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Melphalan-induced toxicity in nude mice following pretreatment with buthionine sulfoximine*

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Summary. Melphalan-induced toxicity in nude mice following pretreatment with a regimen of L-buthionine sulfoximine (BSO), previously shown to enhance the activity of this alkylating agent against rhabdomyosarcoma and glioma xenografts, was examined. Mice were pretreated with i.p. BSO (2.5 mmol/kg ×7 doses at 12-h intervals plus concomitant availability of a 20-mM solution in the drinking water) or vehicle prior to a single i.p. injection of melphalan (35.65 mg/m^2). As compared with control animals who received no BSO pretreatment, mice pretreated with BSO lost weight prior to therapy with melphalan (6.9% weight loss vs 0.3% weight gain; $P < 0.005$) and showed a greater mean nadir weight loss after melphalan (3.8% vs 2.1%; $P = 0.049$). Treatment with melphalan was associated with histologic evidence of reversible gastrointestinal toxicity, reversible myelosuppression, and histologic evidence of acute renal tubular necrosis, with no differences being observed between mice that had been pretreated with BSO and those that had been pretreated with vehicle. No evidence of cardiac, hepatic, or skeletal muscle toxicity was found in melphalan-treated animals. These results suggest that treatment of nude mice with melphalan following BSO-mediated depletion of glutathione does not result in enhanced organ toxicity despite an increase in the antineoplastic activity of this alkylating agent.

Introduction

The observation that the folate antagonist aminopterin induces only transient remissions in children with acute leukemia has demonstrated the clinical consequences of drug resistance [3]. Model using murine leukemia L1210 cells have provided the first opportunity for laboratory generation and quantitation of resistance to chemotherapeutic agents and have enabled mechanistic analysis (and modulation) of drug resistance [2, 20, 24]. As the principal nonprotein intracellular thiol, glutathione protects cells from a variety of exogenous electrophiles, including bifunctional alkylating agents. Studies in murine and human cells have demonstrated that resistance to damage by alkylators may be mediated by an increase in glutathione levels [6, 9, 11, 17, 23]. This observation has led to successful efforts to restore drug sensitivity by depletion of glutathione [6, 11, 17, 23]. Although some agents function exclusively to reverse resistance (e.g., verapamil in pleiotropic drug resistance), glutathione depletion has been shown to increase the activity of alkylating agents against both alkylator-resistant and alkylator-sensitive ovarian cancer cells [9].

Buthionine sulfoximine (BSO) is a selective inhibitor of glutamylcysteine synthetase, the enzyme catalyzing the first step in glutathione biosynthesis [7, 8]. Studies in human ovarian and rhabdomyosarcoma cell lines have demonstrated enhanced alkylator cytotoxicity, particularly for melphalan, when this alkylator was given following BSO-mediated depletion [5, 6, 11, 17, 19, 23]. Although these studies clearly encourage clinical exploitation of BSO-mediated glutathione depletion in tumor chemotherapy, they also raise the concern that systemic depletion of glutathione prior to administration of an alkylating agent could increase systemic toxicity as well as antineoplastic activity.

Preliminary in vivo studies in rodents indicate that the systemic toxicity of melphalan may [10] or may not [18] be increased by pretreatment with BSO. The diverse metabolism and tissue-specific toxicities of bifunctional alkylating agents, coupled with the heterogeneous depletion of

Abbreviations: BSO, L-buthionine-(*SR*)-sulfoximine; CBC, complete blood count; ALC, absolute lymphocyte count; ANC, absolute neutrophil count; ALT, alanine aminotransferase; CPK, creatine phosphokinase; WBC, total white blood cell count

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organ glutathione levels following administration of BSO [15], suggest that toxicity studies should be conducted using BSO/alkylator combinations demonstrating increased (as compared with alkylator alone) antineoplastic activity.

The present studies were designed to determine whether a BSO/melphalan regimen that increases antineoplastic activity against s.c. rhabdomyosarcoma and intracranial glioma xenografts also results in an increase in toxicity. No evidence of increased hematologic, hepatic, gastrointestinal, renal, cardiac, or skeletal muscle toxicity was seen following therapy with BSO plus melphalan versus melphalan alone. Mice that had been pretreated with BSO showed a slight increase in weight loss following therapy with melphalan as compared with animals that received melphalan alone.

