Published in final edited form as:

Toxicology. 2013 June 7; 308: 146–157. doi:10.1016/j.tox.2013.03.017.

Immunomodulatory effects of black cohosh (*Actaea racemosa*) extract in female B₆C₃F₁/N mice

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Abstract

Black cohosh extracts (BCE; *Actaea racemosa*) are being used worldwide as an alternative to hormone replacement therapy for the management of menstrual and menopausal symptoms, yet the effects of BCE on the immune system are largely unknown. Female B₆C₃F₁/N mice were treated daily with BCE (0, 62.5, 125, 250, 500, or 1000 mg/kg) for 28 days by oral gavage. Liver weights were significantly increased (26%–32%) at the 1000 mg/kg dose. Dose-related increases in mean corpuscular volume and mean corpuscular hemoglobin were observed. Decreasing trends were observed in all thymic T cell populations, with the most notable dose-responsive effects on immature thymocytes. In the spleen, dose-related decreases were observed in all cell phenotypes evaluated, reaching the level of statistical significance at the 1000 mg/kg BCE dose. Splenic natural killer (NK) cell numbers were significantly decreased at all BCE doses, with the exception of absolute NK numbers at the 125 mg/kg dose. No effects were observed on T-dependent antibody responses of the humoral immune system, including the antibody-forming cell response to sheep erythrocytes (sRBC) and IgM antibody levels to both sRBC and keyhole limpet hemocyanin. Cytotoxic T cell (T_{CTL}) activity was increased, as was the mixed leukocyte response in one of two studies. Anti-CD3 mediated proliferation and the delayed-type hypersensitivity response were unaffected. No effects were observed on innate immunity or on bone marrow cellularity and colony-forming units. Overall, BCE exposure in B₆C₃F₁/N mice for 28 days at doses up to 1000 mg/kg had minimal immune effects, with the exception of an increased T_{CTL} response.

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Conflict of Interest Statement

Dr. Kimber L. White, Jr. is the owner of a company, ImmunoTox[®], Inc., which conducts immunotoxicological assessments under Good Laboratory Practices, however none of the research presented here involved his company. Dr. Matthew J. Smith is currently an employee of ImmunoTox[®], Inc., however these studies were completed prior to his present employment. This article may, in part, be the work product of an employee or group of employees of the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), however, the statements, opinions or conclusions contained therein do not necessarily represent the statements, opinions or conclusions of NIEHS, NIH or the United States government.

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Keywords

black cohosh extract; *Actaea racemosa*; immunotoxicity; immunomodulation; herbal medicines; immune system

1. Introduction

Black cohosh (*Actaea racemosa*, formerly *Cimicifuga racemosa*) is a tall perennial native to eastern parts of North America. Extracts from the rhizomes of black cohosh (BCE) are used as over-the-counter herbal supplements for the management and treatment of menstrual and menopausal symptoms, including use as an alternative to hormone replacement therapy (Borrelli and Ernst 2008; Lieberman 1998). The recommended daily dose of black cohosh is 40 to 80 mg of an ethanolic or isopropanolic extract containing 2.5% triterpene glycosides, or approximately 1 to 2 mg of 27-deoxyactein daily (Low Dog et al. 2003; Mahady 2001). However, there are a variety of widely available over-the-counter products (capsules, fluid extracts, etc.) that, when taken as directed, provide daily doses exceeding 1600 mg of black cohosh root. Many of these products are not well-characterized as to what extraction procedure (if any) was utilized in their preparation, nor is their 27-deoxyactein content quantified.

The effectiveness of BCE in the treatment of menopausal symptoms has been thoroughly reviewed (Borrelli and Ernst 2008; Lieberman 1998), although determinations regarding its efficacy and mechanisms of action are far from definitive. In one study, BCE treatment was shown to decrease luteinizing hormone (LH) in menopausal women (Duker et al. 1991). However, another study did not observe the same effects on LH, although they reported that BCE treatment showed “weak” estrogenic activity (Wuttke et al. 2006). Several other reports have demonstrated no estrogenic effects following treatment with BCE in humans (Liske et al. 2002; Reed et al. 2008) and in rodents (Mercado-Feliciano et al. 2012). Immunomodulation has been documented following exposure to a number of compounds with estrogenic effects, including drugs and chemicals such as diethylstilbestrol (Bick et al. 1984; Holsapple et al. 1983) and methoxychlor (Guo et al. 2002b; White et al. 2005), as well as the naturally-occurring phytoestrogens genistein and daidzein, which are found in soy products (Guo et al. 2001; Guo et al. 2002a; Zhang et al. 1997).

