Biochemical and Molecular Roles of Nutrients

Splenocyte Glutathione and CD3-Mediated Cell Proliferation Are Reduced in Mice Fed a Protein-Deficient Diet[1,2,3]

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ABSTRACT Protein-energy malnutrition (PEM) is associated with decreased host immune defense. Glutathione (GSH) status is reported to be decreased in PEM, and GSH is important for lymphocyte function. The objective of the present study was to investigate the effects of PEM and dietary repletion (RP) on GSH status in various tissues and splenocytes and on CD3-mediated calcium mobilization and cell proliferation of splenic T-lymphocytes. For the PEM model, mice were fed a 0.5% protein diet (LP group) for 4 or 6 wk, and control mice were fed a 15% protein diet (CP group). In the RP study, LP mice were fed the 15% protein diet for 3 d, 1 wk, 2 wk or 3 wk (RP groups). Glutathione concentrations were significantly lower in liver, lung, heart and spleen of LP mice compared with CP mice at 4 and 6 wk. Splenocytes from LP mice were significantly lower in number and had a lower intracellular GSH concentration, depressed CD3-stimulated T-lymphocyte proliferation in culture media without thiol supplementation (2-mercaptoethanol), and enhanced CD3-stimulated proliferation in thiol-supplemented culture media compared with splenocytes from CP mice. CD3-stimulated calcium mobilization was significantly lower in $CD8^+$, but not $CD4^+$, splenocytes from LP mice. Within 1 wk of dietary repletion, splenocyte GSH concentration was normal and splenocyte numbers were greater, and in vitro sensitivity of CD3-stimulated T-lymphocyte proliferation to thiol was lower, compared with LP mice. Glutathione status in vivo and thiol supplementation in vitro seem to modulate the signal transduction pathway for T-lymphocyte proliferation in mice with PEM. J. Nutr. 127: 44–50, 1997.

KEY WORDS: *glutathione* • *proliferation* • *signal transduction* • *splenocytes* • *mice*

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Protein-energy malnutrition (PEM)[5] is associated with decreased antioxidant and immune defense. In experimental animal models of PEM, increased susceptibility to oxidative stress from xenobiotics and pulmonary oxygen toxicity has been linked to decreased liver and lung concentrations of glutathione (GSH) (Deneke et al. 1985, Jung 1985, Taylor et al. 1992). Children with PEM have compromised GSH status as indicated by significantly decreased blood GSH concentrations that return to control values after treatment and recovery from PEM (Golden and Ramdath 1987, Jackson 1986, Sive et al. 1993). Protein-energy malnutrition is also characterized by a high incidence of opportunistic infections and diarrhea. However, the effects of PEM on GSH status and functioning of the immune system have not been addressed in these studies. In human immunodeficiency virus (HIV)-infected patients with wasting malnutrition, decreased GSH concentrations have been reported in T-lymphocytes, mononuclear cells, plasma and lung epithelial lining fluid (Buhl et al. 1989, Eck et al. 1989). T-lymphocyte GSH concentrations are decreased even though only a small percentage of T-lymphocytes are actually infected with HIV (Staal et al. 1992). However, it is difficult to differentiate the effects of wasting malnutrition and those of HIV or other infections on GSH status and immune function.

The hypothesis that decreased GSH status in PEM may contribute to impaired immune defense is based on several lines of evidence (Bray and Taylor 1994). Spleen GSH concentration is decreased in a rat model of PEM (Bray and Taylor 1994), and a role for GSH in immune function has been demonstrated in a variety of in vitro systems investigating T-lymphocyte activation, proliferation and interleukin-2 (IL-2). For example, depletion of lymphocyte GSH in vitro by conjugation with 1-chloro-2,4-dinitrobenzene or by alkylation with *N*-ethylmaleimide inhibits T-cell receptor–induced mobilization of intracellular calcium and tyrosine phosphorylation of several proteins, including phospholipase Cγ1 (Kanner et al. 1992, Kavanagh et al. 1993). Glutathione supplementation of culture medium modulates the synthesis and turnover of surface IL-2 receptors (Liang et al. 1992). When T-lymphocytes are sorted on the basis of GSH content and stimulated with mitogen, T-lymphocytes with low levels of GSH have a lower capacity to activate and proliferate in vitro (Kavanagh et al. 1990).

[1] Presented in part at the 38th Annual Meeting of the Canadian Federation of Biological Societies, June 14–17, 1995, Saskatoon, SK [Taylor, C. G., Potter, A. J. & Rabinovitch, P. S. (1995) Splenocyte glutathione and CD3-mediated signal transduction in protein-energy malnourished mice. Can. Fed. Biol. Soc. Proc. 38: 328 (abs.)].

[2] Supported by NIH grant AG-01751 and Pilot Award grant NIH DK-35816 through the University of Washington Clinical Nutrition Research Unit.

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[5] Abbreviations used: BrdU, 5′-bromodeoxyuridine; Ca, calcium; CP, 15% protein control group; GSH, glutathione; HIV, human immunodeficiency virus; IL-2, interleukin-2; LP, 0.5% protein group; 2-ME, 2-mercaptoethanol; PEM, protein-energy malnutrition; RP, dietary repletion group.

0022-3166/97 $3.00 © 1997 American Society for Nutritional Sciences.

Manuscript received 16 January 1996. Initial review completed 9 April 1996. Revision accepted 27 September 1996.