Materials and methods

Mice. Male BALB/c mice (nu/nu genotype, 19–28 g; Simonson Labs, Gilroy, Calif.) were housed in groups of five animals per sterile cage under positive-flow Bioclean Units (Lab Products, Maywood, N. J.) and were fed autoclaved food and sterile water. The experiments reported herein were conducted according to the principles set forth in National Institutes of Health (NIH) publication 85–23, "Guide for the Care and Use of Laboratory Animals," and in the Animal Welfare Act of 1966, as amended.

Drugs. L-Buthionine-(*SR*)-sulfoximine (BSO; mol. wt., 222.3 kDa), synthesized as previously described [7], was dissolved in 0.9% NaCl (22.2 mg/ml) and given by i.p. injection at a dose of 2.5 mmol/kg; animals received seven doses of BSO or vehicle at 12-h intervals. Over this 84-h period, animals receiving BSO injections also had access to drinking water containing 20 mM BSO. Melphalan (mol. wt., 305 kDa; Burroughs Wellcome Co., Research Triangle Park, N. C.) was given as a single i.p. dose in a volume of 90 ml/m^2. Animals received 35.65 mg/m^2 in 16.7% dimethylsulfoxide (DMSO), which is 50% of the calculated LD_{10} (dose lethal to 10% of the group) determined as previously described [4].

Measurement of glutathione levels. The glutathione concentrations in the liver, kidney, skeletal muscle (thigh), small intestine, and heart from 2 groups of 12 mice each that had been pretreated with either BSO or saline were measured as previously described [19].

Toxicity analysis. A total of 165 animals were divided into 3 groups: 75 each were placed into 2 experimental groups and were pretreated with BSO or 0.9% saline for 84 h prior to treatment with melphalan; 15 control (untreated) mice used to obtain baseline values received 0.9% saline as pretreatment and were given the melphalan vehicle (16.7% DMSO). Time points for measurement of peripheral blood counts, blood chemistries, and bone marrow cellularity (18, 48, 96, 168, and 240 h) were selected to define the tissue-specific clinical toxicities of the alkylating agents and reflected both acute and subacute toxicity. Gut mucosa was examined at 48 and 168 h and renal tissue at 18, 168, and 240 h. Animals were weighed every 1–2 days and all deaths were noted.

At the specified intervals after treatment, groups of 14–15 mice each were exsanguinated by indirect cardiac puncture after being anesthetized with metaphane. Control animals were killed by injection of 16.7% DMSO at 18 h after treatment. Heparinized blood was used for determinations of hematologic parameters; serum was frozen for later measurement of other toxicity parameters. Mice with internal organs exposed were fixed in 10% phosphate-buffered formalin for subsequent histologic examination of the gastrointestinal tract and kidneys.

Complete blood counts (CBC) were determined using a Baker System 9000 instrument (Baker Instrument Corporation, Pennsylvania). A manual differential blood count was performed to obtain the absolute lymphocyte count (ALC) and the absolute neutrophil count (ANC). Bone marrow, used to assess marrow cellularity, was aspirated from a long bone from five animals per group at each time point. Serum alanine aminotransferase (ALT), creatine phosphokinase (CPK), and creatinine levels were determined by spectrophotometric assay using the DMA kit (Data Medical Associates, Inc., Arlington, Tex.) and a Gilford System 103 spectrophotometer (Gilford Instruments Laboratory, Oberlin, Ohio). Results were expressed as the mean and standard error obtained at each time point.

Gastrointestinal (GI) and renal tissue sections were prepared routinely using the paraffin-embedding technique and were stained with hematoxylin and eosin (H&E). Toxic effects seen in the GI tract were scored by quantitating the total number of focal lesions (necrotic enterocytes) found in the ileal mucosal crypts from three fields of view (magnification, ×400) for each animal; the GI toxicity index represented the mean number of focal lesions per animal for each group at each time point. Sections of renal tissue obtained at 18, 168, and 240 h were reviewed by a single pathologist (D. P. M.) and graded for histologic features (necrosis, karyorrhexis, hyperchromicity) consistent with toxicity. Bone marrow sections from samples obtained at 18, 48, 96, 168, and 240 h were evaluated for cellularity.

