JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2008,** *56,* 9305–9311 **9305**

Immunomodulatory Effects of Cadmium and *Gynostemma pentaphyllum* Herbal Tea on Rat Splenocyte Proliferation

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Gynostemma pentaphyllum Makino (GP) is a herbal tea widely grown in Southeast Asia. However, this herbal tea can be contaminated with some heavy metals, especially cadmium (Cd), from agricultural areas, which may affect human health. The objective of this study is to evaluate the immunomodulatory effects of Cd contaminated in GP herbal tea and inorganic Cd on rat splenocytes. Rats were divided into groups and treated with drinking water (control), high $CdCl_2$ in drinking water (HCd; 0.05 mg/L), GP herbal tea containing 0.05 mg/L Cd (GP-HCd) for 4 months, low $CdCl_2$ in drinking water (LCd; 0.006 mg/L), and GP herbal tea containing 0.006 mg/L Cd (GP-LCd) for 6 months. After the treatments, Cd accumulation in organs and blood was detected by using a graphite furnace atomic absorption spectrophotometer. In spleen, HCd-treated rats had 4-fold higher Cd accumulations than GP-HCd-treated rats. Cd accumulation in liver and kidney in the HCd group also increased significantly. There were no significant changes in total leucocyte and lymphocyte counts; however, these parameters tended to decrease slightly in LCd, GP-LCd, and GP-HCd groups. The HCd group (ex vivo) significantly produced suppressive effects on T cell mitogen-induced splenocyte proliferation, with 1 μg/mL Con A and PHA-P. In addition, 0.5 μg/mL PWM-induced B cell proliferation, through T cell functions, was also significantly inhibited by HCd as compared to the control group, while GP-HCd had no effects. However, both GP-LCd- and LCd-treated rats had a slight increase in Con A-stimulated splenocyte proliferation. This study indicated that high Cd contamination in drinking water alone had suppressive effects on T cell functions, but these effects could not be found with the same Cd level contamination in GP herbal tea.

KEYWORDS: *Gynostemma pentaphyllum*; immunomodulatory; cadmium; splenocyte proliferation; herbal tea

INTRODUCTION

Gynostemma pentaphyllum Makino (Cucurbitaceae) (GP) is a medicinal plant widely distributed in Southern China, Japan, Korea, and Southeast Asia. It has been used in traditional medicine in China (known as Jiaogulan) and Japan (known as Amachazuru). This medicinal plant has been reported to have a wide range of pharmacological effects including hypolipidemia, antigastric ulcer, anticancer, prevention of cardiovascular disease, and immunopotentiating effects (*1*−*5*). The review paper has summarized the effects of GP on the immune system (*6*). A previous study showed that gypenosides, the major active constituents of GP, enhanced the recovery of the spleen weight and cellular immunocompetence in γ-ray irradiated mice (*7*). The total saponins of GP markedly reduced cyclophosphamide-induced atrophy of immune organs, spleen, and thymus (*5*). These saponins also increased the mitogen-stimulated (Con A, LPS, and oval-albumin) splenocyte proliferation in OVA-immunized mice (*8*). Chen et al. (*9*) indicated that GP helped to recover the suppression of mitogen-induced splenocyte proliferation in the γ-ray irradiated mice. The production of antibodies and cytokines was enhanced in Con A-stimulated splenocytes of GP extract-treated mice, suggesting that this extract might promote immune responses through the activation of T and B cells (*10*).

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10.1021/jf801062z CCC: $40.75
Published on Web 09/17/2008