Given that there is considerable evidence that in vitro manipulation of intracellular GSH alters T-lymphocyte function, the goal of the present study was to investigate the effects of in vivo GSH depletion and repletion on tissue GSH concentrations and on T-lymphocyte functions. Synthesis of GSH (L-γ-glutamyl-L-cysteinylglycine) is primarily dependent on availability of cyst(e)ine and methionine in the diet (Taylor et al. 1996). Thus, dietary sulfur amino acid deficiency (0.5% protein) can be used as an in vivo model of tissue GSH depletion (Taylor et al. 1992 and 1996) to investigate the relationship of GSH status (assessed in various tissues and splenocytes) to functional responses of T-lymphocytes. In the present study, immunologically mature young adult mice (3–4 mo) were fed a 0.5% protein diet for 4 or 6 wk until significant weight loss occurred; however, it cannot be assumed that the 0.5% protein dietary regimen in young adult mice will produce depressed thymus-dependent immunity and edematous PEM as previously reported in weanling mice fed a 0.5% protein diet for 21 d (Filteau and Woodward 1987, Woodward and Miller 1991). Thus, the objectives of the present investigation were *1*) to assess GSH status in various tissues and splenocytes, and CD3-stimulated calcium mobilization and cell proliferation of splenocytes from mice fed 0.5% protein (LP group) or 15% protein (CP group) diets for 4 or 6 wk, and *2*) to determine the effects of dietary repletion (3 d, 1 wk, 2 wk and 3 wk) with the 15% protein control diet on GSH concentration, CD3-stimulated calcium mobilization and cell proliferation of splenocytes from mice with PEM.

MATERIALS AND METHODS

Animals and diet. Female C57BL/6 × DBA/2 mice (University of Washington colony), 3–4 mo old, were fed a 0.5% protein diet (LP) or a 15% protein diet (CP) for 4 or 6 wk. For the dietary repletion study, LP mice fed the 0.5% protein diet for 4 wk were then fed the 15% protein control diet for 3 d, 1 wk, 2 wk or 3 wk (RP groups). The diets (**Table 1**) were prepared by Harlan Teklad (Madison, WI). The protocol was reviewed and approved by the University of Washington Animal Care Committee.

Tissue collection and splenocyte preparation. Mice were weighed and then killed by cervical dislocation. Tissues used for GSH analysis were weighed and immediately frozen at −70°C. Spleens were removed by aseptic technique, and splenocytes (i.e., a suspension of mononuclear cells) were prepared by discontinuous gradient centrifugation using Lympholyte M (Cedarlane Laboratories, Hornby, ON). Briefly, spleens were mechanically dissociated into a single cell suspension in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT), layered over Lympholyte M and centrifuged at 400 × *g* for 30 min. Centrifugation over Lympholyte M eliminates polymorphonuclear cells, erythrocytes and dead cells. The band containing the mononuclear cells was washed, pelleted and resuspended in RPMI-1640-5% fetal bovine serum. This suspension of splenic mononuclear cells was defined as the splenocytes. Cells were counted in a hemocytometer. For GSH analysis, an aliquot of splenocytes (2×10^6 cells) was pelleted in a microfuge tube and frozen at −70°C.

Glutathione analysis. Tissue and splenocyte GSH concentrations were determined by the Tietze recycling method as modified for assay in microtiter plates (Baker et al. 1990).

Intracellular calcium mobilization. The intracellular calcium response to stimulation with anti-CD3 (5 mg/L) monoclonal antibody (145-2C11, PharMingen, San Diego, CA) was determined by flow cytometry in indo-1–loaded cells as previously described (Rabinovitch et al. 1986). Alterations in intracellular free calcium (Ca) can be readily quantified in single cells by flow cytometry using indo-1 (Rabinovitch et al. 1986). Indo-1 exhibits large changes in fluorescence emission wavelengths upon Ca binding, and the ratio of intensities at two wavelengths allows the calculation of the intracellular free Ca concentration independent of variability in intracellular dye concentration (Rabinovitch et al. 1986). Splenic mononuclear cells (10^{10}/L) were loaded with indo-1 acetoxymethyl ester (Molecular Probes, Eugene, OR) at a concentration of 3 mg/L for 45 min at 37°C (Grossman et al. 1991). Indo-1 acetoxy-methyl ester is permeable through the cell membrane and is hydrolyzed in the cytoplasm to the impermeant trapped form. Cells were washed and stained with phycoerythrin-conjugated anti- L3/T4 (YTS 191.1) and FITC-conjugated anti-Ly-2 (YTS 169.4) (Caltag, San Francisco, CA) to identify $CD4^+$ and $CD8^+$ cells, respectively, during flow cytometric analyses using an Epics Elite cytometer (Coulter Corporation, Hialeah, FL). After obtaining a baseline resting Ca concentration (Ca_{rest}), intracellular calcium mobilization was initiated by addition of mouse anti-CD3 monoclonal antibody (145-2C11, PharMingen). The maximal intracellular Ca concentration (Ca_{max}) was determined and used to calculate the CD3-stimulated intracellular calcium mobilization as the percentage of increase above baseline resting calcium ($100 \times [Ca_{max} - Ca_{rest}]/Ca_{rest}$). Data were analyzed using the MultiTime software program written by author PRS (Phoenix Flow Systems, San Diego, CA).

Cell proliferation. Round-bottom 96-well tissue culture plates were coated with 10 mg/L anti-CD3 mAb (PharMingen). Splenocytes were cultured in RPMI-1640-10% fetal bovine serum containing 5′-bromodeoxyuridine (BrdU) as previously described (Grossmann et al. 1991) and in the presence or absence of 50 μmol/L 2-mercaptoethanol (2-ME) (Sigma Chemical, St. Louis, MO) or 50,000 U/L IL-2 (R&D Systems, Minneapolis, MN). After 72 h, the cell nuclei were stained with Hoechst 33258 and ethidium bromide, and cell cycle analysis was performed by flow cytometry using an ICP-22 cytometer (Ortho

TABLE 1

Ingredient compositions of the 0.5% (LP) and 15% (CP) protein diets[1]

Ingredient	Diet LP	Diet CP
	g/kg diet	
Casein	5.9	176
DL-Methionine	0.1	3.0
Sucrose	425	338
Cornstarch	424	339
Corn oil	50	50
Cellulose	50	50
Mineral mix[2]	13.4	13.4
Calcium phopshate dibasic[3]	21.8	16.6
Calcium carbonate[3]	0.2	3.9
Vitamin mix[4]	10.0	10.0
Ethoxyquin (antioxidant)	0.01	0.01

[1] Diets were prepared by Harlan Teklad (Madison, WI).