In vitro studies have shown that BCE and some of its primary constituents decreased the production of various cytokines in cultures of whole blood or RAW264.7 macrophages, including TNF- α , IL-6, MIP-2, and IL-8 (Hirabayashi et al. 1995; Sakai et al. 1999; Schmid et al. 2009; Yang et al. 2009). BCE has also been reported to inhibit the release of histamine by rat peritoneal mast cells (Kim et al. 2004). Extracts from other *Cimicifuga* species have demonstrated anti-complement activity (Qiu et al. 2006), as well as suppression of concanavalin A-stimulated spleen cell proliferation (Pan et al. 2009) and the mixed leukocyte response (MLR) (Nishida et al. 2003).

Recent subchronic toxicity studies conducted on behalf of the National Toxicology Program (NTP) indicated that BCE exposure for 90 days by oral gavage resulted in decreased thymus weight (manifested primarily as lymphocyte apoptosis) in young adult female Wistar Han rats but not in female B₆C₃F₁/N mice (Mercado-Feliciano et al. 2012). Furthermore, a treatment-related dysfunction of bone marrow erythropoiesis, manifesting as a non-regenerative macrocytic anemia consistent with a megaloblastic disorder, was observed in both species in those studies, although the mice demonstrated a more severe anemia than the rats.

Based on the use pattern of BCE in humans and on the effects observed on the primary lymphoid organs (i.e. the thymus and bone marrow) reported by Mercado-Feliciano et al., the potential for BCE to induce immunotoxicity is of concern. The present studies have evaluated innate, cell-mediated, and humoral immune functions in adult female B₆C₃F₁/N mice following exposure to BCE for 28 days.

2. Materials and Methods

2.1 Test chemical

The BCE used in these studies (CAS #84776-26-1) was a 1:1 ethanol/water extract of black cohosh purchased from PlusPharma, Inc. (Vista, CA; lot 3012782; relative purity 100.9%). A detailed chemical characterization of this lot of BCE has been previously described (Mercado-Feliciano et al. 2012). BCE dosing solutions were prepared weekly in 0.5% methylcellulose, which was used as the vehicle control for these studies.

2.2 Animals and animal exposure

All animal studies were conducted under a protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Female B₆C₃F₁/N mice, obtained from Taconic Farms, Inc. (Germantown, NY), were quarantined for at least one week prior to being placed on study. Mice were between 8 and 10 weeks of age at the beginning of the studies and were housed four per cage in plastic shoebox cages with hardwood bedding. Animals were maintained on NTP-2000 Laboratory Diet, and tap water access was provided *ad libitum*. Animal rooms were maintained between 18 and 26°C with a relative humidity of 40–70% and a 12-hr light/dark cycle.

Each treatment group consisted of eight animals. Five BCE doses were utilized: 62.5, 125, 250, 500, and 1000 mg/kg. The doses, vehicle, and route of exposure were selected to match the dose levels of the 90-day NTP mouse toxicity studies and to encompass the doses where immune effects were observed. Vehicle control animals were given 0.5% methylcellulose at the same time as the BCE-exposed mice. All animals were dosed once daily for 28 days by oral gavage (*po*) in a volume of 0.1 ml/10 g body weight. Body weights were obtained weekly, and dosing volumes were adjusted accordingly. Unless specifically noted, cyclophosphamide (CPS; Sigma Chemical Co., St. Louis, MO) was utilized as the positive control and was administered daily at 50 mg/kg by intraperitoneal (*ip*) injection to positive control animals during the last 4 days of the exposure period (last 5 days for the keyhole limpet hemocyanin [KLH] study). For the natural killer (NK) cell assay, rabbit anti-asialo GM1 antibody (AAGM1, Wako BioProducts, Richmond, VA), diluted in sterile physiological saline, was utilized as the positive control and was administered by intravenous (*iv*) injection of 0.2 ml at a 1:10 dilution 24 hours prior to evaluation of NK cell activity. Maleic vinyl ether (MVE; Hercules Incorporated, Wilmington, DE, USA) was given as the positive control in the mononuclear phagocytic system (MPS) assay and was administered to the animals in a single *iv* injection at a dose of 50 mg/kg 24 hours prior to evaluation of MPS activity.

