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Role of Glutathione Conjugation in 1-Bromobutane-induced Immunotoxicity in Mice

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Halogenated organic compounds, such as 1-bromobutane (1-BB), have been used as cleaning agents, agents for chemical syntheses or extraction solvents in workplace. In the present study, immunotoxic effects of 1-BB and its conjugation with glutathione (GSH) were investigated in female BALB/c mice. Animals were treated orally with 1-BB at 375, 750 and 1500 mg/kg in corn oil once for dose response or treated orally with 1-BB at 1500 mg/kg for 6, 12, 24 and 48 hr for time course. *S*-Butyl GSH was identified in spleen by liquid chromatography-electrospray ionization tandem mass spectrometry. Splenic GSH levels were significantly reduced by single treatment with 1-BB. *S*-Butyl GSH conjugates were detected in spleen from 6 hr after treatment. Oral 1-BB significantly suppressed the antibody response to a T-dependent antigen and the production of splenic intracellular interlukin-2 in response to Con A. Our present results suggest that 1-BB could cause immunotoxicity as well as reduction of splenic GSH content, due to the formation of GSH conjugates in mice. The present results would be useful to understand molecular toxic mechanism of low molecular weight haloalkanes and to develop biological markers for exposure to haloalkanes.

***Key words*:** 1-Bromobutane, Glutathione, Conjugation, Immunotoxicity, *In vivo*

INTRODUCTION

Due to lower ozone-depleting potency and non-flammability, short chain-halogenated alkanes have been used industrially as chemical intermediates, extraction solvents and co-polymer cross-linking agents (Låg *et al*., 1991). 1-Bromobutane (1-BB) is a colorless liquid that is insoluble in water, but soluble in ethanol. As a primary alkyl halide, it is especially prone to S_N2 type reactions. It is commonly used as an alkylating agent, or in combination with magnesium metal in dry ether to form carbon-carbon bonds.

Due to their structural characteristics, many haloalkanes were known to undergo glutathione (GSH) conjugation (Jones and Walsh, 1979; B'Hymer and Cheever, 2004). For examples, 1- and 2-bromopropane (1- and 2-BP) could be conjugated with GSH (Khan and O'Brien, 1991; Lee *et al*., 2007). Likewise, 1-BB has been known to be conjugated with GSH (James *et al*., 1968). Although GSH plays an important role in detoxifying many electrophilic compounds by conjugate formation or by reducing various oxidizing agents, several compounds are activated following conjugation with GSH (Chasseaud, 1976; Prohaska, 1980; Ozawa and Guengerich, 1983; Van Bladeren, 1983). Moreover, depletion of GSH by treatment with GSH-depleting agents, such as 1-BP and 1,3-dibromopropane could cause hepatotoxicity (Lee *et al*., 2005; 2007).

Of equal importance, GSH depletion by glutathione *S*-transferase substrates could result in lipid peroxidation associated cytotoxicity which could be prevented or delayed by antioxidants or ferric chelators (Anundi *et al*., 1979; Younes and Sieger, 1981; Miccadel *et al*., 1988; Silva and O'brien, 1989). These reports indicated that GSH depletion could promote endogenous oxidative stress, and that GSH might play an antioxidant role (Anundi *et al*., 1979; Younes and Sieger, 1981; Miccadel *et al*., 1988). Moreover, the presence of GSH might be required to maintain normal function of immune system (Elferink and de Koster, 1991; Dröge *et al*., 1994; Wu *et al*., 1994). From our recent study,

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depletion of GSH by treatment with 1-BP induced immunotoxicity in female BALB/c mice (Lee *et al*., 2007b). In addition, our serial studies have implied that formation of GSH conjugates with several bromoalkanes might play an important role in their toxicity (Lee *et al*., 2005, 2007). Most recently, 1-BB could be hepatotoxic in mice due to the depletion of hepatic GSH via conjugate formation of 1-BB with GSH (unpublished data). In the study, it was also observed that spleen and thymus weights were significantly reduced by 1-BB, which might be signs of immunotoxic potential of 1-BB. Nevertheless, neither immunotoxic potential of 1-BB nor formation of its GSH conjugates in immune cells has been investigated until to date.

The aims of present study were to evaluate dose-response and time-course effects of 1-BB on the immune functions, to determine effects of 1-BB on splenic GSH levels in association with formation of GSH conjugates by using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS), and to evaluate a relation between immunotoxicity and depletion of GSH induced by 1-BB *in vivo*.