[2] Teklad #TD79055 mineral mix (calcium phosphate deficient) contained the following (g/kg): sodium chloride 193.7, potassium citrate monohydrate 576.0, potassium sulfate 136.1, magnesium oxide 62.8, manganous carbonate 9.2, ferric citrate (16.7% Fe) 15.7, zinc carbonate 4.2, chromium potassium sulfate 1.44, and (mg/kg diet) cupric carbonate 785, potassium iodate 26.2 and sodium selenite 26.2.

[3] The formulas were adjusted to approximately 0.65% calcium and 0.5% phosphorus based on casein containing approximately 0.02% calcium and 0.7% phosphorus.

[4] Teklad #TD40060 vitamin mix contained the following (g/kg): *p*-aminobenzoic acid 11.0, ascorbic acid (coated, 97.5%) 101.7, Vitamin B-12 (0.1% trituration) 2.97, calcium pantothenate 6.61, choline dihydrogen citrate 349.7, inositol 11.0, menadione 4.96, niacin 9.91, pyridoxine HCl 2.20, riboflavin 2.20, thiamin HCl 2.20, dry retinyl palmitate (500,000 U/g) 3.96, dry all-*rac*-α-tocopheryl acetate (500 U/g) 24.2, cornstarch (diluent) 466.7, and (mg/kg) biotin 44.1, folic acid 198.2, dry cholecalciferol (500,000 U/g) 440.5.

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TABLE 2

Body weight and organ weights of mice fed 0.5% (LP) or 15% (CP) protein diets for 4 or 6 wk[1]

	Treatment group					Probability		
	LP (4 wk)	CP (4 wk)	LP (6 wk)	CP (6 wk)	Pooled SEM	Diet effect	Time effect	Diet × time
					g			
Body	16*	23	12*#	21	0.6	0.0001	0.0001	0.0032
Liver	0.67*	0.96	0.48*#	0.95	0.004	0.0001	0.0035	0.0060
Kidney	0.18*	0.25	0.12*#	0.21#	0.0002	0.0001	0.0001	NS
Lung	0.11	0.12	0.09*	0.13	0.0001	0.0009	NS	0.0024
Heart	0.09*	0.11	0.07*	0.11	0.0001	0.0001	NS	NS
Spleen	0.028*	0.058	0.019*#	0.062	0.002	0.0001	NS	0.0102

[1] Values are means, $n = 5$. * Significant differences ($P < 0.05$) between LP and CP means for the same time period. #Significant differences ($P < 0.05$) between 4 wk and 6 wk means of the same dietary treatment, as determined by the least significant difference method. NS = not significantly different ($P > 0.05$).

Diagnostic Systems, now Becton Dickensen, San Jose, CA) as previously described (Rabinovitch et al. 1988).

Statistical analysis. Plots of data sets exhibited normal distributions and did not exhibit evidence of a pattern of lack of homogeneity; thus data in the two studies were analyzed by two-way (diet and time, or diet and in vitro treatment) ANOVA and one-way (diet) ANOVA, respectively, by the general linear models procedure (SAS software release 6.04, SAS Institute, Cary, NC). The preplanned comparisons between treatment means were LP vs. CP at 4 wk, LP vs. CP at 6 wk, and LP at 4 wk vs. LP at 6 wk; differences between these treatment means were determined by the least significant difference test. The probability level at which the differences were considered significant was $P < 0.05$.

RESULTS

Effects of protein-energy malnutrition. The body weights and organ weights of young adult female mice fed the 0.5% protein diet (LP group) or 15% protein diet (CP group) are presented in **Table 2.** Before the dietary regimen was started, the initial body weight of the mice was 21 ± 1 g (mean ± SEM). The LP mice weighed 30% less at 4 wk and 43% less at 6 wk compared with their respective CP group. Liver, kidney, heart and spleen weights were significantly lower at 4 and 6 wk, and lung weight was significantly lower at 6 wk in LP mice than in CP mice. There was a disproportionaltely large reduction in spleen weight (52–69%) compared with other organ weights (8–49%). The lower spleen weight corresponded with but was exceeded by reduced (85%) splenocyte cell number in LP mice compared with CP mice at 4 and 6 wk (**Table 3**). Surface marker analysis by flow cytometry indicated that LP mice had a higher percentage of $CD4^+$ cells, but not $CD8^+$ cells, compared with CP mice. This resulted in LP mice having a significantly higher $CD4^+/CD8^+$ ratio compared with CP mice.

Glutathione was measured in various organs (**Table 4**) and in gradient-isolated splenocytes (**Fig. 1**). Glutathione concentrations were significantly lower in liver, lung, heart and spleen of the LP group compared with the CP group at both 4 and 6 wk (Table 4). Splenocyte GSH concentration in the LP group was significantly lower by 32% at 4 wk and 64% at 6 wk compared with the CP group (Fig. 1). The close agreement in the reduction of spleen (27%) and splenocyte (32%) GSH concentration in the LP group at 4 wk (Table 4 and Fig. 1) provides evidence that the splenocyte isolation procedure does not significantly alter GSH concentration.

CD3-stimulated intracellular calcium mobilization was measured in $CD4^+$ (T-helper) and $CD8^+$ (T-suppressor) splenocytes (**Table 5**). In the $CD4^+$ subset, CD3-stimulated calcium mobilization was not significantly different in LP and CP mice. In the $CD8^+$ subset, however, less CD3-stimulated intracellular calcium mobilization was observed in splenocytes from the LP group compared with the CP group at both 4 and 6 wk.