Statistical analysis. Student's unpaired *t*-test was used for comparisons of the mean (± SD) weight change, marrow cellularity, and GI toxicity index. Two-way analysis of variance (ANOVA) with appropriate post hoc tests was applied for comparisons of the hematocrit, platelet, and white blood cell (WBC) counts and of determinations of the ANC, ALC, ALT, CPK, and creatinine values obtained between the two treatment groups over time. Fisher's exact test was used to compare categorical data.

Results

Glutathione measurements

The glutathione concentrations in organs taken from mice that had been pretreated with BSO or saline are shown in Table 1. Pretreatment with BSO resulted in depletion of glutathione content to the following percentages of the control values: heart, 13.9%; liver, 92.4%; kidney, 11.5%; skeletal muscle, 7.2%; small intestine, 30.8%.

Table 1. Glutathione concentration[a] in mouse organs following treatment with BSO or saline

Regimen	Heart	Liver	Kidney	Skeletal muscle	Small intestine
Saline	0.57±0.06	6.2 ±1.45	2.8 ±0.38	0.46±0.02	2.2 ±0.32
BSO[b]	0.08±0.01	5.73±1.48	0.33±0.05	0.03±0.01	0.68±0.16

[a] Values represent means ± SD, expressed in μmol/g wet weight

[b] L-buthionine-*SR*-sulfoximine was dissolved in 0.9% saline and given by i.p. injection at a dose of 2.5 mmol/kg; animals received 7 doses at 12-h intervals. Concomitently, 20 mM BSO was available in the drinking water

Table 2. Weight change and GI toxicity of melphalan vs BSO/melphalan

Treatment regimen	Percentage of weight change[a]		GI toxicity index[b]
	Pretreatment	Posttreatment	
Melphalan	+0.3	−2.1	15.1 ± 13.1
BSO/melphalan	−6.9 (P <0.005)[c]	−3.8 (p = 0.049)	12.6 ± 10.3[d]

[a] Pretreatment weight change represents the mean difference between initial weight and weight at the end of the pretreatment period for 75 animals in each treatment group. Posttreatment weight change represents the mean nadir weight loss after treatment with melphalan for the 45 animals in each group that were killed at least 48 h after treatment

[b] GI toxicity index represents the mean (± SD) number of necrotic foci (enterocytes) in the ileal mucosa crypts counted in 3 fields (×400) from animals killed at 48 h after melphalan treatment (15 animals/group). In control animals there was no morphologic evidence of toxicity

[c] Statistical significance was assessed by an unpaired t-test comparing the two treatment groups

[d] No significant difference between groups

Systemic toxicity

We noted 4 acute deaths that were attributable to the injection procedure: 1 of 75 mice died after receiving BSO injections and 3 of 90 succumbed after receiving vehicle injections (P = 0.38). No deaths occurred in either group following treatment with melphalan. During the pretreatment phase, animals receiving BSO showed a mean weight loss of 6.9% as opposed to the mean weight gain of 0.3% displayed by the animals receiving saline (p <0.005; Table 2). After treatment with melphalan, animals that had been pretreated with BSO displayed a greater mean weight loss than did those that had undergone saline pretreatment (3.8% vs 2.1%; P = 0.049). All animals were consistently gaining weight at 5 days after melphalan treatment.

GI toxicity

Sections of the GI tract were examined at 48 and 168 h after melphalan treatment. As seen by light microscopy, lesions were limited to the small and large intestines of animals that were killed at 48 h after the administration of melphalan. No pathologic changes were seen in the stomach; the cecum was not examined. Lesions were focal, necrotic, and limited to the enterocytes of the crypt epithelium. The characteristic pathologic change comprised pyknosis and/or karyorrhexis of single or multiple cells. These necrotic cells were demarcated by a clear peripheral zone and a more eosinophilic cytoplasm. This segregation facilitated the quantitative analysis and differentiation of necrotic cells from those in mitosis (Fig. 1). We found no difference in the GI toxicity index at 48 h after melphalan treatment between animals that had been pretreated with BSO and those that had been given saline (Table 2). No morphologic evidence of toxicity could be detected in control animals or in mice that were examined at 168 h after the administration of melphalan in either treatment group.