MATERIALS AND METHODS

Materials. 1-BB (> 99%) was obtained from Acros Organics (Geel, Belgium). Alserver's solution, DEAE-dextran, agar, bovine serum albumin, 5,5'-dithio-bis(2-nitrobenzoic acid) and 5-sulfosalicylic acid were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum (FBS), Earle's balanced salt solution (EBSS), penicillin G-streptomycin solution, L-glutamine and RPMI 1640 media were purchased from GIBCO (Grand Island, NY, USA). BD Cytofix/Cytoperm Plus™ with GolgiPlug™ kit was obtained from BD Biosciences (San Diego, CA, USA). All antibodies used for flow cytometry were purchased from Pharmingen (San Diego, CA, USA). Sheep red blood cells (SRBCs) were obtained from Colorado Serum Company (Denver, CO, USA). Guinea pig complement was purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA). All other chemicals used were of reagent grade commercially available and used as received.

Animals. Specific pathogen-free female BALB/c mice (19 to 21 g) were obtained from the Orient (Seoul, Korea). Animals received at 4 weeks of age were acclimated for at least 2 weeks. Upon arrival, animals were randomized and housed five per cage. Animal quarters were strictly maintained at $23 \pm 3^{o}C$ and $50 \pm 10\%$ relative humidity. A 12-hr light and dark cycle was used with an intensity of 150-300 Lux. All animal procedures were approved by Institutional Animal Care and Use Committee of Yeungnam University College of Pharmacy based on the guiding principles in the use of animals in toxicology from the Society of Toxicology (USA).

Animal treatment. For dose-response study, 1-BB in corn oil was treated orally to female BALB/c mice at 375, 750 and 1500 mg/kg once. For time-course study, 1-BB at 1500 mg/kg was treated orally once for 6, 12, 24 or 48 hr. Control animals received 10 m*l*/kg of corn oil. Following the blood collection, spleens were removed and homogenized with 4 volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4 for assaying GSH and *S*-butyl GSH, a conjugate of 1-BB. Aliquots of tissue homogenates and sera were stored at $-80^{o}C$ until use.

Antibody response to SRBCs. Thirty min after 1-BB treatment, animals were sensitized intraperitoneally with 5×10^{9} SRBCs/0.5 m*l* EBSS. Four days later, spleens were removed for quantitation of antibody-forming cells (AFCs). Single cell suspensions were prepared in 3 m*l* of EBSS, washed and resuspended in 3 m*l* of EBSS. Spleen cells were then diluted 30-fold by resuspending a 100 μ*l* aliquot of each suspension in 2.9 m*l* of EBSS. The number of AFCs was determined using a modified hemolytic Jerne plaque assay (Jeong *et al*., 1995). Briefly, 0.05% DEAE-dextran was added into melted 0.5% agar in EBSS and maintained at $48^{o}C$ throughout the assay. Then 350 μ*l* of melted agar was dispensed into 12 × 75 mm heated glass tubes (Corning), followed by the addition of 25 μ*l* of an indicator SRBCs, 100 μ*l* of spleen cell suspension and 25 μ*l* of guinea pig complement. SRBCs were washed at least three times with EBSS before use. After thoroughly mixing, a 200 μ*l* aliquot from the tube was then immediately pipetted and the agar solution was covered with a 24 × 40 mm microscope cover slip. The Petri dishes were placed at room temperature for several min to allow the agar to solidify and then, incubated for 3 hr to form hemolytic plaques in a humidified $37^{o}C$ incubator. The cell number of each original sample was determined using a Coulter Counter (Beckman Coulter, Inc.). The plaques that developed were counted using a Stereomaster® plaque viewer (Fisher Scientific, China). The results were expressed as AFCs/10^{6} spleen cells and AFCs/spleen.

Quantitation of splenic and thymic lymphocyte subpopulations. Using the Becton Dickinson FACSCalibur, lymphocyte subpopulations in spleen and thymus were quantitated. Following single oral treatment with 1-BB for 24 hr, splenic and thymic cells were collected and suspended in a staining buffer containing 2% FBS and 0.1% sodium azide in phosphate-buffered saline (PBS), pH 7.4, to a cell density of 1.0×10^{6}/tube. Splenic cells were incubated with anti-mouse CD16/CD32 Fc receptor (1 μg/tube, clone 2.4G2) for 15 min at $4^{o}C$ to prevent nonspecific binding and then labeled with an appropriate monoclonal antibody (mAb) conjugated to a fluorescent probe. Anti-mouse CD3e mAb (clone, 145-2C11) conjugated to peridinin chlorophyll-a protein was used to enumerate T-cells. For T-cell subsets in spleen and thymus, fluorescein isothiocyanate

(FITC)-conjugated anti-mouse CD4 (clone, GK1.5) and phycoerythrin (PE)-conjugated anti-mouse CD8a (clone, 53-6.7) were used.