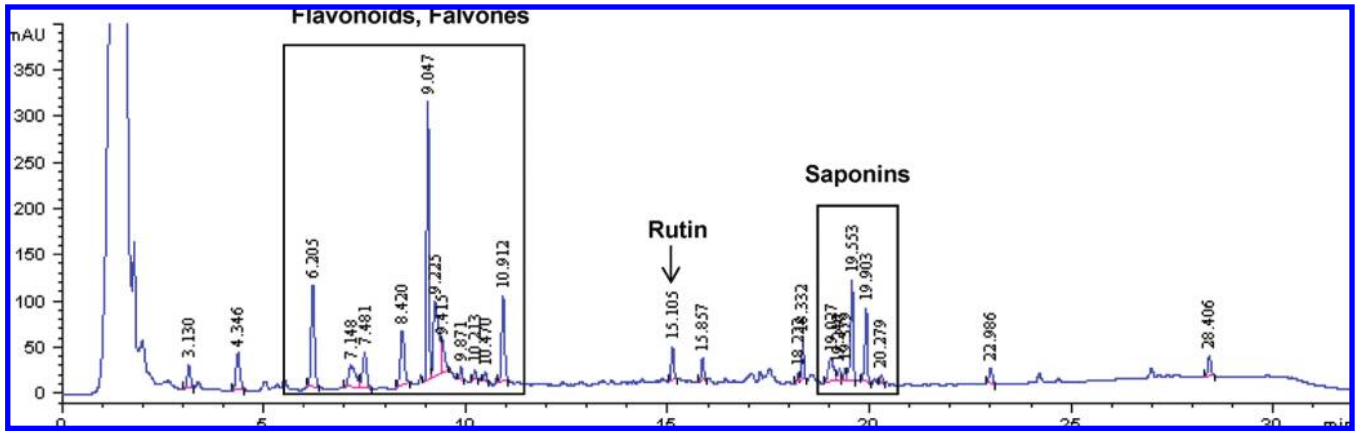

Figure 1. HPLC fingerprint of the dried powder of *G. pentaphyllum* extract.

Table 1. Compositions of the GP Extract

GP extract	% moisture	protein (g/100 g)	total[a] saponins (%)	total[a] gypenosides (%)	cadmium (mg/kg)	iron (mg/kg)	copper (mg/kg)	lead (mg/kg)
GP-HCd	4.4	ND[b]	15.4	6.3	0.70	139.50	10.24	0.76
GP-LCd	4.5	16.7	16.3	7.7	0.09	63.14	9.91	0.10

[a] Standard values of the total saponins and total gypenosides in *G. pentaphyllum* plant are ≥8.0 and ≥4.0%, respectively (Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Thailand). [b] ND = not determined.

Cadmium (Cd) is a major environmental contaminant having a long biological half-life. Human exposure is mainly via oral and inhalation routes. The major sources of cadmium exposure by oral route are drinking water and foodstuff contamination (*11*). Cd not only causes multiorgan toxicity but also is a potent immunotoxicant. Cd affects both humoral and cell-mediated immunity. T and B lymphocyte proliferation was significantly reduced, and macrophage phagocytosis was significantly increased following Cd exposure in drinking water in adult female B6C3F1 mice (containing 10, 50, or 250 mg/L $CdCl_2$) for 90 days (*12*). Its immunosuppressive effects were reported both in vivo (*13*) and in vitro (*14*).

A recent report indicated that Cd was found in medicinal herbs at levels up to 0.74 μg/g (*15*). Medicinal plants can be contaminated with Cd in agricultural areas from soil, fertilizers, water, and pesticides. Our recent study reported that most GP herbal tea products available in Thai markets both locally made and imported were contaminated with Cd, exceeding the maximum permissible content in medicinal plants set by the Ministry of Public Health of Thailand, which is 0.3 mg/kg (*16*). However, the content of Cd in herbal tea has not been regulated. The content of Cd in drinking water should be less than 0.003 mg/L (*11*). The consumption of medicinal plants contaminated with Cd may be another source of Cd exposure, which affects human health. Repeated consumption of GP herbal tea contaminated with Cd may produce alteration of an immune response. Therefore, this study was conducted to investigate the effects on immunomodulatory activity of repeated consumption of GP herbal tea contaminated with low and high Cd contents in comparison to inorganic Cd ($CdCl_2$) in drinking water. The effects of GP extracts on mitogen-induced splenocyte proliferation were also examined. Furthermore, Cd accumulations in blood, immune organs (thymus and spleen), and target organs (liver and kidney) were also determined.

MATERIALS AND METHODS

Chemicals. Cadmium chloride ($CdCl_2$), rutin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), lectin from *Phaseolus vulgaris* red kidney bean (PHA-P), lipopolysaccharide from *Salmonella typhimurium* (LPS), and poke weed (PWM) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Hyclone, Co. (South Logan, UT). RPMI 1640 medium (Gibco BRL Life Technologies Inc., Grand Island, NY) supplemented with 100 units of penicillin−streptomycin/mL, 2 mM L-glutamine, 0.01 mM nonessential amino acid, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (2-Me) (Sigma-Aldrich, St. Louis, MO) and 10% FBS (Hyclone, Co.) were also purchased, but there was no 2-Me in cell culture medium. H_2O_2 and nitric acid were obtained from Merck (Hohenbrunn, Germany) and BDH (VWR International Ltd., Poole, England), respectively.

