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EFFECT OF Acanthopanax senticosus ON THE ACCUMULATION OF CADMIUM AND ON THE IMMUNE RESPONSE OF SPLEEN CELLS

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Exposure to cadmium (Cd) is known to alter immune responses. Acanthopanax senticosus (Rupr. et Maxim.) Harms (AS) extract, an antioxidant-containing complex of phenolic compounds, tetracyclic triterpenoids/steroids, and polysaccharides, is known to produce Cd mobilization and excretion in vivo. Building upon earlier findings, the aim of the study was to evaluate the effects of an AS extract on Cd accumulation and changes in the presence of splenic immune cells in hosts during a chronic metal exposure. Chronic Cd exposure of BALB/c mice was induced by providing them solutions containing different levels of CdCl₂ (25 or 250 mg/L) in double-distilled water, with/without a concurrent presence of AS root extract (approximately 151 g material/L), for 8 wk. At the study end, Cd levels in spleen were measured. Levels of key splenic immune cells, including macrophages, T-lymphocytes, and B-lymphocytes, were determined by immunohistochemistry using, respectively, CD68, CD3, and CD20 antibodies. The results indicated that chronic consumption of AS extract in the presence of the high dose of CdCl₂ led to a significant decrease in Cd levels in mouse spleen. The effects of AS on the lower CdCl₂ dose were less apparent. In addition, the presence of AS and Cd increased the amount of macrophages and both B and T lymphocytes in mouse spleen relative to concentrations that were lowered as a result of chronic metal only intake.

Cadmium (Cd), a known environmental hazard, exerts adverse effects on humans and animals (Waisberg et al., 2003). Cadmium induced changes in the immunoregulatory mechanisms of a host with potentially severe clinical consequences (Krocova et al., 2000). In rodents, Cd produced thymic atrophy and splenomegaly, in addition to modulation of both humoral and cellular immune responses. Morphological alterations including thymic cortical cell depletion and an increase in red pulp with diminished white pulp in spleen were also reported (Pathak and Khandelwal, 2007). Stress protein synthesis was enhanced by Cd prior to cytotoxicity concomitant with a decrease in phagocytic capacity and elevation in tumor necrosis factor (TNF)-α levels (Goering et al. 2000). Cadmium also produced a significant reduction in metabolic activity of phagocytes, and mitogenic activation of lymphocytes in peripheral blood (Nad et al., 2009). Data suggest that circulating lymphocytes might redistribute differentially in lymphoid organs in response to Cd exposure (Ohsawa et al., 1983). Enhanced T-lymphocyte-independent antibody responses that accompany suppressed
T-lymphocyte-dependent responses following Cd exposure appear to be an indication of compensatory mechanisms that are induced within the immune system of exposed hosts (Blakley and Tomar, 1986).

A combination of extracts from adaptogenic plants may boost the suppressed immunity in ovarian cancer patients undergoing chemotherapy (Kormosh et al., 2006). *Acanthopanax senticosus* (Rupr. et Maxim.) Harms (AS, also known as *Eleutherococcus senticosus* or often colloquially referred to as Siberian ginseng) is a species of small woody shrub in the family Araliaceae that is native to northeastern Asia. AS is an adaptogen that seems to alter levels of different neurotransmitters and hormones involved in stress responses, predominantly those mediated by the hypothalamic–pituitary–adrenal (HPA) axis. AS was shown to enhance not just the stress response, but also immune and the endocrine systems, including adrenal glands, spleen, liver, and thymus (Kunimoto et al., 2002; Rogala et al., 2003).

Eleutherosides were found to bind to progestin, estrogen, mineralocorticoid, and glucocorticoid receptor sites in vitro, and thus may potentially exert many pharmacologic actions important in a stress response (Pearce et al. 1982). AS was also shown to possess immunomodulatory properties. Rogala et al. (2003) reported that there is a stimulatory effect of *Acanthopanax* on the humoral response, specifically, antibody production. Bohn et al. (1987) indicated that exposure to this material led to a general enhancement of activation states of T lymphocytes. Lastly, our earlier studies showed that use of this product led to Cd mobilization and excretion in vivo (personal communication). Specifically, in a study wherein exposure to AS was combined with exposure to CdCl₂, the use of AS led to a significant decrease in Cd levels in blood and liver of mice (Pathak and Khandelwal, 2007).