Proliferation assessed by the BrdU-Hoechst assay is shown in **Figure 2.** CD3 monoclonal antibody to the T cell receptor

TABLE 3

Spleen cell numbers and subpopulations of mice fed 0.5% (LP) or 15% (CP) protein diets for 4 or 6 wk[1]

	Treatment group					Probability		
	LP (4 wk)	CP (4 wk)	LP (6 wk)	CP (6 wk)	Pooled SEM	Diet effect	Time effect	Diet × time
No. of splenocytes, $\times 10^6$	12.3*	82.0	9.1*	57.0	7.3	0.0001	NS	NS
Percentage of $CD4^+$	10.0*	7.9	12.1*	7.3	0.8	0.0005	NS	NS
Percentage of $CD8^+$	4.8	5.6	5.1	4.8	0.4	NS	NS	NS
$CD4^+/CD8^+$	2.09*	1.41	2.40*#	1.56	0.08	0.001	0.0144	NS

[1] Values are means, $n = 5$. *Significant differences ($P < 0.05$) between LP and CP means for the same time period. #Significant differences ($P < 0.05$) between 4 wk and 6 wk means of the same dietary treatment, as determined by the least significant difference method. NS = not significantly different ($P > 0.05$).

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TABLE 4

Tissue glutathione (GSH) concentrations of mice fed 0.5% (LP) or 15% (CP) protein diets for 4 or 6 wk[1]

	Treatment group					Probability		
	LP (4 wk)	CP (4 wk)	LP (6 wk)	CP (6 wk)	Pooled SEM	Diet effect	Time effect	Diet × time
					μmol GSH/g wet tissue wt			
Liver	2.46*	9.27	2.63*	11.33	0.32	0.0001	0.0032	0.0097
Kidney	0.35	0.42	0.43	0.39	0.04	NS	NS	NS
Lung	2.34*	3.06	1.74*	2.73#	0.12	0.0001	0.0016	NS
Heart	1.27*	1.63	0.94*	1.68	0.09	0.0001	NS	0.0496
Spleen	3.77*	5.15	ND	ND	0.02	0.0001		

[1] Values are means, $n = 5$. *Significant differences ($P < 0.05$) between LP and CP means for the same time period. #Significant differences ($P < 0.05$) between 4 wk and 6 wk means of the same dietary treatment, as determined by the least significant difference method. NS = not significantly different ($P > 0.05$). ND = not determined.

was used to stimulate T cell proliferation of splenocytes cultured in media with and without supplementation of 2-ME or IL-2 for 72 h. In the absence of 2-ME, T-lymphocyte proliferation was significantly lower in the LP group than in the CP group at both 4 and 6 wk. Thiol supplementation of culture media with 2-ME increased T cell proliferation in splenocytes from both LP and CP groups. The percentage of increase in T cell proliferation in the presence of 2-ME was greater in splenocytes from the LP group (188–225%) than in splenocytes from the CP group (53–75%), and this resulted in a higher percentage of proliferating cells in the LP group than the CP group under this condition. Addition of IL-2 to the culture media did not alter the proliferation rates of splenocytes from LP and CP mice in the absence or presence of 2-ME. Similar trends were observed when cell proliferation was assessed by [^{3}H]thymidine incorporation (data not shown).

Effects of dietary repletion. Dietary repletion (RP) with the 15% protein control diet was investigated in LP mice previously fed the 0.5% protein diet for 4 wk. At all time points, RP groups had significantly greater body weight, spleen weight, spleen to body weight ratio and splenocyte number than LP mice (**Table 6**). After 3 d RP, there was an acute increase in body weight, followed by transitory weight loss at 1 wk RP. By 3 wk RP, there was a sustained increase in body weight that was not significantly different from that for the CP group. Similarly, there was a sudden increase in spleen weight after 3 d RP to a weight significantly greater than that for the CP group. After 1 wk RP, the spleen weight had decreased and was not significantly different from that of the CP group during the remainder of the study. The spleen:body weight ratio followed a similar pattern of change, being significantly greater at 3 d and 1 wk RP and not significantly different at 2 wk and 3 wk RP compared with the CP group. Splenocyte number was increased 1.6-fold after 3 d RP compared with LP mice. Splenocyte number then increased more gradually to 3.3-, 3.7- and 4.1-fold of the LP baseline at 1, 2 and 3 wk RP, respectively. However, at 3 wk RP, splenocyte number was still significantly lower than in the CP group (70% recovery).

Splenocyte GSH concentration (**Fig. 3**) and functional variables, including CD3-stimulated Ca mobilization (**Table 7**) and proliferation (**Fig. 4**), were determined during the RP experiment. Glutathione concentration was significantly lower in splenocytes from LP mice than in splenocytes from CP mice (Fig. 3). Dietary RP for 1 wk or longer significantly increased splenocyte GSH concentration to levels equivalent to that of the CP group. Similar results were obtained when splenocyte GSH concentration was expressed per cell number (Fig. 3) or per milligram of protein (data not shown). CD3-stimulated intracellular Ca mobilization in $CD4^+$ or $CD8^+$ T-lymphocytes was not significantly altered by LP or RP (Table 7). The CD3-stimulated proliferation response of the splenocytes was assessed in vitro using the BrdU-Hoechst proliferation assay. Thiol supplementation of culture media with 2-ME produced a significantly higher percentage of proliferating cells regardless of dietary treatment (Fig. 4). The proliferation response after 2-ME supplementation was greater in splenocytes from LP mice (183%) than splenocytes from CP mice (61%). After 3 d RP, the proliferation response in the presence of 2-ME was similar (83%) to that for the splenocytes from the CP group (61%).

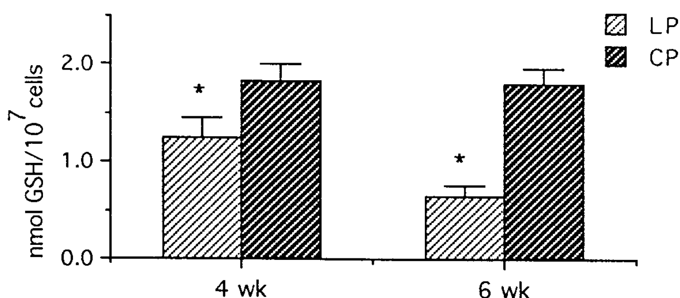

FIGURE 1 Glutathione (GSH) concentration of splenocytes from mice fed 0.5% (LP) or 15% (CP) protein diets for 4 or 6 wk. Values are means ± SEM, $n = 5$. An asterisk indicates a significant difference between LP and CP means for the same time period. The probability value for a diet effect was $P = 0.0001$; time and diet × time effects were not significant ($P > 0.05$).

