathways (Sagone *et al.*, 1978; Lawrence, 1981).

Studies in other systems have indicated that the

major factor conveying resistance to oxidative insults such as γ -irradiation is intracellular GSH. For example, GSH-deficient mutants of *Escherichia coli* have been shown to be sensitive to much lower doses of ionizing irradiation than are GSH⁺ strains (Morse & Dahl, 1978). In eukaryotes, loss of GSH can result in the generation of lipid peroxides (Anundi *et al.*, 1979), inability to reduce membrane disulphide groups (Sutherland & Pihl, 1968), increase in cell lysis (Anundi *et al.*, 1979), and alterations of cytoskeletal functions (Oliver *et al.*, 1976) and cell proliferation (Holmgren, 1976).

The purpose of the present investigation was to develop an assay to measure picomolar quantities of GSH for use in determining GSH in 0.5×10^5 – 2.5×10^5 lymphocytes. Previous assays required over 10^6 cells, and were not efficient when limited numbers of selected cell types were to be assayed. By using this new assay, the GSH contents of purified mouse T- and B-cell preparations, as well as mouse and human peripheral-blood lymphocytes and human erythrocyte preparations, were determined and significant differences noted. Correlations between thiol-mediated enhancement, oxidative inhibition and GSH status of different cell types were observed.

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione; T-cells, thymus-derived lymphocytes; B-cells, bone-marrow-derived lymphocytes; PBS, phosphate-buffered saline (0.14 M-NaCl, 0.01 M-sodium phosphate, pH 7.2).

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Materials and methods

Materials

Diamide [diazenedicarboxylic acid bis-(*NN*-dimethylamide); Kosower, 1978], GSH, L-cysteine and recrystallized concanavalin A (type IV) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *N*-[2-³H]Ethylmaleimide (692 Ci/mol; New England Nuclear Corp., Boston, MA, U.S.A.) in pentane was maintained at 4°C, and 1.08 ml of PBS containing 0.66% Nonidet P40 (Particle Data Laboratories, Elmhurst, IL, U.S.A.) and 0.1 mM-EGTA (Eastman Kodak Co., Rochester, NY, U.S.A.) was added immediately to the vial after opening. The pentane was evaporated from the vial by agitation under a stream of nitrogen. *N*-[³H]-Ethylmaleimide prepared in this manner was used the same day, since prolonged storage in an aqueous solvent resulted in the formation of breakdown products.

Cell preparations

Lymphocytes from the peripheral blood of normal human donors were isolated as follows: 15 ml of whole heparinized blood (1500 units/60 ml of blood) was diluted with 15 ml of PBS and layered on 12 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ, U.S.A.) and centrifuged at 1000 *g* for 10 min at 4°C. The buffy coat was isolated and washed once with PBS. The pellet was resuspended in 5 ml of 0.83% NH₄Cl and incubated for 4 min at 22°C. The cells were washed twice more in PBS. Cells prepared in this manner were over 95% viable, as judged by Trypan Blue exclusion, and were free of erythrocytes. Human spleen fragments were aseptically obtained and teased apart in a balanced salt solution (Mishell & Dutton, 1967). The teased cells were transferred to sterile 50 ml tubes and allowed to settle for 5 min on ice. The supernatant single-cell suspension was further purified as described for human peripheral-blood lymphocytes. CBA/J mouse (Jackson Laboratories, Bar Harbor, ME, U.S.A.) spleen cells and peripheral-blood lymphocytes were isolated by treatment with 0.83% NH₄Cl and Ficoll/Metrizoate sedimentation as described previously (Davidson & Parish, 1975). T-cells were negatively selected from mouse spleen cells by affinity adherence on rabbit anti-(mouse Fab)-coated Petri dishes as described by Wysocki & Sato (1978). B-cells were isolated from mouse spleen cells after treatment with anti-(Thy 1.2)+ complement as reported by Noelle & Lawrence (1980a).

Cell cultures

Cultures of mouse and human spleen cells and human peripheral-blood lymphocytes were established by using techniques described by Mishell & Dutton (1967), with the following modification:

medium contained 50 μ M-2-mercaptoethanol (Eastman Kodak) where indicated. For the proliferative studies, 2×10^5 cells/200 μ l per micro-well were cultured in the presence of 2 μ g of concanavalin A/ml with or without 2-mercaptoethanol.