B-cells and macrophages in splenocytes were stained with anti-mouse CD45R/B220 mAb (clone, RA3-6B2) conjugated to PE and FITC-conjugated anti-mouse CD11b (clone, M1/70), respectively. An isotype control was used for each antibody. Cell suspensions were incubated with appropriate antibodies at 1 μg/tube for at least 30 min on ice in the dark. After incubation, the 1 × FACS Lysing solution (Becton Dickinson, San Jose, CA, USA) was treated for 10 min for hemolysis. The cells were washed twice, resuspended in PBS containing 2% FBS and 0.1% sodium azide in total volume of 0.5 m*l*, and analyzed using the FACSCalibur® flow cytometry with CellQuest® software (Becton Dickinson, San Jose, CA, USA).

Assay of intracellular IL-2 production. To simplify the fixation and permeabilization of cells for immunofluorescent staining of intracytoplasmic IL-2, the BD Cytofix/Cytoperm Plus™ with GolgiPlug™ kit was used. For *in vitro* activation, single cell suspensions (1×10^6 splenocytes/m*l*) from mice treated with 1-BB for 24 hr were cultured with 1 μg/m*l* of Con A in the presence of recombinant IL-2 (rIL-2, Roche Applied Science, Mannheim, Germany) at 37°C in 5% CO_2 for 12 hr. After incubation, the splenocytes were stimulated again with the rIL-2 and Con A for 5 hr. Brefeldin A (10 μg/10^6 cells) was added in this stage. Then the cells were washed with a staining buffer, and blocked the nonspecific binding through adding anti-mouse CD16/CD32 Fc receptor (1 μg/10^6 cells/tube) for 20 min on ice. The $CD4^+$ cells were identified by using FITC-conjugated anti-mouse CD4 antibody (0.5 μg/tube; clone, GK1.5), which was suspended in the staining buffer and incubated for 30 min on ice in a dark condition. To fix and permeabilize cells, Cytofix/Cytoperm solution (100 μ*l*/tube) was added and incubated on ice for 20 min in dark. And then 1 × Perm/Wash solution (1 m*l*/tube) was added for washing.

To stain intracellular cytokine, the fixed/permeabilized cells were incubated with 100 μ*l* of Perm/Wash solution containing PE-conjugated anti-mouse IL-2 antibody (0.2 μg/tube; clone, JES6-5H4) on ice for 30 min in dark. After washing with 1 × Perm/Wash solution twice, the cells were resuspended in staining buffer (300 μ*l*/tube) and performed flow cytometric analysis.

Splenic content of reduced GSH. Splenic GSH levels were determined by a previous method (Ellman, 1959), with the normalization through determination of splenic proteins according to the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Sample preparation for LC/ESI-MS. To identify *S*-butyl GSH, spleen homogenates were extracted by addition of 2 volumes of acetonitrile. After vortex mixing for 10 min and centrifugation at 15,000 ×g at 15°C for 10 min to remove proteins, the resulting supernatant was evaporated under a stream of nitrogen in a water bath maintained at 60°C. The resulting residue was reconstituted in a mixture of 50/50 of 0.1% aqueous formic acid/methanol by vortexing and a subsequent centrifugation at 15,000 ×g at 15°C for 10 min. Then a 10 μ*l* aliquot was used for LC/MS analysis.