DISCUSSION

The major finding of the present study was that splenocytes from LP mice were significantly reduced in number (Table 3), with a lower GSH concentration (Fig. 1) and lower CD3-stimulated proliferation in culture media without 2-ME (Fig. 2), compared with splenocytes from CP mice. Within 1 wk, dietary repletion with the 15% protein control diet increased splenocyte number (Table 6), restored splenocyte GSH concentration (Fig. 3) and decreased the thiol sensitivity of CD3-stimulated T-lymphocyte proliferation in vitro (Fig. 4).

The LP mice had reduced GSH status as indicated by lower GSH concentrations in liver, lung, heart, spleen and spleno-

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TABLE 5

CD3-stimulated intracellular calcium mobilization in $CD4^+$ or $CD8^+$ splenocytes from mice fed 0.5% (LP) or 15% (CP) protein diets for 4 or 6 wk[1,2]

	Treatment group					Probability		
	LP (4 wk)	CP (4 wk)	LP (6 wk)	CP (6 wk)	Pooled SEM	Diet effect	Time effect	Diet × time
					% increase			
$CD4^+$	45.3	61.7	58.4	59.1	6.9	NS	NS	NS
$CD8^+$	72.1*	96.2	67.4*	86.4	6.4	0.0039	NS	NS

1 Values are means, $n = 5$. *Significant differences ($P < 0.05$) between LP and CP means for the same time period. NS = not significantly different ($P > 0.05$).
2 CD3-stimulated intracellular calcium mobilization was calculated as the percentage of increase above baseline resting calcium ($100 \times [Ca_{max} - Ca_{rest}]/Ca_{rest}$).

cytes (Table 4). The 0.5% protein dietary regimen has been used previously as a model for tissue GSH depletion in rats (Taylor et al. 1992). The present study demonstrates that depletion of tissue GSH in this form of PEM includes depletion of GSH in mouse spleen and splenocytes (Table 4, Fig. 2 and 4). Glutathione concentration was assayed in the entire splenic mononuclear population using the Tietze recycling assay (Baker et al. 1990). Decreased T-lymphocyte GSH has been reported in HIV patients with wasting malnutrition; however, only the relative intracellular GSH content of T-lymphocytes, not absolute GSH concentration, was obtained using a flow cytometric assay (Roederer et al. 1993).

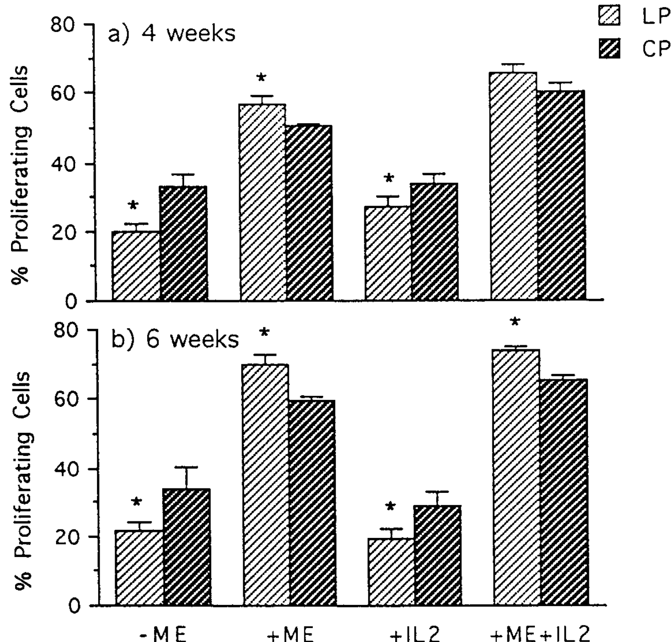

FIGURE 2 CD3-stimulated proliferation response of splenocytes from mice fed 0.5% (LP) or 15% (CP) protein diets for 4 wk (*a*) or 6 wk (*b*). Splenocytes were cultured with or without 2-mercaptoethanol (ME) and/or interleukin-2 (IL-2) for 72 h as described in Materials and Methods. Results are expressed as means ± SEM, $n = 5$. An asterisk indicates significant differences between LP and CP means for each in vitro treatment. Diet was not a significant ($P > 0.05$) main effect, but the probability value was $P = 0.0001$ for an in vitro treatment effect and $P = 0.0001$ for a diet × in vitro treatment effect.

T-lymphocyte proliferation in vitro and splenocyte cell number in vivo seem to be associated with GSH status. The LP mice had significantly lower splenocyte cell numbers (Tables 3 and 6), lower splenocyte GSH concentrations (Fig. 1 and 3) and lower CD3-stimulated T-lymphocyte proliferation in unsupplemented culture media (Fig. 2 and 4). Although the entire splenic mononuclear population was used in the proliferation assay, the CD3 monoclonal antibody would stimulate only T-lymphocytes. Decreased lymphocyte proliferation and/or increased apoptosis probably occurs in vivo in mice with PEM as indicated by the substantial reduction of spleen cell numbers in LP mice (Tables 3 and 6). Dietary protein repletion restored both splenocyte GSH concentration (Fig. 3) and receptor-mediated T-lymphocyte proliferation in unsupplemented culture media (Fig. 4) by 1 wk RP. Splenocyte number increased 1.6-fold after 3 d RP, but interestingly, the splenocyte number had recovered to only 70% of that for CP mice after 3 wk RP (Table 6). Significant reductions in nucleated spleen cells and primary acquired cell-mediated immunity (assessed by delayed hypersensitivity to sheep red blood cells) have been reported in a mouse model of preadolescent wasting PEM (Woodward et al. 1992). However, tissue or lymphocyte GSH status has not been previously reported in investigations of immunological function in animal models of PEM. The intracellular GSH concentration seems to be important for the proliferation of lymphocytes and other cell types in in vitro proliferation assays. When $CD4^+$ T-lymphocytes from normal human donors were sorted by flow cytometry on the basis of GSH content, the proliferative response was lower in $CD4^+$ T-lymphocytes containing lower levels of GSH (Kavanagh et al. 1990). Also, fibroblasts and endothelial cells have decreased intracellular GSH concentrations during quiescence compared with the proliferative growth phase (Mallery et al. 1993, Shaw and Chou 1986).