Measurement of [³H]thymidine incorporation

Cultures were pulsed for 6 h with 0.5 μ Ci of [³H]thymidine (6.7 Ci/mmol; New England Nuclear) on the days specified, harvested with a multiple cell harvester, and incorporated radioactivity (c.p.m.)/culture was measured by liquid-scintillation spectrometry as described by Noelle & Lawrence (1980a). All results presented are the means for triplicate cultures, expressed as c.p.m./culture. The results were considered significant when $P < 0.01$ by Student's *t* test.

Preparation of sample for GSH micro-determination

Cell suspensions (10⁷ cells/ml) were sampled in 200 μ l batches into 1.8 ml conical centrifuge tubes (Sarstedt, Princeton, NJ, U.S.A.), 1.0 ml of PBS was added, and the tubes were centrifuged at 200 *g* for 10 min in a Beckman TJ-6R instrument. The supernatant was removed and the pellet of cells resuspended in 1.0 ml of PBS and centrifuged again. The pellet was resuspended in 80 μ l of PBS, and three 10 μ l batches (2.5×10^5 cells/10 μ l) were transferred into 0.5 ml microfuge tubes (Sarstedt) containing 15 μ l of *N*-[³H]ethylmaleimide in 0.66% Nonidet P-40 (prepared as described above). The tubes were vigorously agitated and centrifuged for 10 s in a Beckman Microfuge B to ensure that all reactants were at the bottom of the tube. Tubes were incubated for 2 h at 22°C or 24 h at 4°C, both of which were determined to give identical and optimal results. Each tube then received 5 μ l of filtered 60% (w/v) trichloroacetic acid (Fisher Chemical Co., Fairlawn, NJ, U.S.A.), and the tubes were again agitated and centrifuged for 10 s in a Beckman Microfuge. All samples were deproteinized after 15 min at 4°C by centrifugation for 10 min in a Beckman Microfuge B.

Thin-layer chromatography of prepared samples

Samples (5 μ l) of the trichloroacetic acid supernatant of cell lysates (prepared as described above) were spotted on to cellulose MN-300 t.l.c. sheets (Brinkman Instruments, Westbury, NY, U.S.A.). The spots were dried and the trichloroacetic acid was extracted by immersion of the t.l.c. sheets in diethyl ether. The sheets were then chromatographed vertically in a butan-1-ol/pyridine/acetic acid/water system (20:10:3:10, by vol.) originally described by States & Segal (1969). During the summer, if the humidity is high, the proportions 6:2:1:2 (by vol.) are preferable. The solvent front was allowed to run to 1–1.5 cm from the top of the

sheet. Each lane was cut into 1 cm × 2 cm pieces and individual pieces were placed into mini-vials. To each mini-vial, 0.2 ml of water was added, and the vials were capped and incubated at 22°C for 1 h to extract the adducts of *N*-[³H]ethylmaleimide. Finally, to each vial 4 ml of Ultrafluor (National Diagnostics, Somerville, NJ, U.S.A.) was added, the vials were cooled at 4°C and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer model 3330.

Quantitative estimates of GSH were determined from GSH standard curves as well as by calculations of counting efficiency by using adducts of [³H]GSH (New England Nuclear) and *N*-ethylmaleimide spotted on 1 cm × 2 cm pieces of t.l.c. sheets and processed as described above. All GSH measurements are the arithmetic means for at least triplicate samples, ± S.D.

Results

Quantification of GSH by the t.l.c./[³H]*N*-ethylmaleimide technique

The technique developed for the measurement of GSH in lymphocytes was an application and extension of a technique originally described by States & Segal (1969) for the qualitative detection of adducts of *N*-ethylmaleimide. Briefly, GSH or a detergent lysate of cells was incubated with *N*-[³H]ethylmaleimide for 2 h at 22°C, deproteinized with trichloroacetic acid, then the acid supernatant was chromatographed on cellulose-coated

chromatography sheets. The location on the sheet corresponding to the GSH-*N*-[³H]ethylmaleimide adduct was excised and the amount of this adduct quantified by liquid-scintillation spectrometry.