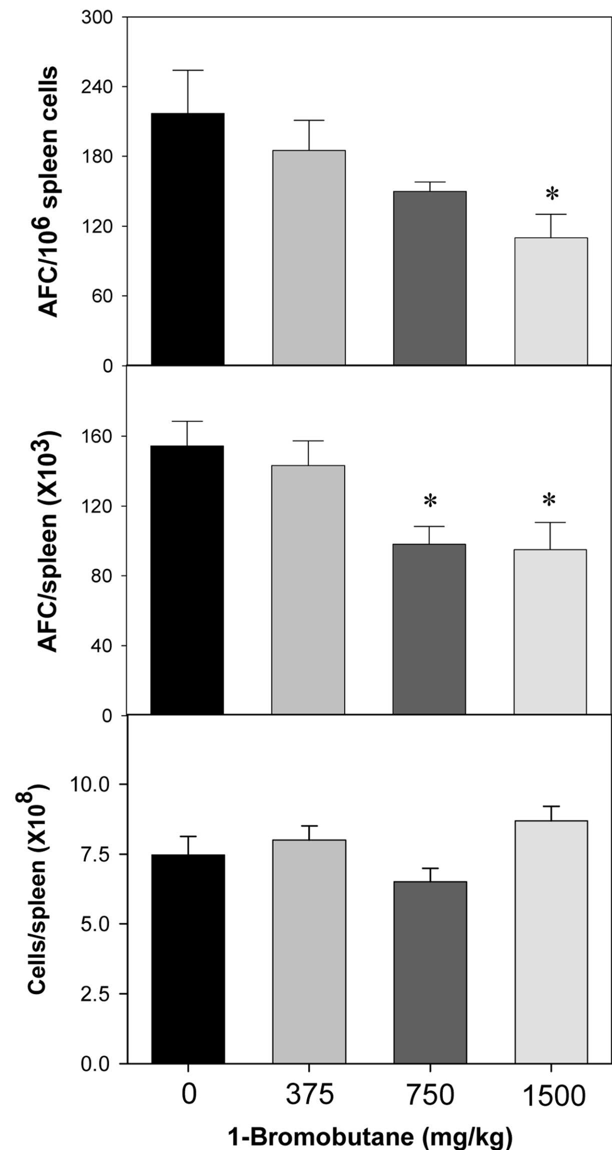

Fig. 1. Effects of 1-bromobutane on T-dependent antibody response in female BALB/c mice. Mice were treated orally with 1-bromobutane in corn oil at 10 ml/kg once. Thirty min later, mice were immunized with SRBCs intraperitoneally. Four days later, the number of antibody-forming cells (AFCs) was enumerated. Each bar represents mean ± S.E. of five animals. Asterisks indicate the value significantly different from vehicle control at $P < 0.05$ (*).

LC/ESI-MS. The HPLC consisted of a surveyor system (Thermo Finnigan, San Jose, CA, USA) with the LCQ advantage trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source. The column used was the Atlantis dC18 (3.0 × 100 mm, 3 μm) for analysis of 1-BB conjugates. The HPLC mobile phases consisted of 100% acetonitrile in 0.1% formic acid (A) and 0.1% aqueous formic acid, pH 4.0 (B). For GSH conjugates of 1-BB, a gradient program was used for HPLC separation with a flow rate of 300 μ*l*/min. The linear gradient was as follows: 0~5 min, 5% A; 5~15 min, 5% to 70% A. For MS analysis, nitrogen was used both as sheath gas at 40.0 *l*/min and as auxiliary gas at 10.0 *l*/min with a capillary temperature of 300°C and the spray voltage set to 4.5 kV. The mass spectrometer was operated in the positive ion mode in *m/z* range 100~400. Helium was used as collision gas for MS experiments, followed by the isolation of ions over a selected mass window of 1 Da.

Statistics. The mean value ± standard error (S.E.) was determined for each treatment group of a given experiment. Dunnett's t-test was used to compare statistical significance of data (SPSS 12.0, SPSS Institute, USA). The significant values at either $P < 0.05$ (*) or $P < 0.01$ (**) were represented as asterisks.

RESULTS

Immunotoxic potential of 1-BB in vivo. Due to the limited information on immunotoxic potential of 1-BB, effects of 1-BB on immunotoxic parameters were initially studied. To evaluate effects of 1-BB on T-dependent antibody response, 1-BB-treated mice were sensitized intraperitoneally with SRBCs and the number of AFCs was enumerated 4 days later (Fig. 1). Single treatment with 1-BB significantly suppressed the antibody response to SRBC in a dose-dependent manner. 1-BB could suppress antibody response at 1500 mg/kg without any changes in numbers of splenocytes. To determine cellular target of 1-BB-induced immunosuppression, mice were administered with the same doses of 1-BB without immunization, followed by splenic and thy-