The aim of this study was to determine the ability of an AS extract to mitigate immunotoxic effects from Cd. The effects of the presence of AS on accumulation of Cd as well as on levels of macrophages, T lymphocytes, and B lymphocytes in mouse spleen after chronic treatment with Cd as CdCl₂ in drinking water were investigated.

**MATERIALS AND METHODS**

**Preparation of the Extract From *Acanthopanax senticosus* (AS) Roots**

The extract from roots of AS was prepared from raw material (Filaretov and Bogdanova, 1986) imported from Poland. Briefly, 1 kg dried AS roots was ground into 3-mm particles that were then evenly moistened with 1 L 40% aqueous ethanol, and then were placed in a closed vessel for 24 h. The moistened roots were then placed in a percolator to which 1 L of 40% aqueous ethanol was then poured to yield a 1:1 extract. The materials were heated for 24 h, after which the inert fibrous part of the material was removed and pressed; the pressed liquid was then mixed with materials that had been solubilized. The final material was then filtered. Yield analyses indicated that 1 ml of extract contained 0.151 g dried material (i.e., 151 mg AS/ml = final concentration).

**Animals**

Experiments were performed using male BALB/c mice (6 wk old, weighing 20–25 g) obtained from the Vivarium of the Institute Immunology of Vilnius University (Vilnius, Lithuania). All mice were housed in facilities maintained at 22 ± 1°C with a 55 ± 10% relative humidity and a 12-h light/dark cycle. All mice had ad libitum access to standard rodent food during the studies. Mice were weighed weekly and euthanized 8 wk after treatments according to the rules defined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. All protocols here were approved by the Lithuanian Commission for Ethics in Laboratory Animal Use, the State Food and Veterinary Service (license number 0028).

**Experiment Protocol**

BALB/c mice were acclimatized for 1 wk before being randomly allocated to 6 treatment
Acanthopanax EFFECTS ON Cd CONCENTRATION AND SPLEEN CELLS

groups (n = 15/group): (1) CdCl₂, 25 μg/ml (such that mice received 0.145 mg Cd²⁺ daily based on expected intake of approximately 5.8 ± 0.2 ml water/d); (2) CdCl₂, 250 μg/ml (1.45 mg Cd²⁺ daily); (3) AS extract at 0.529 mg AS root/ml (equivalent of approximately 3.07 mg AS root daily) [Note: The dose of AS used here (i.e., 0.12 g/kg/d) was selected to be far less than an earlier derived oral LD₅₀ value for the powdered AS (approximately 30 g/kg; Baldwin et al., 1986)]; (4) CdCl₂ (25 μg/ml) + AS extract; (5) CdCl₂ (250 μg/ml) + AS extract; or (6) double-distilled water only. In all cases, mice were exposed for a period of 8 wk. The water bottles were removed each morning and total volumes consumed were recorded; any losses from spillage, seepage, and so on were also noted. Each morning, the bottles were replaced with ones containing freshly prepared solution. All glassware was soaked in nitric acid and rinsed with double-distilled water prior to the next filling to ensure there was no carryover contamination or traces of Cd from a previous filling.

Weight gains were monitored during the exposure period. The body weight of 6 randomly selected mice/group was measured daily throughout the 8 wk of exposures. From these data, average weight gain per week was calculated. At the end of the exposure period, mice were euthanized via cervical dislocation and spleens were extracted, weighed, and processed for analyses. From among all the spleens recovered in each group, one subset was used for analyses of Cd content and the other for immunohistochemical analyses of cell populations.

Detection of Cd in Spleen

The modified method of Schlemmer (1989) was employed for analysis of Cd in the spleen samples. Each specimen was weighed and then digested in 1 ml of 0.125 M NaOH at 90°C until completely solubilized. At that point, the digest received an equal volume of double-distilled water. Levels of Cd in each spleen were then determined using a Perkin-Elmer/Zeeman 3030 electrothermal graphite furnace atomic absorption spectrophotometer (Perkin Elmer, Wellesley, MA) and analyzing for Cd at 228.8 nm.