The first aspect of developing a quantitative t.l.c./*N*-[³H]ethylmaleimide technique was to define the *R_F* values for the GSH-*N*-ethylmaleimide adduct as well as other common non-protein thiol adducts that might be present in the trichloroacetic acid supernatant of cell lysates. GSH has been reported to represent over 95% of the total non-protein thiol in erythrocytes, followed by L-cysteine, representing approx. 2.3% (Kosower, 1978). Since GSH and L-cysteine comprised over 98% of the non-protein thiol found in cell lysates, GSH and L-cysteine standards were chromatographed and the *R_F* values of the *N*-ethylmaleimide adducts ascertained. As shown in Fig. 1(a), the commercial preparation of *N*-[³H]ethylmaleimide had approx. 7–10% contaminating breakdown products that were non-extractable by ether and which accumulate on storage of the radiochemical in an aqueous solution. As illustrated by Fig. 1(b), 96% of the GSH-*N*-[³H]ethylmaleimide adduct migrates between 4 and 5 cm from the origin, which represents an *R_F* of 0.28. The L-cysteine-*N*-[³H]ethylmaleimide adduct migrates at 8–10 cm from the origin (Fig. 1c) and had an *R_F* value of 0.64. This system allowed for clear separation of *N*-[³H]ethylmaleimide adducts of GSH and L-cysteine from mixtures of GSH and L-cysteine (Fig. 1d). Next, verification of the t.l.c./*N*-[³H]ethylmaleimide method for quantifi-

Fig. 1. Thin-layer-chromatography profiles of *N*-[³H]ethylmaleimide (a), *N*-[³H]ethylmaleimide + GSH (b), *N*-[³H]ethylmaleimide + cysteine (c) and *N*-[³H]ethylmaleimide + GSH + cysteine (d)

Samples were prepared as described in the Materials and methods section and chromatographed. Each lane was cut into 1 cm pieces and the quantity of *N*-[³H]ethylmaleimide or its adducts was determined by liquid-scintillation spectrometry.

Fig. 2. Quantitative measurement of GSH by N -[^3H]-ethylmaleimide/t.l.c. method

To graded concentrations of GSH, 5 nmol of N -[^3H]ethylmaleimide in 0.66% Nonidet P-40 in PBS was added, acidified with trichloroacetic acid and chromatographed as described in the Materials and methods section. Each lane was cut into 1 cm pieces and the quantity of N -[^3H]ethylmaleimide-GSH in each sample determined by liquid-scintillation spectrometry. Each point represents the arithmetic mean $\pm$ S.D. for triplicate samples.

cation of GSH in prepared solutions as well as in lysates of lymphocytes was necessary. Fig. 2 illustrates that a linear equimolar relationship exists between the amount of GSH added to the assay and the number of mol of N -[^3H]ethylmaleimide-GSH adduct detected at an R_F of 0.28 (4–5 cm from the origin). The slope of the line presented in Fig. 2 was 0.93 and had a correlation coefficient of 0.99. When spleen cell lysates were prepared and N -[^3H]ethylmaleimide was added, the t.l.c. profile obtained was as portrayed in Fig. 3(a). To confirm that the radioactivity in the spleen cell preparations moving 4–6 cm from the origin was GSH, exogenous GSH was added. As shown in Fig. 3(b), GSH added to spleen cell lysates increased the radioactivity observed at 4–6 cm from the origin. When measured, 110% of the GSH added was detected in the lysate plus GSH mixture. Exogenously added L-cysteine increased the radioactivity detectable 8–10 cm from the origin, and 99% of the added L-cysteine was detectable by subtracting values obtained from $100 \times [\text{d.p.m. (L-cysteine + cells)} - \text{d.p.m. (cells)}] / \text{d.p.m. (L-cysteine)}$. Further control experiments indicated that the detection of GSH linearly increased with increasing cell number between 1.5×10^5 and 2.0×10^6 cells/assay tube containing 10 nmol of N -[^3H]ethylmaleimide. Half-maximal saturation of GSH from 5×10^5 spleen cells was