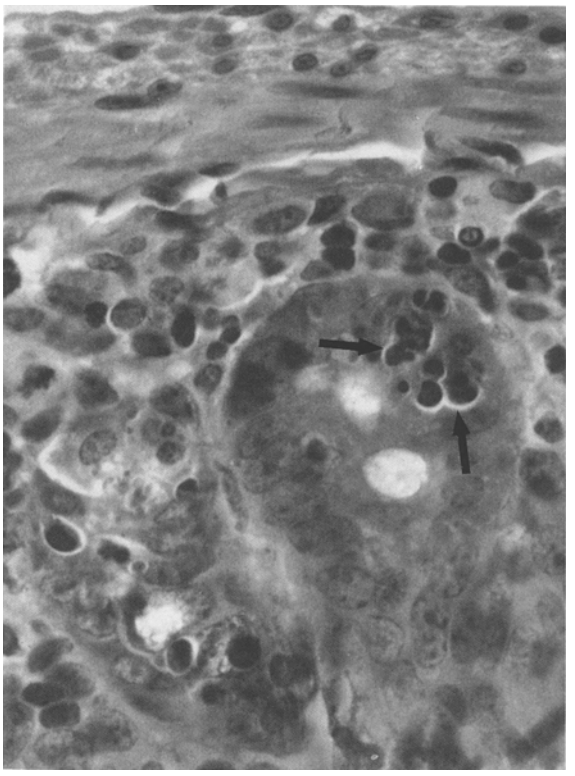

Fig. 1. Photomicrograph of a section of GI tract displaying typical necrotic enterocytes. H&E, ×360

Hematologic toxicity

The changes in hematocrit and platelet count observed in the two treatment groups are displayed in Fig. 2. The mean nadir hematocrit was 28.3% and 29.2% for animals treated with melphalan and those given BSO/melphalan, respectively; the platelet count showed a significant increase at 240 h after melphalan treatment ($P \leq 0.05$; ANOVA with the Tukey post hoc test). There was no difference between treatment groups for hematocrit or platelet count at any time point.

The total WBC, ANC, and ALC values obtained for each treatment group are displayed in Fig. 3. Animals that had been pretreated with BSO appeared to show a more rapid depletion of neutrophils and a greater depletion of lymphocytes at 48 and 96 h after treatment, but these differences were not statistically significant. Although there was no apparent rebound in the peripheral WBC at 10 days after the administration of melphalan, examination of bone marrow aspirates revealed increased cellularity at 10 vs 2 days after melphalan treatment (86.1% ± 11.7% vs 45.5% ± 29.2%; P = 0.002), with no difference occurring between treatment groups.

Hepatic and skeletal/cardiac muscle toxicity

The mean ALT value obtained for untreated animals was 87.7 ± 31.4 IU/l; the mean CPK value found for control animals was 1,195.5 IU/l. There were no significant

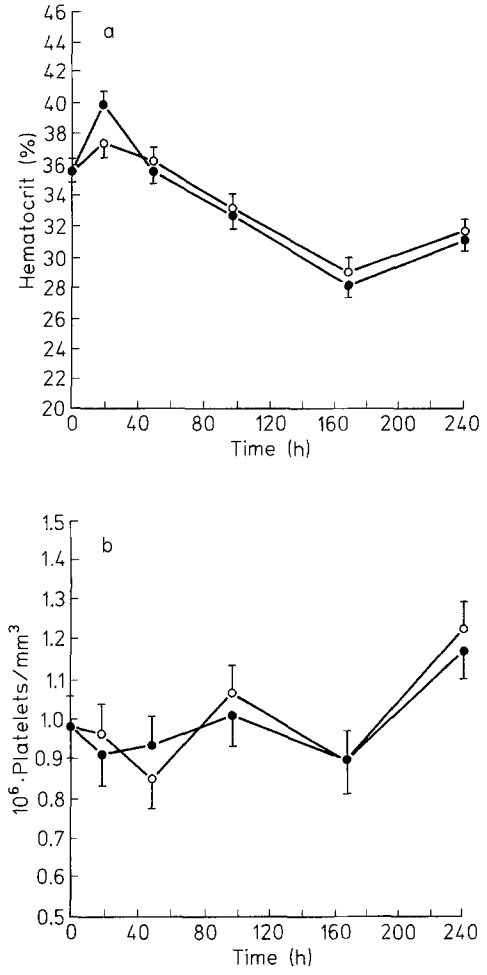

Fig. 2. The hematocrit (*upper panel*) and peripheral platelet count (*lower panel*) in animals treated with melphalan (●) and BSO/melphalan (○). Animals received melphalan at $t = 0$. Values represent the mean ± SE of 11–15 animals

changes at any time points in animals treated with BSO/melphalan or melphalan alone (Table 3).