Immunohistochemical Detection of Splenic Macrophages and T and B lymphocytes

Each second subset spleen was fixed in 10% neutral-buffered formalin for 48 h, then placed in paraffin blocks and sectioned at 3 μm on a silanized surface. Sections were then deparaffinized and rehydrated using a Varistain Gemini slide stainer (Thermo Scientific, Runcorn, UK). The sections were then washed with distilled water and heated in Tris/ethylenediamine tetraacetic acid (EDTA) buffer, pH 9, for 8 min at 110°C in a Histoprocessor RHS-1 microwave (Milestone, Sorisole, Italy). A Shandon coverplate system (Thermo Scientific) was then employed for immunohistochemical labeling. Specifically, after blocking the activity of endogenous peroxidases with a specific blocking solution, the slides were incubated with primary antibody (Dako Cytomation, Glostrup, Denmark) for 1 h. The specific rabbit anti-mouse antibodies (all from Abcam, Cambridge, UK) used were a 1:300 dilution of anti-CD68 (clone KP1); a 1:300 dilution of anti-CD3; or a 1:200 dilution of anti-CD20 (clone EP459Y). After primary incubation and rinsing away of unbound antibody, the samples underwent sequential 30-min incubations with Advance HRP Link and Advance HRP Enzyme reagents (Dako). Binding of the primary antibodies was detected using a Liquid DAB+ Substrate-Chromogen System (Dako). Lastly, each section was counterstained with Mayer’s hematoxylin (J. T. Baker, Phillipsburg, NJ) and then mounted using xylene-based medium (ConsulMount TM; Thermo Scientific). Each slide was examined by light microscopy, and histological images were taken using a DP-70 Olympus digital camera (Olympus Corp., Tokyo, Japan). Analysis of the cells in each slide was then performed using Image-Pro AMS 6.0.0 software (Figure 1).


Statistical Analysis

Results were expressed as mean ± standard error of mean. A Student’s t-test with a Bonferroni correction was applied for the comparisons of Cd levels. Nonparametric Kruskal–Wallis and Mann–Whitney tests were used for evaluation of differences in the areas (%) of macrophages and T and B lymphocytes in spleens of mice in each group. The level of statistical significance was set at \( p \leq 0.05 \).

RESULTS

Cd\(^{2+}\) Concentration in Mouse Spleen

The concentrations of Cd\(^{2+}\) in mouse spleen were measured 8 wk after oral administration of low and high doses of CdCl\(_2\) (25 and 250 mg/L, respectively) and AS extract solutions. The combined administration of high-dose CdCl\(_2\) with AS significantly decreased Cd\(^{2+}\) levels in spleen approximately 39-fold relative to concentrations seen when high-dose CdCl\(_2\) was given alone. Intake of the AS solution exerted no significant effect on splenic Cd\(^{2+}\) levels when administered in combination with low-dose CdCl\(_2\) (Figure 2).

Immunohistochemical Evaluation of Macrophages and T and B Lymphocytes in Spleen

Immunohistochemical evaluation of macrophages, T lymphocytes, and B lymphocytes in spleen.
spleen of mice was conducted 8 wk after oral administration of low- and high-dose CdCl₂ (25 and 250 mg/L, respectively), with and without AS extract. Administration of AS alone or AS together with low or high doses of CdCl₂ significantly increased the amount of macrophages compared to levels noted in organs of control mice (Figure 3). Combined administration of low and high doses CdCl₂ with AS solution significantly elevated the amount of splenic T-lymphocytes compared to controls, Cd25 only, and Cd250 only (Figure 4). Administration of AS alone exerted no marked effect on T-lymphocyte levels.

**FIGURE 3.** Total area of macrophages in spleen (as %) of mice. Mice were administered various Cd ± AS solutions (or AS-only solution or double-distilled water) for 8 wk. One day after the final exposure mice were euthanized and spleens obtained for immunohistochemical analyses. Asterisks indicate value significantly different from the *water control, **Cd 25 mg/L group, or ***Cd 250 mg/L group (p < .05).

**FIGURE 4.** Total area of T-lymphocytes in spleen (as %) of mice. Mice were given various Cd ± AS solutions (or AS-only solution or double-distilled water) for 8 wk. One day after final exposure mice were euthanized and spleens extracted for immunohistochemical analyses. Asterisks indicate value significantly different from *water control or **Cd 250 mg/L group (p < .05).

**FIGURE 5.** Total area of B-lymphocytes in spleen (as %) of mice. Mice were treated with various Cd ± AS solutions (or AS-only solution or double-distilled water) for 8 wk. One day after final exposure mice were euthanized and spleens obtained for immunohistochemical analyses. Asterisks indicate value significantly different from *water control or **Cd 250 mg/L group (p < .05).

The presence of B lymphocytes in spleen was stimulated by AS. The numbers of these types of lymphocytes were significantly higher in mice that were given AS solution relative to concentrations noted in controls. The added presence of AS solution also exerted a significant effect on levels of B-lymphocytes in mice given high doses of metal. The combined administration of high-dose CdCl₂ with AS significantly elevated B-lymphocyte levels in spleen compared to high-dose CdCl₂ alone (Figure 5).