Fig. 3. Thin-layer-chromatography profiles of N -[^3H]-ethylmaleimide-labelled acid supernatants of mouse spleen cells

Cells were suspended alone (a) or with glutathione (b) or with cysteine (c) in 0.66% Nonidet P-40 in PBS containing 5 nmol of N -[^3H]ethylmaleimide, acid precipitated, and samples of each were chromatographed, as described in the Materials and methods section. Each lane was cut into 1 cm pieces and the quantity of N -[^3H]ethylmaleimide adduct was determined by liquid-scintillation spectrometry.

achieved with 1 nmol of N -[^3H]ethylmaleimide. For routine measurements of GSH, a saturating concentration of 5 nmol of N -[^3H]ethylmaleimide was added/ 2.5×10^5 cells.

A variety of means for cell disruption were initially tested to ascertain the optimal means for lysing cells. The method that yielded the highest estimate of GSH was cell disruption in 0.33% Nonidet P-40 at pH 7.2 for 2 h at 22°C. Other methods tested included freeze-thawing, boiling, or high pH in the presence or absence of detergent. All of these methods yielded GSH values 12–22% lower than by lysis with Nonidet P-40 at pH 7.2 at 22°C (results not shown).

GSH content of different cell preparations

Since human erythrocyte GSH content has been previously documented (Hardin *et al.*, 1954; Ellman, 1959; Tietze, 1969), it was of interest to compare values obtained by the t.l.c./N-ethylmaleimide method with values previously reported and obtained by independent means. Previously reported values for GSH in human erythrocytes were 440 amol/cell (Ellman, 1959), 230 amol/cell (Hardin *et al.*, 1954) and 240 amol/cell (Tietze, 1969). The value obtained by the method reported in the present paper compares favourably with the two last methods. The content of GSH in human peripheral-blood lymphocytes has also been determined and is similar to that reported for the total non-protein thiol content of these cells (Kosower, 1978). Mouse spleen cells and mouse peripheral-blood lymphocytes contained approx. 30% of the GSH found in human peripheral-blood lymphocytes, as did mouse spleen T- and B-cells (Table 1). On incubation at 37°C in a balanced salt solution containing 0.1% glucose (Mishell & Dutton, 1967), human peripheral-blood lymphocytes and mouse B-cells showed 1.43- and 1.36-fold increases, respectively,

in the content of GSH; however, only marginal increases in intracellular GSH were noted in the other cell preparations. Incubation of cells for 30–120 min at 37°C did not increase GSH values above those reported at 30 min (results not shown). The intracellular GSH concentrations in human peripheral-blood lymphocytes and mouse spleen cells, respectively, were 1430 ± 240 ($n = 4$) and 400 ± 60 ($n = 12$) amol/cell (means $\pm$ S.E.M.). The mouse lymphocytes consistently had approx. one-third as much GSH as did human peripheral-blood lymphocytes.

Sensitivity of the concanavalin A-induced response of mouse and human spleen cells to 2-mercaptoethanol

Since human peripheral-blood lymphocytes contained 3 times as much GSH as did mouse spleen cells, mouse peripheral-blood lymphocytes, or T- and B-cells, it was decided to examine if the concanavalin A-induced responses of human peripheral-blood lymphocytes and human spleen cells were as sensitive to oxidation and reduction as were those of mouse cells. As presented in Table 2,

Table 1. *Glutathione content of various lymphoid cell preparations*

GSH values are expressed as the arithmetic means $\pm$ S.D. for quadruplicate determinations. Cells were prepared as described in the Materials and methods section. After isolation, cells were incubated in balanced salt solution (containing glucose) for 0 or 30 min at 37°C. Abbreviation: ND, not done.