Thiol supplementation of culture media with 2-ME substantially increased the receptor-mediated proliferation response of T-lymphocytes regardless of dietary treatment (Fig. 2 and 4). However, T-lymphocytes from LP mice displayed a significantly greater enhancement of proliferation by in vitro thiol supplementation than did T-lymphocytes from RP or CP mice (Fig. 2 and 4). The greater proliferative response of splenocytes from LP mice than of splenocytes from CP mice in thiol-supplemented culture media (Fig. 2) may represent either greater intrinsic proliferative capacity of the supplemented LP cells, or alternatively may be due to the slightly greater proportion of $CD4^+$ cells in the spleens of LP mice (Table 3). It has been demonstrated previously that 2-ME facilitates the

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TABLE 6

Effect of dietary repletion (RP) on body weight, spleen weight and splenocyte number in mice previously fed the 0.5% protein diet (LP) for 4 wk compared with mice fed the 15% protein diet (CP) for 7 wk[1]

	Treatment group[2]							Probability
	LP (4 wk)	RP (3 d)	RP (1 wk)	RP (2 wk)	RP (3 wk)	CP (7 wk)	Pooled SEM	Diet effect
Body weight, *g*	15.4*	25.2	20.6*	22.8*	24.2	25.8	0.9	0.0001
Spleen weight, *mg*	30.3*	87.1*	68.0	62.5	60.6	65.2	4.3	0.0001
Spleen weight/body weight, *mg/g*	1.97*	3.45*	3.31*	2.75	2.50	2.54	0.17	0.0001
Splenocyte number, $\times 10^6$	11.0*	29.3*	36.1*	41.1*	45.3*	64.9	6.7	0.0004

[1] Values are means, $n = 5$. An asterisk indicates that a treatment group is significantly different ($P < 0.05$) from the CP control group, as determined by the least significant difference method.
[2] The LP group was fed the 0.5% protein diet for 4 wk. The RP groups were fed the 0.5% protein diet for 4 wk and repleted with the 15% protein diet for 3 d to 3 wk. The CP group was fed the 15% protein diet for 7 wk.

transport of cyst(e)ine from culture media into cells as the mixed disulfide of 2-ME and cysteine (Ishii et al. 1981). Addition of 2-ME or cysteine to culture media increases transport of cysteine into resting or activated lymphocytes and retards time-dependent decreases in intracellular GSH during in vitro culture (Ishii et al. 1987, Zmuda and Friedenson 1983). Coaddition of 2-ME (50 μmol/L) and cysteine (500 μmol/L) acts synergistically to significantly increase intracellular GSH concentration and the proliferative response of concanavalin A–stimulated mouse splenocytes compared with adding either 2-ME or cysteine alone (Zmuda and Friedenson 1983). In agreement with our results that T-lymphocytes from LP mice display greater in vitro sensitivity to 2-ME during proliferation (Fig. 2 and 4), Noelle and Lawrence (1981) reported that 2-ME exhibits the greatest enhancement of concanavalin A–stimulated cell proliferation on lymphocytes with relatively lower concentrations of intracellular GSH.

Thiol supplementation of culture media may increase the proliferative response of lymphocytes by additional mechanisms. Many of the genes involved in cell proliferation are regulated by transcription factors, such as NFkB, that are sensitive to thiol redox status for DNA binding activity (Droge et al. 1991). It has also been reported that the synthesis and turnover of surface IL-2 receptors may be sensitive to thiol redox status (Liang et al. 1992). In the present study, however, the CD3-stimulated proliferation response of T-lymphocytes from LP or CP mice was not altered by addition of IL-2 to culture media in the presence or absence of 2-ME (Fig. 2). Thus, it seems that the amount of IL-2, the number of IL-2 receptors and binding of IL-2 to IL-2 receptors were not limiting factors for in vitro proliferation of T-lymphocytes from LP or CP mice in this study. The enhanced proliferation response of T-lymphocytes from LP mice in the presence of 2-ME suggests that accessory cells from LP mice were not a limiting factor for the proliferation response measured in this assay.

The early steps of the T-lymphocyte signal transduction pathway can be assessed by mobilization of calcium, an important second messenger. In this study, CD3-mediated Ca mobilization in $CD4^+$ T-lymphocytes, but not $CD8^+$ T-lymphocytes, was maintained in LP mice (Table 5). In other studies, depletion of lymphocyte GSH in vitro by conjugation with 1-chloro-2,4-dinitrobenzene or by alkylation of thiol groups with the permeant sulfhydryl-reactive nonpolar maleimide *N*-ethylmaleimide inhibited receptor-induced mobilization of intracellular calcium and tyrosine phosphorylation of several proteins, including phospholipase Cγ1 and an associated 35-kDa protein (Kanner et al. 1992, Kavanagh et al. 1993). In

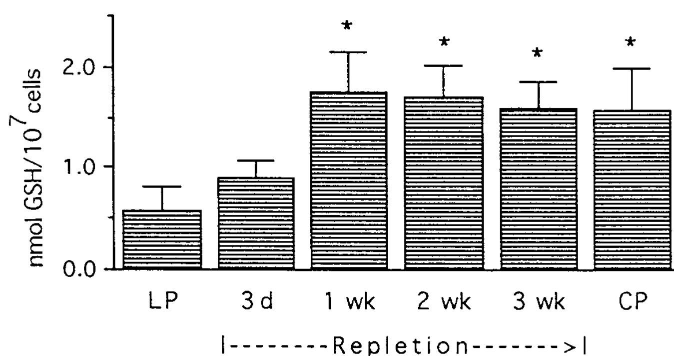

FIGURE 3 Effects of dietary repletion (15% protein diet for 3 d to 3 wk) on glutathione (GSH) concentrations in splenocytes from mice previously fed the 0.5% protein diet (LP) for 4 wk compared with mice fed the 15% protein diet (CP) for 7 wk. Values are means ± SEM, $n = 5$. An asterisk indicates significant differences between means for the LP group and other dietary treatments. Diet was a significant main effect ($P < 0.05$).