Renal toxicity

Light microscopic examination of kidney sections did not demonstrate morphologic evidence of renal toxicity at 18 h after melphalan administration in either treatment group. At later time points, morphologic evidence of a variable degree of acute tubular necrosis was seen in kidney sections from 12 of 15 and 8 of 15 mice at 168 h after treatment with melphalan and BSO/melphalan, respectively ($P > 0.05$) and in 10 of 15 and 8 of 15 animals at 240 h after treatment with melphalan and BSO/melphalan, respectively ($P > 0.05$). The lesions were characterized by nuclear and cytoplasmic hyperchromicity, nuclear atypia, and shrinkage and flattening of affected cells. Occasional foci demonstrated necrotic cells with karyorrhexis and some histiocytic and fibroblastic interstitial response. These lesions were focal and widely scattered and predominantly involved the ascending loop of Henle and collecting tubules in the lower third of the cortex (Fig. 4).

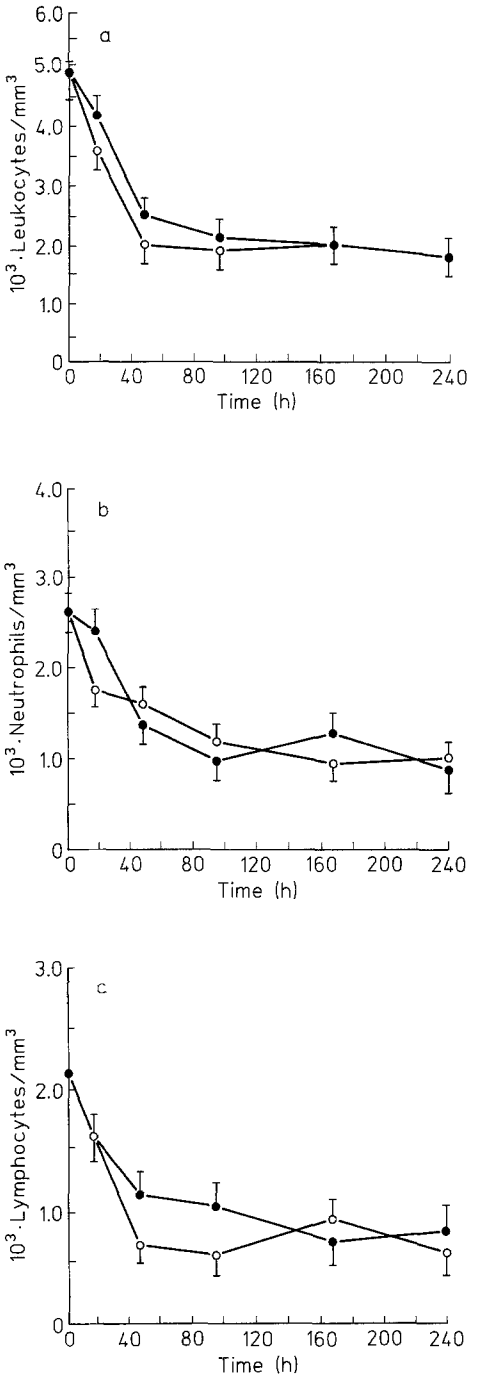

Fig. 3. The peripheral total white blood cell count (*upper panel*), absolute neutrophil count (*middle panel*), and absolute lymphocyte count (*lower panel*) in animals treated with melphalan (●) and BSO/melphalan (○). Animals received melphalan at $t = 0$. Values represent the mean ± SE of 11–15 animals

At all time points following therapy with melphalan, the creatinine concentrations found in animals that had been pretreated with BSO were similar to those determined in mice that had received saline. Creatinine concentrations in control animals (0.26 mg/dl at 18 h after treatment with saline/16.7% DMSO) were not statistically different from those in animals treated with BSO/melphalan or melphalan alone (Table 3).