Preparation of Plant Extracts. GP plants were freshly harvested from the field (Ratchaburi province, Thailand) after growing for 3−4 months, washed, and then air-dried. Dried plants were ground into powder and kept in a dry place until use. One kilogram of dried powder of GP plant was extracted with 6 L of hot water (70−75 °C) for 1 h (the extraction ratio was 1:6). The extract was then filtered and collected. The residue was re-extracted twice with 6 L of hot water each time. The collected water extracts were then combined and spray dried. The % yield of extracts was between 17 and 20%. Plant extracts, weighing 22−25 mg, were dissolved in 3.0 mL of hot water (70−75 °C) and vigorously shaken. The extracts were left at room temperature until they cooled down and were then filtered through a 0.45 μm nylon membrane (13 mm, Orange Scientific, Braine-I'Alleud, Belgium) prior to high-performance liquid chromatography (HPLC) analysis (HP1100, Baudrats, Germany). A reverse phase column, Hypersil BDS C_{18} 5 μm, 150 mm × 4 mm i.d. (Thermo Electron Co., Southend-on-Sea, United Kingdom), was used in this study. The mobile phase consisted of water (solvent A) and acetonitrile (Merck, Darmstadt, Germany) (solvent B) with a gradient system from 0% solvent B to 90% in 28 min (flow rate at 1.0 mL/min). The total run time was 32 min. Rutin was used as an external standard for the standardization of GP extract with retention time at 15.10 min. All extracts were examined for chemical fingerprints by HPLC analysis at 205 nm. The HPLC fingerprint of GP extract is shown in **Figure 1**. The compositions of the GP extracts are shown in **Table 1**. The rutin contents of two GP extracts (both low and high Cd contents) were 1.16 ± 0.21 and 1.04 ± 0.11 mg/g dry weight, respectively.

Animals. Male Sprague−Dawley rats, weighing 200−230 g, were used in this study. The animals were supplied by the National Laboratory Animal Center, Thailand. The care and use of animals were

Table 2. Cd Contents in Dried GP Extract Powder, GP Extract Dissolved in Drinking Water, and the Estimated Daily Consumption of Additional Cd from Drinking Water and GP Herbal Tea in Rats[a]

time of exposure treatment	dried GP extract (mg/kg)	drinking water[b] or GP extract in drinking water (mg/L)	estimated daily consumption of Cd ($\times 10^{-3}$ μg/day) (mean ± SEM)
		4 months	
HCd		0.05[b]	21.39 ± 1.22
GP-HCd	0.70	0.05	21.53 ± 1.39
		6 months	
LCd		0.006[b]	2.89 ± 0.14
GP-LCd	0.09	0.006	2.69 ± 0.12

[a] For the control groups, rats received Cd only from drinking water and food (<0.0001 mg/L and 0.157 ± 0.006 mg/kg, respectively). The average daily food intake was 38−40 g/kg BW. The estimated daily Cd intake from drinking water and food was approximately 0.0061−0.0064 mg/kg BW. [b] $CdCl_2$ was dissolved in drinking water.

in accordance with National Guidelines and Chulabhorn Research Institute Animal Care and Use Committee (CRI-ACUC). They were housed under controlled housing conditions (temperature, 23 ± 2 °C; humidity, 50%) with a 12:12 h light:dark cycle in the animal facility of Chulabhorn Research Institute. Animals had free access to extruded food (C.P feed number 082, Thailand) and tap water ad libitum throughout the 7 days acclimatization period. The Cd contents in animal drinking water and food were <0.0001 mg/L and 0.157 ± 0.006 mg/kg, respectively, by inductively coupled plasma-mass spectrometer (ICP-MS; Agilent Technologies 7500c, Palo Alto, CA) as the method previously described (*16*). The animals were randomly assigned to receive drinking water (control group), $CdCl_2$ in drinking water at high dose of 0.05 mg/L (HCd group), and GP herbal tea (prepared as GP extract in drinking water) contaminated with Cd 0.05 mg/L (GP-HCd group) for 4 months or $CdCl_2$ in drinking water at low dose of 0.006 mg/L (LCd group) and GP herbal tea contaminated with Cd 0.006 mg/L (GP-LCd group) for 6 months (**Table 2**). The number of rats in each group was six. The animal sample size of this experiment was determined by using relevant literature and statistical power analysis (resource equation method) to estimate the animal sample size (*17*). Two GP herbal teas in this study were prepared as concentrated extracts (instant tea powders) from GP products contaminated with Cd at different levels. One bag of this GP instant tea contains 300 mg of extract (per cup). The selected doses of GP herbal tea corresponded to daily human consumption (600 mg or 2 bags per day/60 kg body weight), which was extrapolated to a dose of 70 mg extract/kg/day in rats (*18*). The GP extracts, in the form of instant tea, were then dissolved in drinking water and measured for Cd contents. The GP drinking teas used in our experiment contained Cd levels at 0.006 and 0.05 mg/L, for low and high doses of Cd, respectively. The daily Cd consumption of treated rats was measured, and the amount of Cd intake was estimated as shown in **Table 2**. Rats treated with both GP groups received higher Cd contamination in drinking tea than the maximum permissible value of Cd in drinking water (0.003 mg/L; *11*). At the end of the treatment, rats were sacrificed by using 100% CO_2, and then, blood and organs (liver, kidney, spleen, and thymus) were collected. Total leucocytes and lymphocyte counts and Cd contents in organs and blood were determined.