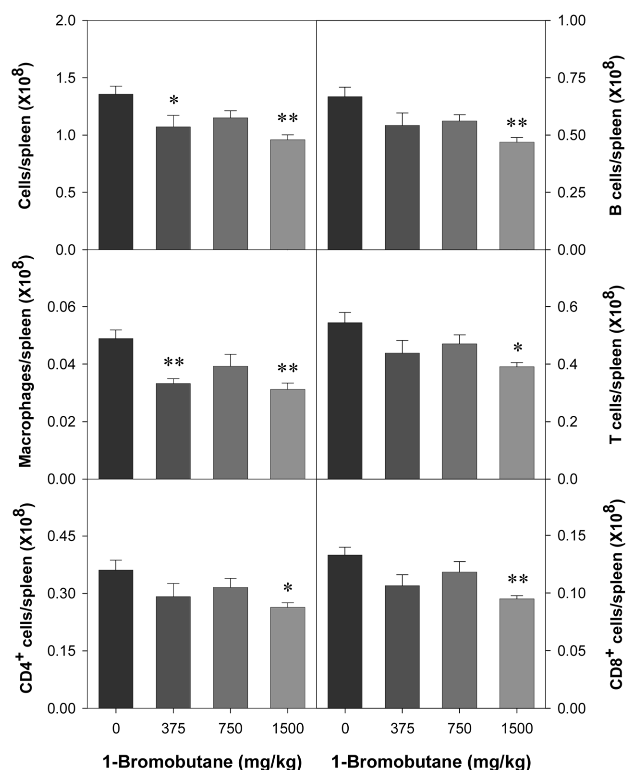

Fig. 2. Effects of 1-bromobutane without an immunization on splenic lymphocyte subpopulations in female BALB/c mice. Mice were treated orally with 1-bromobutane in corn oil at 10 ml/kg once. All animals were subjected to necropsy 24 hr after the treatment. Each bar represents mean number of cells ± S.E. of five animals. Asterisks indicate the value significantly different from vehicle control at either $P < 0.05$ (*) or $P < 0.01$ (**).

Table 1. Effects of 1-bromobutane without an immunization on percentage of splenocyte and thymocyte phenotypes

		1-Bromobutane (mg/kg)			
		VH	375	750	1500
Spleen (%)	T cells	40.06 ± 0.64	40.91 ± 1.03	40.88 ± 1.28	40.79 ± 0.63
	$CD4^+$ cells	26.51 ± 0.52	26.33 ± 0.90	27.03 ± 0.70	26.58 ± 0.49
	$CD8^+$ cells	9.82 ± 0.13	9.60 ± 0.31	10.47 ± 0.52	9.69 ± 0.71
	B cells	49.17 ± 0.76	49.67 ± 0.71	47.90 ± 0.49	45.36 ± 1.88
	Macrophages	3.64 ± 0.25	3.22 ± 0.31	2.29 ± 0.17**	1.68 ± 0.20**
	CD4/CD8	2.70 ± 0.08	2.72 ± 0.08	2.69 ± 0.11	2.78 ± 0.08
Thymus (%)	$CD4^-CD8^+$	2.78 ± 0.38	3.30 ± 0.34	3.87 ± 0.33	3.23 ± 0.47
	$CD4^+CD8^+$	83.71 ± 0.81	79.81 ± 1.26	77.46 ± 1.18	79.50 ± 1.13
	$CD4^-CD8^-$	1.98 ± 0.22	3.41 ± 0.38*	3.62 ± 0.44**	2.70 ± 0.16
	$CD4^+CD8^-$	11.53 ± 0.30	13.47 ± 0.84	15.05 ± 0.62**	14.57 ± 0.71*

Each value represents mean ± S.E. of five animals. Asterisks indicate the values significantly different from vehicle control (VH) at either $P < 0.05$ (*) or $P < 0.01$ (**).