**DISCUSSION**

Oxidative stress was found to play a major role in chronic Cd-induced hepatic and renal toxicity (Shaikh et al., 1999). Cadmium, a possible human carcinogen, is also a potent immunotoxicant and potentiates oxidative stress, an outcome often followed by induction of mitochondrial-caspase dependent apoptosis (Smalinskiene et al., 2009). It was previously shown that exposure to Cd produced marked suppression of T- and B-lymphocyte activities in lymphoid organs of guinea pigs, and also suppressed the metabolic activity of peritoneal macrophages in these
hosts. Other studies demonstrated that Cd intoxication also results in inhibition of RNA- and DNA-related processes in immune cells, and, ultimately impacts antibody synthesis (Boroskova and Dvoroznakova, 1997). These types of effects helped establish that Cd negatively impacts cellular and humoral immunity (Daum et al., 1993). Several studies noted that circulating lymphocytes differentially redistribute in lymphoid organs in response to Cd exposure (Ohsawa et al., 1983; Fujimaki, 1987; Lafuente et al., 2004).

*Acanthopanax senticosus* (Rupr. et Maxim.) Harms (AS) was found to increase resistance of animals and humans to stress factors (Dardymov, 1976). AS contains a plethora of compounds including antioxidants, immunostimulants, cell proliferation stimulators, and anti-inflammatory agents. Chemically, AS contains complex phenolic compounds and tetracyclic triterpenoids and steroids. The phenolics include phenylpropanoids and lignans, such as eleutheroside E (Phillipson and Anderson, 1984). These agents are structurally similar to catecholamines—mediators of the sympathoadrenal system (SAS) involved in activation of the stress system in the early stages of stress responses. The tetracyclic triterpenoids, such as eleutheroside A and daucosterol, structurally resemble corticosteroids that act as stress hormones involved in the protective inactivation of the stress system (Pearce et al., 1982). Elevation in levels of glutathione (GSH) and heat-shock proteins by AS was previously noted (Pearce et al., 1982; Wiegant et al., 2009). AS appears to also impart protective effects on activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Hong et al., 2009). Our results showed that treatment with AS reduced levels of Cd in spleen that were chronically exposed to this metal. An increase in levels seen in mice given Cd only in GPx and CAT activities indicates that the AS extract was able to restore levels of molecules critical to the function of these enzymes, such as NAD(H) and GSH (Hong et al., 2009). A disappearance of reduced GSH in situ is most likely a result of binding of Cd$^{2+}$ to the thiol (-SH) group on the molecule. This Cd–SH interaction also may explain how metallothionein (MT) (containing four thiolate groups; Andersen, 1984), like GSH, plays a protective role against Cd-induced toxicity in vivo (Ochi et al., 1988).

Panossian and Wikman (2009) proposed that adaptogens help an organism adapt to a stressor (i.e., render it less sensitive to, in the case here, Cd). This is postulated to be achieved by the adaptogen acting rather like a low-molecular-weight “vaccine” to induce a mild activation of the stress system such that, in turn, the host may be able to cope with a more severe stress. In this sense, adaptogens act as challengers and mild stressors (i.e., “stress-mimetics”), giving rise to adaptive and stress-protective effects mainly associated with the HPA axis (a part of the stress system). It is now widely accepted that circulating serum Hsp70 and Hsp72 act as danger signals, that danger theory postulated that immune activation involves danger/non-danger molecular recognition schemes, and that innate immune cells are activated by danger signals derived from stressed or damaged self-proteins (Gallucci and Matzinger, 2001). On this basis, one may conclude that decreases in Cd-related toxicities observed here were mediated, in part, by AS-induced increases in expression of heat-shock proteins Hsp70 and Hsp72. Further study is necessary to verify whether levels of these two HSP are indeed modulated by AS treatment in Cd-exposed hosts.

In conclusion, studies showed that the AS influenced cellular and humoral immune responses in Cd-intoxicated hosts. Specifically, data demonstrated that AS led to increases in the amounts of immune cells macrophages and B and T lymphocytes in host spleens treated chronically with Cd. Whether this outcome was due to an effect of AS on the cells themselves or simply a result of less Cd being present in the organ to exert toxicity remains to be determined.

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DECLARATION OF INTEREST

The authors declare no conflicts of interest. The authors alone are responsible for the content of this article.

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