Cd Contents in Organs and Blood. Blood and tissue samples (liver, kidney, spleen, and thymus) were collected, and tissues were weighed after sacrifice. Tissues were dried by using a freeze-dryer (Labconco, Kansas city, MO). Heparinized blood was diluted in 0.02% TX-100 in 0.02% HNO_3. Tissue samples were digested by using microwave digestion. Dried samples were weighed, 0.2 g, and then, 6.0 mL of concentrated nitric acid and 2.0 mL of H_2O_2 were added. Each digested sample was diluted to 50 mL with deionized water. Cd contents in the organs and blood were determined by using a graphite furnace atomic absorption spectrophotometer (GFAAS) (AAnalyst600, Perkin-Elmer, Bodenseewerk, Germany). The accuracy and precision of the method were tested with DORM-2 dog fish muscle (National Research Council Canada, Ottawa, Ontario). The determined Cd content in DORM-2 dog fish was 0.045 ± 0.001 mg/kg (certified value is 0.043 ± 0.008 mg/kg).

Mitogen-Induced Splenocyte Proliferation Assay. Spleens from treated rats were aseptically isolated and then separated into single cell suspensions in complete medium [m-med; RPMI1640 supplemented with 10% heat-inactivated FBS (10% FBS), 100 unit penicillin: streptomycin/mL, and 50 μM 2-mercaptoethanol (2-Me)] by passing through a fine stainless steel mesh and needle #21 three times. The cell suspension was layered on IsoPrep (Robbins Scientific Co., Norway) and centrifuged (800*g* at 18 °C for 30 min). The white band was collected into m-med and then centrifuged again. The erythrocytes were lysed with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) and incubated for 5 min at room temperature. After centrifugation (200*g* at 18 °C for 10 min), the pellets were washed in m-med. The pellets were centrifuged again (180*g* at 18 °C for 5 min) and washed in 1 mL of m-med three times and resuspended in culture medium without 2-Me (c-med). The cell numbers were counted with a hemacytometer by the tryphan blue dye exclusion technique. Cell viability always exceeded 95%. The splenocytes were adjusted to 4×10^6 cells/mL in c-med and transferred in 50 μL to 96 well microtiter plate (Costar, Cambridge, MA). Triplicate cultures were treated with 50 μL of different mitogens (1 μg/mL Con A, 1 μg/mL PHA-P, 5 μg/mL LPS, and 0.5 μg/mL PWM). Con A and PHA-P stimulated the proliferation of T lymphocytes, while PWM and LPS stimulated B-lymphocytes. The cells in these experiments were cultured at 37 °C in 5% CO_2, 95% atmosphere for 68 h. The splenocytes were added with 10 μL/well of MTT (1 mg/mL) and incubated for 4 h. The reactions were stopped by adding 25 μL of 10% SDS in 0.01 N HCl. The absorbance value (OD) was measured at a wavelength of 600 nm by microplate spectrophotometer (SPECTRAmax 384 plus, Molecular Devices, Sunnyvale, CA) after overnight incubation. The stimulation index (SI) was calculated based on the following formula:

$$\mathrm{SI} = \frac{\mathrm{OD\ (mitogen\text{-}stimulated\ cultures)} - \mathrm{OD\ (medium)}}{\mathrm{OD\ (nonstimulated\ cultures)} - \mathrm{OD\ (medium)}}$$

Total Leucocyte and Lymphocyte Counts. Blood was collected by heart puncture. The whole blood samples were gently mixed with EDTA. The total leucocytes and lymphocytes were counted by a hematology analyzer (Coulter JT, Fullerton, CA).

Statistical Analysis. Data were expressed as means ± SEM. Differences were tested for statistical significance by using Student's *t* test and one-way analysis of variance (ANOVA) on repeated measurements when appropriate. Multiple comparisons between groups were performed by least significant differences test (SPSS software, Version 11); a p value <0.05 was regarded as statistically significant.