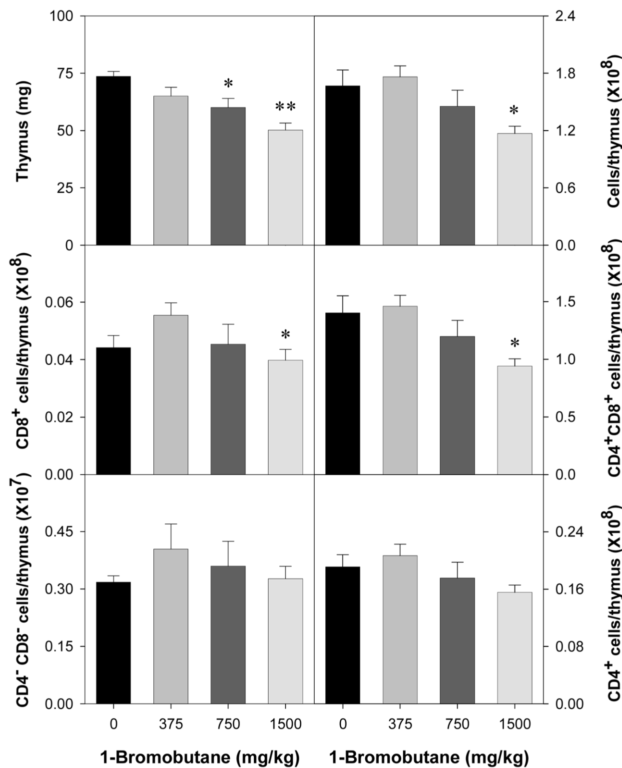

Fig. 3. Effects of 1-bromobutane without an immunization on thymocyte subpopulations in female BALB/c mice. Mice were treated orally with 1-bromobutane in corn oil at 10 ml/kg once. All animals were subjected to necropsy 24 hr after the treatment. Each bar represents mean number of cells ± S.E. of five animals. Asterisks indicate the value significantly different from vehicle control at either $P < 0.05$ (*) or $P < 0.01$ (**).

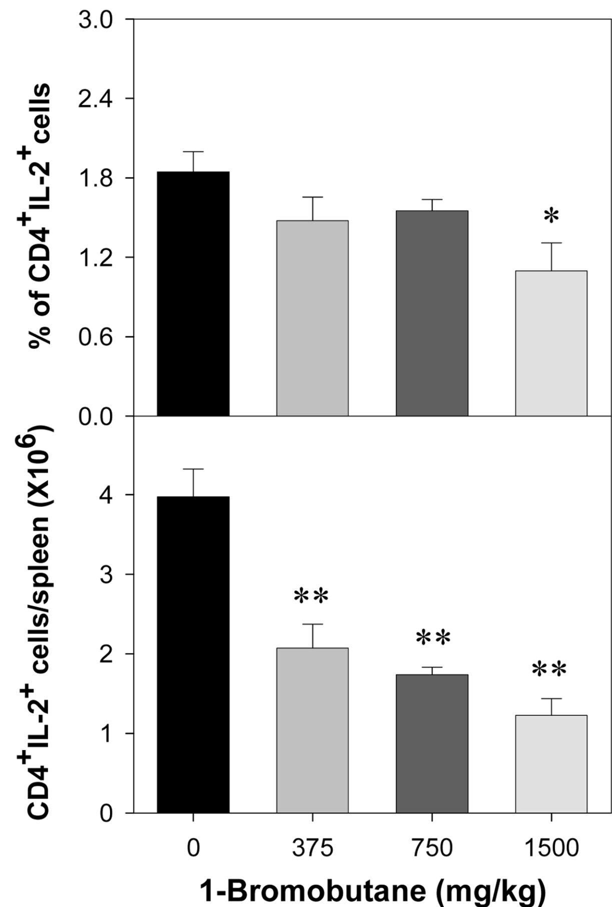

Fig. 4. Effects of 1-bromobutane on splenic intracellular IL-2 production in female BALB/c mice. Splenocytes isolated from 1-bromobutane-treated mice for 24 hr were cultured for 12 hr in the presence of concanavalin A. Each value represents mean ± S.E. of five animals. Asterisks indicate the value significantly different from vehicle control at either $P < 0.05$ (*) or $P < 0.01$ (**).

mic lymphocyte phenotypings and determination of IL-2 producing cells by using flow cytometry.

Mice exposed to 1-BB showed a significant decrease in percentage of macrophage cells among total splenocytes (Table 1). The percentages of $CD4^-CD8^-$ and $CD4^+CD8^-$ cells to total thymocytes were increased by 1-BB. Meanwhile, the changes in absolute numbers of splenocytes and thymocytes were somewhat different. Mice treated with 1-BB significantly decreased in the numbers of all subpopulations in spleen, and immature $CD4^+CD8^+$ cells and $CD4^-CD8^+$ cells in thymus (Figs. 2 and 3). The reduction of absolute cell numbers was associated with decreases in splenic and thymic cellularity.

Subsequently, mice were treated orally with 375, 750 and 1500 mg/kg of 1-BB to determine immunotoxic mechanism of 1-BB. Twenty four hr later, spleen cells were aseptically isolated and incubated in the presence of Con A, a T-cell mitogen. Then, the T-cells producing IL-2 were determined flow cytometrically. The production of splenic intracellular IL-2 in response to Con A (1 μg/m*l*) was decreased dose-dependently in 1-BB-treated mice (Fig. 4). The number of $CD4^+IL\text{-}2^+$ cells was reduced approximately 48, 59 and 66%, following exposure of mice to 375, 750 and 1500 mg/kg of 1-BB, respectively.