TABLE 7

Effect of dietary repletion (RP) on intracellular calcium mobilization in mice previously fed the 0.5% protein diet (LP) for 4 wk compared with mice fed the 15% protein diet (CP) for 7 wk[1,2]

	Treatment group[3]						
	LP (4 wk)	RP (3 d)	RP (1 wk)	RP (2 wk)	RP (3 wk)	CP (7 wk)	Pooled SEM
	% increase						
$CD4^+$	51.0	49.8	52.7	52.3	53.1	57.4	2.2
$CD8^+$	49.4	45.8	54.5	54.0	56.1	58.7	3.0

[1] Values are means, $n = 5$. There was no diet effect ($P > 0.05$).
[2] CD3-stimulated intracellular calcium mobilization was calculated as % increase above baseline resting calcium ($100 \times [Ca_{max} - Ca_{rest}]/Ca_{rest}$).
[3] The LP group was fed the 0.5% protein diet for 4 wk. The RP groups were fed the 0.5% protein diet for 4 wk and repleted with the 15% protein diet for 3 d to 3 wk. The CP group was fed the 15% protein diet for 7 wk.

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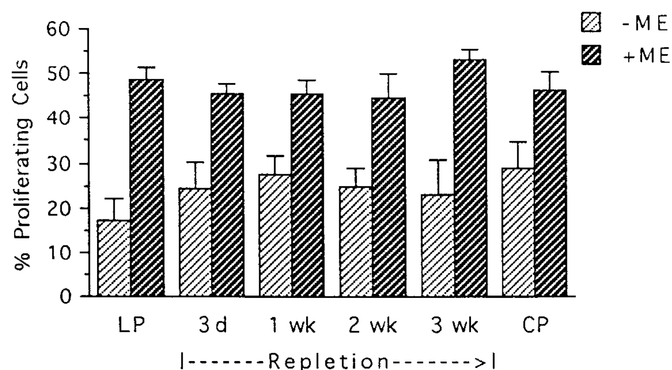

FIGURE 4 Effects of dietary repletion (15% protein diet for 3 d to 3 wk) on CD3-stimulated proliferation response of splenocytes from mice previously fed 0.5% protein diet (LP) for 4 wk compared with mice fed 15% protein diet (CP) for 7 wk. Splenocytes were cultured with or without 2-mercaptoethanol (ME) for 72 h as described in Materials and Methods. Results are expressed as means ± SEM, $n = 5$. The ME treatment was a significant main effect ($P < 0.05$), whereas diet and diet × ME treatment were not significant ($P > 0.05$) main effects.

the setting of weight loss (wasting malnutrition), decreased spleen cell numbers and decreased splenocyte GSH (especially after 6 wk of consumption of the LP diet), it is perhaps surprising that lymphocyte proliferation was not reduced to a greater extent than observed and that Ca mobilization in $CD4^+$ T-lymphocytes was unaffected. We hypothesize that the in vivo survival of lymphocytes in PEM may be determined in part by selection of lymphocytes that maintain the functional capacity to respond to receptor stimulation, mobilize Ca and proliferate. We speculate that this selection process may be an adaptive response to minimize loss of immune cell function during wasting malnutrition and might be mediated by apoptotic death of those cells least capable of maintaining cellular redox status (Sato et al. 1995).

ACKNOWLEDGMENTS

The authors acknowledge the expert technical assistance of S. C. Kim, A. Dayton, M. Shen, K. Roellich and P. Otto.