Cell type	Time of incubation ...	GSH content (amol/cell)	
		0 min	30 min
Mouse spleen cells		334 ± 25	390 ± 11
Mouse splenic T-cells		332 ± 40	336 ± 28
Mouse splenic B-cells		356 ± 22	483 ± 46
Human peripheral-blood lymphocytes		810 ± 65	1160 ± 87
Mouse peripheral-blood lymphocytes		412 ± 19	ND
Human erythrocytes		240 ± 17	250 ± 22

Table 2. *Sensitivity of the concanavalin A-induced response of mouse and human lymphoid cells to 2-mercaptoethanol*
Human spleen cells (HSC), human peripheral-blood lymphocytes (HPBL), mouse spleen cells (MSC) or mouse peripheral-blood lymphocytes (MPBL) were cultured at 2×10^5 cells/200 μ l per micro-well, with 2 μ g of concanavalin A (Con A)/ml with or without 5 μ M-2-mercaptoethanol (2-ME). Cell cultures were pulsed from 18 to 24 h (day 2), 66 to 72 h (day 3), or 90 to 96 h (day 4) after initiation of culture with 0.5 μ Ci of [3 H]thymidine, and the results are representative of three separate experiments.

Day of culture	[3 H]Thymidine incorporation (c.p.m./culture)							
	HSC		HPBL		MSC		MPBL	
	Con A	Con A + 2-ME	Con A	Con A + 2-ME	Con A	Con A + 2-ME	Con A	Con A + 2-ME
2	20140	22634	6192	5967	49874	106539	27314	86793
	± 915	± 3668	± 732	± 161	± 2267	± 10206	± 3006	± 5214
3	33515	39541	32129	30275	115975	180511	54512	93506
	± 1904	± 2123	± 2936	± 1605	± 2255	± 8729	± 4630	± 5671
4	48021	59709	35095	39557	84873	47451	73926	60115
	± 3601	± 6419	± 2075	± 7193	± 6575	± 3823	± 5128	± 3079

Table 3. *Effect of oxygen partial pressure on the concanavalin A-induced response of human spleen cells*

Spleen-cell and peripheral-blood-lymphocyte cultures were pulsed from 66 to 72h after initiation of culture with 0.5 μ Ci of [3 H]thymidine, and the results are expressed as the arithmetic means $\pm$ s.d. for triplicate cultures. The cells were placed in culture under the oxygen partial pressures listed for the entire culture period. The results are representative of three separate experiments.

Cell cultures	Culture conditions	[3 H]Thymidine incorporation (c.p.m./culture)	
		7% O ₂	17% O ₂
Mouse spleen cells		66 120 $\pm$ 876	3456 $\pm$ 281
Mouse peripheral-blood lymphocytes		51 421 $\pm$ 3791	5800 $\pm$ 347
Human spleen cells		18 644 $\pm$ 766	34 249 $\pm$ 3004
Human erythrocytes		24 496 $\pm$ 1488	33 756 $\pm$ 3487

the day-2 concanavalin A responses of mouse spleen cells and mouse peripheral-blood lymphocytes were enhanced 2.1-fold and 3.2-fold, respectively, in the presence of 2-mercaptoethanol, but the addition of the latter to the corresponding human cell cultures did not substantially enhance the concanavalin A-induced response. On day 3, the responses to concanavalin A plus 2-mercaptoethanol of mouse spleen cells and mouse peripheral-blood lymphocytes were enhanced 1.6-fold and 1.7-fold respectively, but the response of the corresponding human cells still was unaffected. As previously reported (Noelle & Lawrence, 1980a), 2-mercaptoethanol depressed the late (day-4) concanavalin A-induced response. The day-4 responses of human cultures were not enhanced or depressed by 2-mercaptoethanol.

It has been reported previously (Noelle & Lawrence, 1979) that mouse spleen and T-cells are unresponsive to concanavalin A if cultured at 17% O₂, unless 2-mercaptoethanol was present. To assess whether human peripheral-blood lymphocytes or human spleen cells share a similar sensitivity to high O₂ partial pressure, these cells were cultured under 7% or 17% O₂. As shown in Table 3, 17% O₂ did not diminish the concanavalin A-induced response of these cells, but rather enhanced their responses.