Table 3. Serum ALT, CPK, and creatinine levels in mice treated with melphalan or BSO plus melphalan

Regimen	Parameter	Time points				
		18 h	48 h	96 h	168 h	240 h
Control	ALT[a]	87.7[c]				
	CPK[a]	1,195.5				
	C[b]	0.26				
Melphalan	ALT	155.5	99.5	150.3	81.6	186.1
	CPK	2,117.7	1,544.5	1,688.5	559.3	1,188.8
	C	0.3	0.19	0.18	0.42	0.39
BSO + melphalan	ALT	142.8	52	120.6	151.9	137.4
	CPK	1,830.5	877.5	1,611.7	1,066	1,425.5
	C	0.23	0.23	0.21	0.28	0.4

C, Creatinine
[a] Values are expressed in IU/l
[b] Values are expressed in mg/dl
[c] All values shown represent the mean of 8 samples

Discussion

Although the therapeutic benefits of BSO-mediated glutathione depletion prior to treatment with melphalan have been demonstrated in vivo using murine neoplasms and models of human ovarian cancer, rhabdomyosarcoma, and glioma [5, 10, 13, 16], few studies have explored the host toxicity of this combination [11, 17, 18, 22]. Our data demonstrate that a BSO regimen shown to enhance the activity of melphalan against human rhabdomyosarcoma and glioma xenografts [5, 19] did not result in increased melphalan toxicity. BSO-mediated glutathione depletion increases weight loss but does not increase the acute GI, hematologic, hepatic, cardiac, or skeletal muscle toxicity of melphalan.

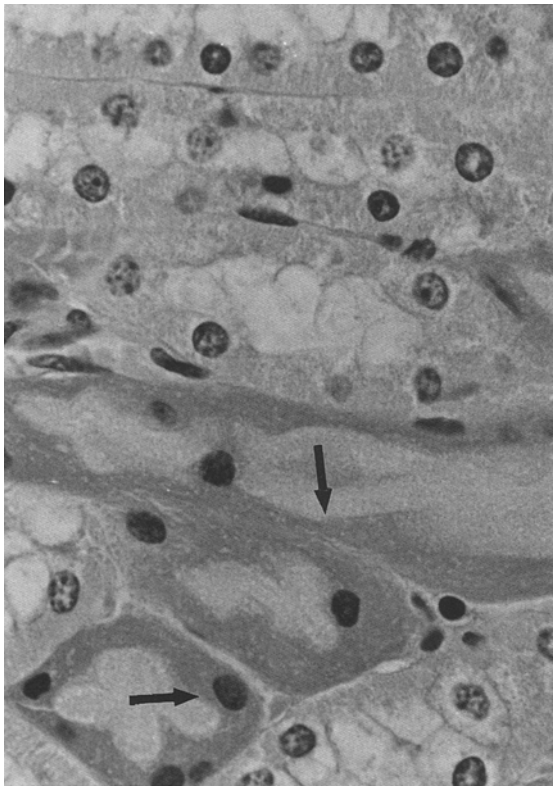

Fig. 4. Photomicrograph of renal tissue sections, demonstrating typical pathologic changes in an animal treated with BSO/melphalan. *Arrows* depict hyperchromic, degenerate tubules with epithelial cells that reveal atypical nuclei. H&E, × 720

Animals pretreated with BSO lost significantly more weight than did controls (Table 2). Soble and Dorr [22] reported a similar degree of weight loss (6%–7%) in DBA/2J mice that received a slightly larger dose of BSO (5 g/kg over 5 days). Martensson and Meister [13] used a regimen very similar to ours for 21 days and found no weight gain in Swiss-Webster mice. Ozols et al. [17] have suggested that this BSO-induced weight loss is a function of decreased oral intake in animals given the BSO solution. However, Martensson et al. [14] have described severe degeneration of the epithelial cells of the jejunum and colon in Swiss-Webster mice that were treated with BSO for 7–14 days.