LITERATURE CITED

Baker, M. A., Cerniglia, G. J. & Zaman, A. (1990) Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Anal. Biochem. 190: 360–365.
Bray, T. M. & Taylor, C. G. (1994) Enhancement of tissue glutathione for antioxidant and immune functions in malnutrition. Biochem. Pharmacol. 47: 2113–2123.
Buhl, R., Holroyd, K. J., Mastrangeli, A. & Cantin, A. M. (1989) Systemic glutathione deficiency in symptom-free HIV-seropositive individuals. Lancet 2: 1294–1298.
Deneke, S. M., Lynch, B. A. & Fanburg, B. L. (1985) Effects of low protein diets or feed restriction on rat lung glutathione and oxygen toxicity. J. Nutr. 115: 726–732.
Droge, W., Eck, H. P., Gmunder, H. & Mihm, S. (1991) Modulation of lymphocyte functions and immune responses by cysteine and cysteine derivatives. Am. J. Med. 91: 140S–144S.
Eck, H. P., Gmunder, H., Hartman, M., Petzoldt, D., Daniel, V. & Droge, W. (1989) Low concentrations of acid-soluble thiol (cysteine) in the blood plasma of HIV-1 infected patients. Biol. Chem. Hoppe-Seyler 370: 101–108.
Filteau, S. M. & Woodward, B. D. (1987) Influence of severe protein deficiency and of severe food intake restriction on serum levels of thyroid hormones in the weanling mouse. Nutr. Res. 7: 101–107.
Golden, M.H.N. & Ramdath, D. (1987) Free radicals in the pathogenesis of kwashiorkor. Proc. Nutr. Soc. 46: 53–68.
Grossman, A., Maggio-Price, L., Jinneman, J. C. & Rabinovitch, P. S. (1991) Influence of aging on intracellular free calcium and proliferation of mouse T-cell subsets from various lymphoid organs. Cell. Immunol. 135: 118–131.
Ishii, T., Bannai, S. & Sugita, Y. (1981) Mechanism of growth stimulation of L1210 cells by 2-mercaptoethanol in vitro. J. Biol. Chem. 256: 12387–12392.
Ishii, T., Sugita, Y. & Bannai, S. (1987) Regulation of glutathione levels in mouse spleen lymphocytes by transport of cysteine. J. Cell. Physiol. 133: 330–336.
Jackson, A. A. (1986) Blood glutathione in severe malnutrition in childhood. Trans. R. Soc. Trop. Med. Hyg. 80: 911–913.
Jung, D. (1985) Disposition of acetaminophen in protein-calorie malnutrition. J. Pharm. Exp. Ther. 232: 178–182.
Kanner, S. B., Kavanagh, T. J., Grossmann, A., Hu, S.-L., Bolen, J. B., Rabinovitch, P. S. & Ledbetter, J. A. (1992) Sulfhydryl oxidation down-regulates T-cell signalling and inhibits tyrosine phosphorylation of phospholipase $C\gamma1$. Proc. Natl. Acad. Sci. U.S.A. 89: 300–304.
Kavanagh, T. J., Grossmann, A., Jaecks, E. P., Jinneman, J. C., Eaton, D. L., Martin, G. M. & Rabinovitch, P. S. (1990) Proliferative capacity of human peripheral blood lymphocytes sorted on the basis of glutathione content. J. Cell. Physiol. 145: 472–480.
Kavanagh, T. J., Grossmann, A., Jinneman, J. C., Kanner, S. B., White, C. C., Eaton, D. L., Ledbetter, J. A. & Rabinovitch, P. S. (1993) The effect of 1-chloro-2,4-dinitrobenzene exposure on antigen (CD3)-stimulated transmembrane signal transduction in purified subsets of human peripheral blood lymphocytes. Toxicol. Appl. Physiol. 119: 91–99.
Liang, S.-M., Lee, N., Chen, Y. Y. & Liang, C.-M. (1992) Effects of glutathione on the synthesis and turnover of interleukin-2 receptors. Cell. Immunol. 144: 131–142.
Mallery, S. R., Lantry, L. E., Laufman, H. B., Stephens, R. E. & Brierley, G. P. (1993) Modulation of human microvascular endothelial cell bioentergetic status and glutathione levels during proliferative and differentiated growth. J. Cell. Biochem. 53: 360–372.
Noelle, R. J. & Lawrence, D. A. (1981) Determination of glutathione in lymphocytes and possible association of redox state and proliferative capacity of lymphocytes. Biochem. J. 198: 571–579.
Rabinovitch, P. S., June, C. H., Grossman, A. & Ledbetter, J. A. (1986) Heterogeneity among T cells in intracellular free calcium responses after mitogen stimulation with PHA or anti-CD3: simultaneous use of indo-1 and immunofluorescence with flow cytometry. J. Immunol. 137: 952–961.
Rabinovitch, P. S., Kubbies, M., Chen, Y. C., Schindler, D. & Hoehn, H. (1988) BrdU-Hoechst flow cytometry: a unique tool for quantitative cell cycle analysis. Exp. Cell Res. 174: 309–318.
Roederer, M., Staal, F.J.T., Anderson, M., Rabin, R., Raju, P. A., Herzenberg, L. A. & Herzenberg, L. A. (1993) Disregulation of leukocyte glutathione in AIDS. Ann. N. Y. Acad. Sci. 677: 113–125.
Sato, N., Iwata, S., Nakamura, K., Hori, T., Mori, K., & Yodoi, J. (1995) Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. J. Immunol. 154: 3194–3203.
Shaw, J. P. & Chou, I.-N. (1986) Elevation of intracellular glutathione content associated with mitogenic stimulation of quiescent fibroblasts. J. Cell. Physiol. 129: 193–198.
Sive, A. A., Subotzky, E. F., Malna, H., Dempster, W. S. & Heese, H.D.V. (1993) Red blood cell antioxidant enzyme concentrations in kwashiorkor and marasmus. Ann. Trop. Paediatr. 13: 33–38.
Staal, F.J.T., Roederer, M., Herzenberg, L. A. & Herzenberg, L. A. (1992) Glutathione and immunophenotypes of T and B lymphocytes in HIV-infected individuals. Ann. N.Y. Acad. Sci. 651: 453–463.
Taylor, C. G., Bauman, P. F., Sikorski, B. & Bray, T. M. (1992) Elevation of lung glutathione by oral supplementation of L-2-oxothiazolidine-4-carboxylate protects against oxygen toxicity in protein-energy malnourished rats. FASEB J. 6: 3101–3107.
Taylor, C. G., Nagy, L. E. & Bray, T. M. (1996) Nutritional and hormonal regulation of glutathione homeostasis. Curr. Top. Cell. Regul. 34: 189–208.
Woodward, B. D. & Miller, R. G. (1991) Depression of thymus-dependent immunity in wasting protein-energy malnutrition does not depend on an altered ratio of helper ($CD4^+$) to suppressor ($CD8^+$) T cells or on a disportionately large atrophy of the T-cell relative to the B-cell pool. Am. J. Clin. Nutr. 53: 1329–1335.
Woodward, B. D., Woods, J. W. & Crouch, D. A. (1992) Direct evidence that primary acquired cell-mediated immunity is less resistant than is primary thymus-dependent humoral immunity to the depressive influence of wasting protein-energy malnutrition in weanling mice. Am. J. Clin. Nutr. 55: 1180–1185.
Zmuda, J. & Friedenson, B. (1983) Changes in intracellular glutathione levels in stimulated and unstimulated lymphocytes in the presence of 2-mercaptoethanol or cysteine. J. Immunol. 130: 362–364.

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