Taken together, single 1-BB would have immunotoxic potential via suppression of IL-2 production.

Splenic GSH levels and formation of GSH conjugates with 1-BB. Quantitative analyses of GSH conjugates have been possible from the selective scan functions available on MS/MS system (Baillie and Davis, 1993). MS/MS may also be employed as a highly sensitive and selective tool for the detection of unknown GSH conjugates in complex samples of biological origin. The collision induced dissociation of parent ion species has proven to be an effective mean of extracting information on the structures of GSH conjugates. Based on the product ion spectra of protonated GSH, previous study identified the routes of fragmentation for the protonated GSH (Haroldsen *et al.*, 1988). By the experi-

Table 2. Effects of 1-bromobutane on content of GSH and formation of *S*-butyl GSH in spleen: dose-response

Dose (mg/kg)	GSH (nmole/mg protein)	*S*-Butyl GSH (pmole/mg protein)
0	55.7 ± 2.5	N.D.
375	46.7 ± 4.3	1.6 ± 0.9
750	48.3 ± 3.8	1.3 ± 0.2
1500	38.5 ± 3.8**	2.7 ± 0.7

Mice were treated orally with 375, 750 and 1500 mg/kg of 1-bromobutane in corn oil at 10 ml/kg once. All animals were subjected to necropsy 24 hr after the treatment. Each value represents mean ± S.E. of five animals. Asterisks indicate the value significantly different from vehicle control at $P < 0.01$ (**) for GSH. N.D., not detected.

Table 3. Effects of 1-bromobutane on content of GSH and formation of *S*-butyl GSH in spleen: time-course

Time (hr)	GSH (nmole/mg protein)	*S*-Butyl GSH (pmole/mg protein)
0	55.7 ± 2.5	N.D.
6	59.5 ± 5.0	N.D.
12	33.7 ± 1.2**	N.D.
24	35.1 ± 2.0**	2.7 ± 0.7
48	33.6 ± 0.8**	11.3 ± 10.3

Mice were treated orally with 1500 mg/kg of 1-bromobutane in corn oil at 10 ml/kg once. All animals were subjected to necropsy 6, 12, 24 and 48 hr after treatment. Each value represents mean ± S.E. of five animals. Asterisks indicate the value significantly different from the 0 hr control at $P < 0.01$ (**) for GSH. N.D., not detected.

mental condition developed recently (unpublished data), *S*-butyl GSH could be successfully detected in splenic homogenates at the dose of 1-BB tested in the present study.

Following oral treatment of mice with 375, 750 and 1500 mg/kg of 1-BB once for 24 hr, splenic homogenates were analyzed for determining GSH levels and the presence of *S*-butyl GSH (Table 2). Splenic content of GSH was slightly decreased by 375 and 750 mg/kg of 1-BB and significantly decreased to 69.1% of control by 1500 mg/kg of 1-BB. Amounts of *S*-butyl GSH in splenic homogenates formed by single treatment with 1500 mg/kg of 1-BB for 24 hr were 2.7 pmole/mg protein (Table 2).

When the production of GSH conjugate was investigated in spleen following oral treatment with 1500 mg/kg of 1-BB for 6, 12, 24 and 48 hr, *S*-butyl GSH was detected from 24 hr after the treatment (Table 3). These results were consistent with the results that splenic GSH levels were significantly reduced from 12 hr after single dose of 1-BB at 1500 mg/kg (Table 3).

DISCUSSION

Halogenated organic compounds, such as 1-BB, have widely been used in industry. Recently, hepatotoxic effects of 1-BB and its conjugation with GSH were investigated in female BALB/c mice (unpublished data). In the study, three kinds of GSH conjugates, including *S*-butyl GSH, *S*-butyl cysteine, and (hydroxybutyl)mercapturic acid, were identified in livers by LC/ESI-MS. Particularly, S-butyl GSH conjugate was detected maximally 6 hr after treatment with 1-BB in liver with marked reduction of hepatic GSH levels. At the same time, 1-BB increased in serum activities of alanine aminotransferase and aspartate aminotransferase, suggesting that 1-BB could cause hepatotoxicity as well as depletion of GSH content, due to the formation of GSH conjugates in mice (unpublished data). During the study, the reduction of spleen and thymus weights were observed, indicating possible immunotoxic potential of 1-BB. Therefore, the present study was designed to investigate the immunotoxic potential of 1-BB with a particular emphasis on the production of GSH conjugates in spleen.