BSO-mediated glutathione depletion in the intestinal mucosa may further predispose the gut to melphalan-induced cellular injury. In preclinical therapeutic trials of BSO, a significant increase in weight loss following treatment with melphalan has not been reported [5, 11, 17–19, 22]. However, the use of tumor-bearing animals in those studies complicates the interpretation of weight loss as a marker of GI toxicity. In our study using non-tumor-bearing animals, there was a small but significant increase in mean nadir weight loss: 3.8% vs 2.1% in animals treated with BSO and melphalan vs those given melphalan alone ($P = 0.049$). This was not associated with significant histopathologic differences as measured by a GI toxicity index (Table 2) and sections of GI mucosa from animals that were killed at 168 h after melphalan administration were normal in both treatment groups. Thus, despite the statistically significant differences in weight loss, no histologic evidence of differential GI toxicity was apparent.

The effects of glutathione depletion on hematopoietic stem cells remain unresolved. Ono and Shrieve [16] demonstrated that BSO (5 mmol/kg ×4 doses) depleted bone marrow glutathione to 31% of control values. Kramer et al. [11] reported that a single dose of BSO (450 mg/kg) did not significantly deplete the bone marrow glutathione content but did delay the recovery of the peripheral WBC following treatment with melphalan at doses of 2.5–

13 mg/kg. Several investigators have found that BSO pretreatment in vitro and in vivo does not alter melphalan-induced marrow toxicity as measured by the splenic colony-forming unit (CFU) assay [17, 18, 22]. Carmichael et al. [1] have reported different baseline and post-cyclophosphamide glutathione concentrations in erythrocytic, granulocytic, and lymphocytic cells in mice. The effects of BSO on different hematopoietic stem-cell fractions have not been reported. Using cell counts from peripheral blood as markers of marrow toxicity, we observed a similar magnitude of depletion and a similar rate of rebound in the hematocrit, total WBC, ANC, ALC, and platelet counts in animals that received melphalan plus BSO or melphalan alone and demonstrated that our BSO regimen does not enhance melphalan-induced toxicity to erythrocytic, granulocytic, lymphoid, or megakaryocytic stem cells (Figs. 2, 3).

The glutathione content of the liver is high relative to that of other murine tissue and is rapidly depleted following the administration of BSO [8]. Soble and Dorr [22] have demonstrated that BSO enhances melphalan-induced hepatotoxicity as measured by an elevated serum aspartate aminotransferase level; however, the dose of melphalan used was not specified and statistical analysis was not presented. Martensson and Meister [13] found that prolonged administration of BSO in a regimen similar to that used in the present study resulted in mitochondrial damage in skeletal muscle, but these investigators did not report any corresponding change in CPK activity. We used serum ALT and CPK activity as markers of toxicity and could detect no increase in the acute hepatic, cardiac, or skeletal muscle toxicity of melphalan that was attributable to pretreatment with BSO.

The effects of BSO on melphalan-induced renal damage have recently been demonstrated [12], with renal tubular necrosis being seen in 80% of male mice and 20% of female mice receiving BSO (1,600 mg/kg every 4 h for 6 doses) prior to melphalan treatment (15 mg/m^2). However, although pretreatment with lower doses of BSO produced similar degrees of glutathione depletion, it was not associated with enhanced renal toxicity secondary to melphalan. We observed variable degrees of acute tubular necrosis in animals that were treated with melphalan, with no evidence for enhanced toxicity being seen in animals that had been pretreated with BSO.

Our results demonstrate that a regimen of BSO previously shown to enhance the antineoplastic activity of melphalan at both the subcutaneous and intracranial sites of athymic mice [5, 19] does not enhance murine toxicity. These studies, in conjunction with prior reports in the literature [14, 21], show that BSO-mediated enhancement of melphalan toxicity may be dependent on the delivered dose of BSO and emphasize the importance of determining the smallest BSO dose required for maximal glutathione depletion in the target tumor. Although similar information defining dose-response relationships and glutathione levels in different organs in humans would enhance the rational use of BSO/alkylator therapy, the practical problems associated with such studies preclude the generation of such data. Nevertheless, selection of BSO regimens that enhance alkylator therapy without increasing toxicity may be feasible, particularly by the evaluation of appropriate preclinical models.

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