RESULTS

Our previous study showed that GP herbal tea products contained high contents of Cd in the range of 0.02−4.77 mg/kg (n = 16; average, 1.27 mg/kg) (*16*), with 14 of 16 GP herbal tea products containing Cd contents over the maximum permissible value for medicinal plants, 0.3 mg/kg (*11*, Ministry of Public Health, Thailand). A high Cd level was detected in both local and imported GP products. Cd contamination in GP plant may result from using contaminated water or fertilizer for agriculture. However, when this plant is used as a herbal drinking tea, Cd released into the infusion is less than in leaf powders. The Cd contents in GP infusions in our previous study were in a range of 0.001−0.023 mg/L (% release = 18.6) (n = 8). The lowest and highest Cd levels in these GP infusions were 0.001 and 0.023 mg/L, respectively, with an average at 0.007 mg/L. On the basis of these results, our present study prepared GP extract contaminated with Cd in the form of instant tea and then dissolved in drinking water. Cd levels in GP drinking tea used for our experiment were 0.006 mg/L for GP-LCd group and 0.05 mg/L for GP-HCd group. These Cd levels in GP

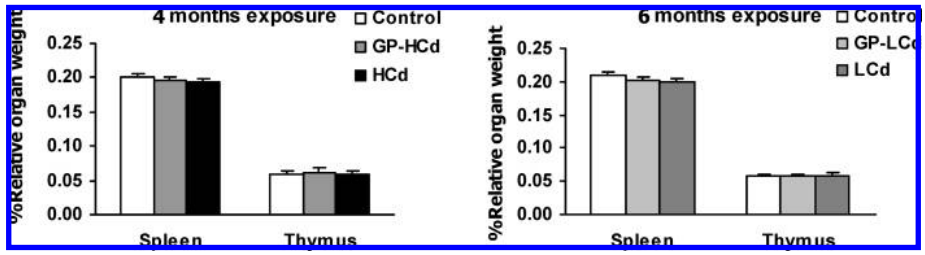

Figure 2. Relative thymus and spleen weights of rats exposed to $CdCl_2$ in drinking water and GP herbal tea contaminated with Cd. Rats were exposed for 4 months to HCd (high $CdCl_2$ in drinking water) and GP-HCd (GP herbal tea contaminated high Cd) and for 6 months to LCd (low $CdCl_2$ in drinking water) and GP-LCd (GP herbal tea contaminated low Cd). Data were expressed as % relative organ weight, means ± SEM ($n = 6$).

Table 3. Changes in Body Weights of Rats[a]

	body weight (g) (mean ± SEM)	
treatment	day 0	day 120
	4 months exposure	
control	294.85 ± 3.94	514.85 ± 8.91
HCd	294.08 ± 4.58	494.85 ± 7.75
GP-HCd	290.54 ± 3.42	503.85 ± 6.42
	day 0	day 180
	6 months exposure	
control	288.54 ± 3.49	523.92 ± 6.74
LCd	291.07 ± 3.50	532.46 ± 5.89
GP-LCd	290.46 ± 4.79	544.85 ± 7.53*

[a] The values are presented as means ± SEM ($n = 6$). Significant differences with the control group are designated as *$p < 0.05$.

drinking tea were also close to a moderate Cd level (0.007 mg/L) and the highest Cd level (0.023 mg/L) in GP infusions in our previous report (*16*). It is noted that both values are higher than the standard maximum value of Cd in drinking water, 0.003 mg/L (*11*).

Effect on Body Weight and Organ Weight. Repeated consumption of GP extract contaminated with high Cd (GP-HCd group) and high Cd alone (HCd group) in drinking water for 4 months and GP extract contaminated with low Cd (GP-LCd group) and low Cd alone (LCd group) in drinking water for 6 months did not significantly alter the relative thymus and spleen weights as compared to the control rats (**Figure 2**). There was no change in the body weight of high Cd treatments; however, there was a trend in increasing body weight in the low Cd-treated group (**Table 3**). For a high dose of Cd (HCd-group), the experiment was terminated at 4 months since the basal systolic blood pressure of rats in this group significantly increased and started from 3 months (data not shown).

Effect on Cd Accumulation in Organs and Blood. Cd accumulation in organs and blood collected from all treated rats was determined at the end of the exposure times. The results showed that HCd group produced a significantly higher Cd accumulation in liver (4.8-fold) and kidney (3.4-fold) than that of the control and GP-HCd groups ($p < 0.001$) (**Table 4**). There was no significant difference in Cd contents in liver and kidney, isolated from LCd and GP-LCd groups. The HCd group had 4-fold higher Cd accumulation in spleen than GP-HCd group ($p < 0.05$). However, Cd contents in thymus and spleen were not detected in both GP-LCd and LCd groups. The Cd content in blood exhibited a significant increase in HCd group as compared to the control group. Unexpectedly, there were significantly less Cd contents in the blood in both GP-LCd and LCd groups than in the control group. For both control groups, Cd contents in blood, liver, and kidney were mainly due to its presence in the animal food (0.157 ± 0.006 mg/kg).