Regeneration of intracellular GSH after oxidative insult

The capacity of a cell type to recover from an oxidative insult (such as 17% O₂) may be reflected in its static intracellular GSH content, but recovery may also be represented by its ability to regenerate intracellular stores of GSH (Kosower, 1978). To examine the relative sensitivity to an oxidant and the relative capacity of different cell types to recover from oxidative attack, the following system of regeneration was employed: cells (mouse T- or B-cells, human peripheral-blood lymphocytes, and human erythrocytes as an established comparative control; Kosower *et al.*, 1969) were treated with 100 μ M-diamide in PBS at 37°C for 30 min (oxi-

Fig. 4. *Capacity of T-cells, B-cells, human peripheral-blood lymphocytes and human erythrocytes to regenerate intracellular stores of GSH*

Cell preparations (10⁷ cells/ml) were treated with 0.1 mM-diamide for 30 min at 37°C in PBS. After this treatment, each cell preparation was washed twice and resuspended in a balanced salt solution at 37°C (arrow), and the amount of GSH in T-cells (●), B-cells (▲), human peripheral-blood lymphocytes (○) and human erythrocytes (■) was determined at various times after diamide treatment and expressed as a percentage of the initial GSH in each cell preparation. Each point represents the mean of quadruplicate samples, and the variance was less than 10%.

dative period), then washed free of the diamide and placed in a balanced salt solution (glucose-containing) at 37°C (regenerative period). As shown in Fig. 4, the total GSH content of T-cells, B-cells and human erythrocytes was diminished to less than 11% of their original content. The GSH content of human peripheral-blood lymphocytes was decreased by only 58% by this treatment. Human erythrocytes were able to regenerate all of their GSH, and there appeared to be a slow but linear increase in the GSH content of human peripheral-blood lymphocytes. However, mouse T- and B-cells only produced a transient increase in GSH, and even at their

maximum point of GSH regeneration (30 min), B-cells only had 58% of their initial amount. At 75 min, mouse T-cells and B-cells had only 63% and 32% respectively of their initial GSH. Since human peripheral-blood lymphocytes have approx. 3 times as much GSH as do human erythrocytes and mouse T- and B-cells, this may account for their greater resistance to diamide oxidation. Permeability differences also could account for the differential resistance to diamide. All GSH determinations (mol/cell) were done on the cells recovered after the various incubations; therefore the differences do not reflect a loss in cell number or viability. A differential resistance to diamide between human peripheral-blood lymphocytes and mouse spleen cells also was apparent in the proliferative assay (Fig. 5), in that 10–100 μ M-diamide inhibited proliferation of the latter cells significantly more than that of the human cells.

Fig. 5. Effects of diamide on proliferation of human peripheral-blood lymphocytes (●) or mouse spleen cells (■) induced by concanavalin A

Lymphocyte cultures were pulsed on day 3 of culture with 0.5 μ Ci of [³H]thymidine, and incorporation (c.p.m./culture) was assessed. Control cultures were not pretreated with diamide, and the untreated human peripheral-blood lymphocytes and mouse spleen cells had 37139 ± 2876 and 52536 ± 4870 c.p.m. respectively. Treated lymphocytes were incubated with various concentrations of diamide for 30 min at 37°C, washed, and added to the cultures (2×10^5 viable cells/well). Each point represents the mean $\pm$ s.d. for three experiments.

Discussion

Thiol-reactive compounds have been shown to modulate mouse lymphocyte activities (Kwock *et al.*, 1976; Field *et al.*, 1978; Goldberg *et al.*, 1978; Redelman & Hudig, 1980). It has been suggested that mouse lymphocyte reactivities can be inhibited by surface oxidation (Noelle & Lawrence, 1980b, 1981), and GSH may be involved in reversing oxidative inhibitory effects (Sagone *et al.*, 1978; Lawrence, 1981). Since GSH has been previously implicated in the maintenance and repair of membranes and membrane functions and comprises 90% of the cellular non-protein thiol pool (Kosower, 1978), it was of interest to investigate the amount of GSH in lymphocytes.

Large numbers of T- and B-cells are difficult to obtain; therefore an assay to quantify GSH in picomolar quantities was developed. The GSH assay described utilized 2.5×10^5 cells/GSH determination, with an average coefficient of variation of less than 11% for triplicate determinations. The GSH content of human erythrocytes has been previously estimated by measurement of total non-protein thiols (Hardin *et al.*, 1954), as well as total GSH (Tietze, 1969). Tietze (1969) reported that human erythrocytes contain 240 amol of GSH/cell, which is comparable with the GSH content of human erythrocytes reported here (Table 1). The GSH content of human peripheral-blood lymphocytes is estimated at 1.4 fmol/cell, which is also in close agreement with published results (Kosower, 1978; Hardin *et al.*, 1954). Mouse spleen cells contained approx. 33% as much GSH as human peripheral-blood lymphocytes, as did mouse T- and B-cells.