For 1-BB-induced immunotoxicity studies, T-dependent antibody response, splenic and thymic lymphocyte subpopulations, and IL-2-producing cells were employed following single treatment with 1-BB. These parameters have long been implicated and employed as sensitive parameters for determining xenobiotic-induced immunotoxicity (Luster *et al*., 1988). As results, it was found that 1-BB could clearly suppress all three parameters, indicating that 1-BB would be immunotoxic compounds and that the immunotoxicity might be due to, at least partially, suppression of IL-2 production by T-cells. Subsequently, a role of GSH conjugation in 1-BB-induced immunotoxicity was investigated by analyzing the changes in splenic GSH levels and the production of GSH conjugates in spleen.

As observed in livers (unpublished data), the decrease in cellular GSH and the production of GSH conjugate were also observed in spleen cells by treatment with 1-BB in the present study (Tables 2 and 3). In addition, 1-BB could suppress immune functions at the same doses that showed the decrease in GSH levels (Table 1, Figs. 1 to 4), indicating a possible role of GSH in 1-BB-induced immunotoxicity. In fact, the reports that immune function could be affected by GSH in immune cells are elsewhere (Elferink and de Koster, 1991; Dröge *et al*., 1994; Wu *et al*., 1994). The effector phase of cytotoxic T cell response and IL-2-dependent functions were inhibited even by a partial depletion of intracellular GSH pool (Dröge *et al*., 1994). In addition, the stimulation of DNA synthesis in lymphocyte populations strongly depended on the intracellular GSH level, so that intracellular GSH depletion could inhibit IL-2-dependent T cell growth (Gmunder *et al*., 1990). The present results were consistent with these reports. The DNA synthesis in mitogenically stimulated splenic T cell cultures was also a GSH-dependent function, so that the suppressed cytotoxic T lymphocytes in the late phase of mixed lymphocyte cultures by depletion of GSH could be overcome by high extracellular concentrations of GSH (Roth

and Dröge, 1991; Roth *et al*., 1991).

Moreover, immunotoxicity by depletion of GSH and, thereby, increased production of reactive oxygen species could be demonstrated in many previous literatures on Cd-induced immunotoxicity (Yamada *et al*., 1981; Pathak and Khandelwal, 2006a, b, 2007). Cd, a well known carcinogenic and immunotoxic metal, affected both humoral and cell mediated immunity (Pathak and Khandelwal, 2007). Cd caused oxidative stress and apoptosis in immune cells including both B- and T-cells (Pathak and Khandelwal, 2006a, b). In addition, T-cell apoptosis by Cd was related to marked depletion of intracellular GSH, in association with suppression of IL-2 and interferon-g expression (Pathak and Khandelwal, 2007).

When the amounts of GSH depletion and the production of GSH conjugates in liver and spleen were compared following 1-BB treatment, liver showed very dramatic changes in both GSH depletion and conjugate production (unpublished data; Tables 2 and 3). Changes in cellular GSH and GSH conjugates in spleen were significant, but relatively marginal when compared to the change in liver. Although the production of all GSH conjugates other than *S*-butyl GSH was not determined in the present study, these results indicate that the cellular level of GSH in immune cells might be very critical in modulation of immune functions, such as IL-2 production by T-cells.

Because 1-BB could also be metabolized by CYPs (James *et al*., 1968), certain phase I metabolite(s) might contribute to 1-BB-induced hepatotoxicity. For example, we recently reported that phase I metabolism of 1-BP by CYPs, at least in partial, might contribute to its toxicity, in addition to oxidative stress by GSH depletion via conjugate formation with GSH (Lee *et al*., 2010).

In conclusion, the present study demonstrated that 1-BB might induce immunotoxicity via suppression of IL-2 production in mice, and that 1-BB-induced immunotoxicity might be closely related to the depletion of GSH. Although the possible mechanism of oxidative stresses in 1-BB-induced immunotoxicity remains to be elucidated further in the near future, a recent report on 1-BP, a structural analog of 1-BH, suggested the macrophage activation via NF-κB transactivation and ERK1/2 MAP kinase phosphorylation (Han *et al*., 2008).

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