Effect on Total Leucocyte and Lymphocyte Counts. GP contaminated with high Cd (GP-HCd) produced a decrease in total leucocyte and lymphocyte counts, but this effect was not significant (**Table 5**). In addition, both GP-LCd and LCd groups also induced a decrease in these parameters, but it was also not significantly different in all treatments.

Effect on Mitogen-Induced Splenocyte Proliferation. The present study determined the immunomodulatory effect of GP herbal tea extract contaminated with different levels of Cd on the lymphocyte proliferation, which responds to four different mitogens (**Table 6**). Con A and PHA-P are T-lymphocyte mitogens, whereas PWM and LPS are B lymphocyte mitogens. Lymphocyte proliferation in subchronic exposure of HCd group (4 months) (ex vivo) exhibited significantly suppressive effects of T cell mitogen-induced splenocyte proliferation, 1 μg/mL Con A and PHA-P, at $p < 0.01$ and $p < 0.05$, respectively, and 0.5 μg/mL PWM-induced B cell proliferation, through the release of soluble factors by T cells at $p < 0.05$ (**Table 6** and **Figure 3**). However, the GP-HCd group had almost as much splenocyte proliferation response to mitogens (Con A, PHA-P, and PWM) as the control group. HCd and GP-HCd groups did not alter splenocyte proliferation to 5 μg/mL LPS, B cell-dependent. These results suggested that the active compounds in GP extract may be a potent activator of T cell proliferation.

Table 4. Cd Contents in Various Organs of Rats (mg/kg)[a]

treatment	thymus	spleen	liver	kidney	blood (μg/L)
			4 months of exposure		
control	0.00 ± 0.00	0.00 ± 0.00	0.036 ± 0.004	0.274 ± 0.022	0.643 ± 0.064
GP-HCd	0.00 ± 0.00	0.001 ± 0.001	0.033 ± 0.007###	0.248 ± 0.032###	0.734 ± 0.073
HCd	0.002 ± 0.001	0.004 ± 0.001*	0.158 ± 0.025***	0.853 ± 0.079***	0.948 ± 0.127*
			6 months of exposure		
control	0.00 ± 0.00	0.00 ± 0.00	0.037 ± 0.008	0.335 ± 0.023	0.414 ± 0.012
GP-LCd	0.00 ± 0.00	0.00 ± 0.00	0.034 ± 0.005	0.329 ± 0.029	0.369 ± 0.011**
LCd	0.00 ± 0.00	0.00 ± 0.00	0.046 ± 0.008	0.422 ± 0.043	0.358 ± 0.015**

[a] The values are presented as means ± SEM ($n = 6$). Significant differences with normal group are designated as *, **, and *** ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). Significant differences between GP-Cd and Cd groups are designated as ### ($p < 0.001$).

Table 5. Changes in Leucocyte Numbers ($\times 10^6$ Cells/mL)[a]

treatment	WBC	lymphocytes	monocytes	granulocytes
		4 months of exposure		
control	8.46 ± 0.69	6.88 ± 0.54	1.04 ± 0.09	0.54 ± 0.09
GP-HCd	6.91 ± 0.62	5.76 ± 0.54	0.76 ± 0.06	0.38 ± 0.06
HCd	8.30 ± 0.50	6.74 ± 0.36	1.04 ± 0.09	0.60 ± 0.11
		6 months of exposure		
control	8.20 ± 0.54	6.45 ± 0.47	1.21 ± 0.17	0.55 ± 0.11
GP-LCd	7.24 ± 0.57	5.87 ± 0.43	0.97 ± 0.12	0.41 ± 0.07
LCd	7.29 ± 0.25	5.87 ± 0.19	0.98 ± 0.08	0.45 ± 0.04

[a] The values are presented as means ± SEM ($n = 6$).

Table 6. SI of Mitogen-Induced Splenocyte Proliferation[a]

treatment	Con A (1 μg/mL)	PHA-P (1 μg/mL)	PWM (0.5 μg/mL)	LPS (5 μg/mL)
		4 months of exposure		
control	3.56 ± 0.27	1.03 ± 0.03	1.80 ± 0.13	1.26 ± 0.07
GP-HCd	3.13 ± 0.33	0.96 ± 0.02	1.68 ± 0.10	1.31 ± 0.07
HCd	2.32 ± 0.29**	0.94 ± 0.02*	1.42 ± 0.12*	1.18 ± 0.14
		6 months of exposure		
control	4.86 ± 1.06	1.03 ± 0.40	2.40 ± 0.22	1.46 ± 0.19
GP-LCd	5.92 ± 0.89	1.00 ± 0.03	2.48 ± 0.18	1.37 ± 0.06
LCd	5.55 ± 0.76	1.05 ± 0.04	2.18 ± 0.20	1.32 ± 0.09

[a] The values are presented as means ± SEM ($n = 6$). Significant differences with control groups are designated as *$p < 0.05$ and **$p < 0.01$.