Since a significant difference in GSH content existed between human peripheral-blood lymphocytes and mouse spleen cells or mouse peripheral-blood lymphocytes, the sensitivity of the proliferative responsiveness to thiol enhancement of each cell type was examined. As shown in Table 2, the addition of 2-mercaptoethanol to cultures of human peripheral-blood lymphocytes or human spleen cells did not significantly alter the concanavalin A-induced responsiveness. In contrast, the concanavalin A-induced response of mouse spleen cells or mouse peripheral-blood lymphocytes was enhanced over 2-fold on day 2 and depressed on day 4. Further potential redox differences between human and mouse lymphocytes were apparent. Human peripheral-blood lymphocytes cultured under 17% O₂ were hyper-responsive to concanavalin A (Table 3), whereas in 17% O₂, mouse spleen cells and mouse T-cells were rendered unresponsive to concanavalin A (Noelle & Lawrence, 1981; Table 3). The fact that human peripheral-blood lymphocytes have significantly more GSH than do

mouse spleen cells may be an explanation for why these human cells were less sensitive to oxidation and thiols. This possibility is supported by the data, which indicate that human peripheral-blood lymphocytes were less sensitive to diamide inhibition of proliferation (Fig. 5). Although the amounts and reactivities of GSH-related enzymes, such as GSH peroxidase and GSH reductase, need to be assessed in the lymphocyte preparations, preliminary results show that the GSH content of the lymphocytes may be correlated with the ability of oxidants and thiols to modulate their proliferation.

The ability of lymphocytes to offset oxidative damage also was measured by determining the capacity of the cells to regenerate intracellular stores of GSH. Mouse T-cells were more active than B-cells in regenerating GSH. The lower ability of B-cells to regenerate GSH may be partially responsible for their greater radiosensitivity (Anderson & Warner, 1977). It also may be pertinent to note that 2-mercaptoethanol alone permits or promotes B-cells, but not T-cells, to proliferate (Noelle & Lawrence, 1980a). Therefore the hypersensitivity of B-cells to oxidation (radiation-induced) and selective thiol compounds may be related to their lower capacity to regenerate and maintain GSH *in vitro*. The transient regeneration of GSH in mouse lymphocytes is not understood at present. The GSH could be reoxidized rapidly to GSSG and/or secreted into the media, as has been reported for mouse lymphoid cells (Kosower, 1978). Alternatively, the GSH could be utilized in repairing the oxidative damage to cellular proteins such as the formation of disulphide cross-linkages of membrane proteins. Evidence suggests that surface oxidation of lymphocytes by copper-phenanthroline can alter thiol-containing membrane proteins, that these alterations affect their reactivities, and that 2-mercaptoethanol and extracellular GSH can modulate these effects (Noelle & Lawrence, 1980b, 1981). Since mouse lymphocyte GSH is approx. 30% of that found in human peripheral-blood lymphocytes, this may explain why mouse lymphocytes are hypersensitive to oxidative products.

There is indirect, suggestive evidence which links the GSH = GSSG content of cells with cell activation. The uptake of Ca^{2+} by lymphocytes has been suggested to be involved in the triggering of lymphocyte activation (Kaplan, 1977), and Ca^{2+} fluxes in cells have been shown to be influenced by the GSH = GSSG content of cells (Valverde & Malaisse, 1980). Furthermore, the redox state of the lymphocyte can modulate the intracellular concentration of cyclic GMP (Goldberg *et al.*, 1978), which is known to be intimately involved in lymphocyte proliferation (Strom *et al.*, 1977). In addition, thiol regulation of cell proliferation has been known for 50 years (Hammett, 1929), and

intracellular GSH has been suggested to be involved in regulating the cell cycle as well as protecting the cell from radiation (Ohara & Terasima, 1969).

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