The GP-LCd and LCd groups had a slight increase in splenocyte proliferation with Con A as compared to the control group. In addition, a slight increase in splenocyte proliferation response to PWM was found in the GP-LCd group, but this response was slightly reduced in the LCd group. Both GP-LCd and LCd groups showed a slightly decreased splenocyte proliferation response to LPS, but there was no alteration on this response to PHA-P at 0.5 μg/mL in both groups.

DISCUSSION

The phytochemical compounds of this plant have been identified about 90 dammarane type glycosides (called gypenosides), and some of these compounds were closely related to the ginseng saponins (*19*). Total saponins and total gypenosides of GP extracts used in our study were 15.4 and 6.3%, respectively, for GP-HCd and 16.3 and 7.7%, respectively, for GP-LCd. The total saponin extracted from GP (10 and 30 mg/kg), subcutaneously, enhanced Con A-induced splenic T lymphocyte and LPS-induced splenic B lymphocyte proliferation and increased splenic cell IL-2 formation (*5*). In the present study, there were no changes in the body weights of all treatment groups. Shibutani et al. (*20*) also reported that rats fed diet containing Cd-polluted rice (1.1 mg/kg Cd) and $CdCl_2$, 1.1, 5, 20, and 40 mg/kg for 22 months, did not show any changes in body weight and mean food intake during the feeding experiment. We did not measure food consumption during our experiment. However, on the basis of previous study in our laboratory, the average daily food intake in normal rat at 3 months was 38−40 g/kg/day. In our control group, rats received Cd only from drinking water and food (<0.0001 mg/L, and 0.157 ± 0.006 mg/kg, respectively). Therefore, the estimated daily Cd intake from drinking water and food in control rats was approximately 0.0061−0.0064 mg/kg BW (baseline). Additional Cd intake for the treatment groups was from $CdCl_2$ in drinking water and GP drinking herbal tea (**Table 2**). The estimated daily Cd intake in the GP-HCd group (GP-HCd) was about 10 times higher than the GP-LCd group.

After long-term exposure (6 months) to low level of Cd, unexpectedly significant decreases of Cd in blood ($p < 0.01$) were found in LCd and GP-LCd groups, but there were no significant differences of Cd levels in the other tissues. These results showed that the low Cd contamination in GP drinking herbal tea did not result in Cd accumulation in any rat tissues. It is known that initially after exposure, Cd in blood is bound to plasma protein, mainly albumin, and high molecular weight proteins with a small fraction of Cd bound to metallothionein (MT) (*21*). Cd is also transported to the liver and induces MT synthesis. After forming a Cd−MT complex, it is then released back to the blood and transported to the kidney. In the kidney, the Cd−MT complex passes through the glomeruli and is reabsorbed by the proximal tubules or excreted in urine and feces. In the present study, a significant decrease of Cd in blood in rats exposed to low Cd may be due to the rate of MT synthesis being sufficient to trap all Cd entering in the body and detoxifying it. However, in the case of high Cd intake, MT synthesis and binding may be saturated or insufficient to prevent Cd absorption. Foulkes and McMullen (*22*) reported that at low Cd concentration, the endogenous Cd-binding capacity sufficed to trap almost all Cd taken up in the intestinal mucosa, and at higher Cd levels, the induction of MT synthesis by Zn also increased fractional trapping of Cd. The mice occasionally exposed to high Cd (2100 μg Cd/kg) once a week for 5 weeks had higher Cd levels in blood and a higher Cd accumulation in the liver and kidney as compared to the continuously exposed mice (300 μg Cd/kg) (*23*). These results indicated that in mice subjected to a low and continuous Cd exposure, the rate of basal MT production is sufficient to sequester the Cd entering the epithelial cells but for a high intake of Cd for a short period, this MT production might be too slow to delay or prevent absorption, which led to an increase in the Cd absorption. The present results also showed that after 4 months of exposure to a high level of Cd, significant increases of Cd in blood, spleen, kidney, and liver were detected only in the HCd group. It is interesting to find that there was a slight decrease of Cd in blood in GP-HCd group as compared to the HCd group; however, Cd

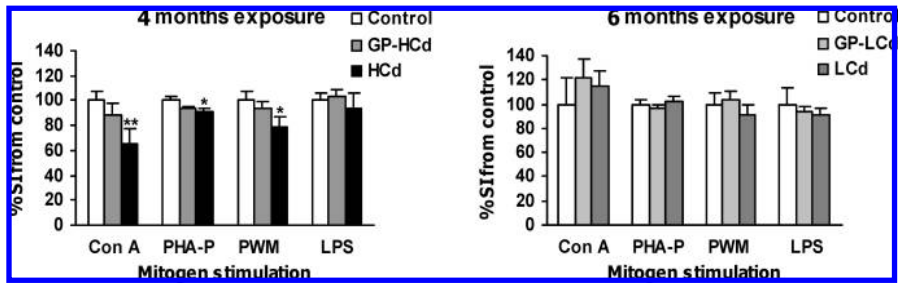

Figure 3. Suppressive effects on mitogen-induced splenocyte proliferation in ex vivo. Treated rat splenocytes were cultured with mitogens (1 μg/mL ConA and PHA-P, 0.5 μg/mL PWM, and 5 μg/mL LPS), at 37 °C, 5% CO_2, for 68 h. Splenocyte proliferation was analyzed by MTT assay, and the SI was calculated as % of control ($n = 6$). Significant differences with normal groups were designated as *$p < 0.05$ and **$p < 0.01$.

accumulations in tissues especially spleen, liver, and kidney of HCd group were 4-, 4.8-, and 3.4-fold higher, respectively, than those of the GP-HCd group (**Table 4**). The mechanism for these results remains unclear. Therefore, the effects of GP on Cd accumulation in blood and organs should be studied further. The GP extract contained the active compounds, gypenosides, plant fiber, polysaccharides, protein, and amino acids. Currently, we are investigating the nutritional value of GP extract, and we have found that it contained total protein about 17−20 g/100 g and some amino acids with thiol groups, for example, methionine at 0.157 mg/g. These compounds can also bind to free Cd; therefore, the bioavailability of Cd from drinking herbal tea may be less than $CdCl_2$ in drinking water, leading to less Cd absorption and accumulation in target organs. Nyberg and Zhou (*24*) reported that Cd could bind to polypeptide extracted from Cd exposed algae and *Agrostis* root.

Previous studies on immunomodulatory effects of Cd reported that Cd simulated IL-2 production only in preactivated T cells (Jurkat T cells) (*25*) and also strongly inhibited the proliferative responses of Con A-stimulated thymocytes in both male and female adult rats 5 weeks after cessation of Cd exposure (*13*). The present study aimed to understand the effects of Cd and GP on T and B lymphocyte functions using several lymphocyte mitogens. Con A stimulates preferentially T lymphocytes, LPS activates B lymphocytes, and PWM and PHA-P induced both T and B lymphocytes. However, B lymphocytes proliferate mainly in the presence of T lymphocytes. The results from the present study indicated the significant suppression of T lymphocyte proliferation of HCd group to mitogens, Con A, PHA-P, and PWM but revealed no effects on LPS-stimulated B lymphocyte proliferation. However, the same levels of Cd contamination in GP herbal tea had no effects on both T and B lymphocyte proliferations. There were no changes in numbers of total leucocytes for both GP-Cd treatment groups. The present study showed interesting results concerning both the action of the active compounds in GP extract and the Cd causing the alterations of T lymphocyte function but not in the number of leucocytes. One possible explanation is that the active compounds in GP extract may directly modify T cell responses. Huang et al. (*10*) also reported that the intraperitoneal injection of GP extract into mice enhanced IL-2 and IFN-γ production from Con A-stimulated splenocytes in a dose-dependent manner (0.05, 0.5, and 5 g/kg/day) and T cells from mice receiving the highest dose of GP (5 g/kg) secreted more IL-10, an important mediator of T regulatory cells, than lower doses of GP (0.05 and 0.5 g/kg). Therefore, GP extract may improve the immune responses, especially from suppressed T lymphocytes, caused by Cd contaminated in GP herbal tea. However, Chavalittumrong et al. (*26*) found that the water extracts of GP at the doses of 50, 200, and 400 mg, twice daily, given to healthy volunteers for 2 months neither induced cytokine secretion nor enhanced NK cell activity. Their results suggested that GP extract (800 mg), taken daily for 2 months by healthy persons, did not alter the immune function.

In conclusion, our results reported that rats receiving GP drinking tea contaminated with moderate to high Cd levels at 0.006 and 0.05 mg/L, respectively, daily for 4−6 months did not alter the immune system, while high $CdCl_2$ in drinking water alone (at 0.05 mg/L) had significant suppressive effects on T cell function. However, the long-term toxic effects of GP herbal tea contaminated with Cd on other body functions such as cardiovascular and nervous systems should be studied further in detail.

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Received for review April 4, 2008. Revised manuscript received July 25, 2008. Accepted July 31, 2008.

JF801062Z