2.3 Toxicological Studies

2.3.1 Body and organ weights—Animals were weighed on Day 1 (the first day of treatment) and on Days 8, 15, 22 and 29. On Day 29, animals were euthanized by CO₂ inhalation followed by cervical dislocation, and the liver, spleen, lungs, thymus, kidneys, and adrenals were removed, cleaned of connective tissue, and weighed.

2.3.2 Hematology parameters—Blood was obtained from the retro-orbital sinus of mice under CO₂ anesthesia and collected in ethylenediaminetetraacetic acid (EDTA) tubes. The

following hematological parameters were assessed using a Hemavet 1500S (Drew Scientific, Waterbury, CT, USA): the numbers of erythrocytes and leukocytes, leukocyte differentials, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets. The percentage of reticulocytes was evaluated by flow cytometry after staining with Retic-COUNT[®] (BD Biosciences, San Jose, CA, USA).

2.4 Immunological Studies

2.4.1 Thymocyte and splenocyte phenotyping by flow cytometric analysis

Single-cell suspensions of thymocytes and splenocytes were analyzed by flow cytometry to enumerate the various cell populations, using a method previously described (Auttachot et al. 2009). Thymocytes were analyzed for T-lymphocytes and T-lymphocyte subsets only. Splenocytes were analyzed for B-lymphocytes, total T-lymphocytes, T-lymphocyte subsets, NK cells, and macrophages. Antibodies utilized were fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) for enumerating B-lymphocytes, phycoerythrin (PE)-conjugated anti-mouse CD4 for enumerating CD4⁺ T-lymphocytes, FITC-conjugated anti-mouse CD8 for enumerating CD8⁺ T-lymphocytes, Mac-3 antibody conjugated to FITC for macrophages, and anti-mouse NK1.1 conjugated to PE and hamster anti-mouse CD3e conjugated to FITC for NK cells and total T-lymphocytes. Cells that were NK1.1⁺CD3⁻ were identified as NK cells. CD4⁺CD8⁻ cells were identified as T-helper (T_H) lymphocytes, CD4⁻CD8⁺ cells were identified as cytotoxic T-lymphocytes (T_{CTL}), and CD4⁺CD8⁺ cells were identified as immature T-lymphocytes. Isotype-matched irrelevant antibodies were used as controls. Enumeration was conducted on a Becton Dickinson FACScan Flow Cytometer in which log fluorescence intensity was read by setting a live gate on red fluorescence propidium iodide (PI) to eliminate non-viable cells. The data were analyzed using CellQuest software v. 3.2.1 (Becton Dickinson, San Jose, CA). Five thousand cells were counted for each sample.

2.4.2 Spleen IgM antibody-forming cell (AFC) response to the T-dependent

antigen sheep red blood cells (sRBC)—The primary IgM AFC response to sRBC was conducted using the hemolytic plaque assay of Jerne et al. (Jerne et al. 1963) as modified by White et al. (White et al. 2010). The data were expressed in terms of specific activity (AFC/10⁶ splenocytes) and total spleen activity (AFC/spleen).

2.4.3 Serum IgM antibody titers to the T-dependent antigens sRBC and KLH—

An enzyme-linked immunosorbent assay (ELISA) system, developed in our laboratory (Temple et al., 1993), was used with slight modification (Auttachot et al. 2009) to determine serum anti-sRBC IgM antibody titers in the same animals evaluated for the AFC response. Sample starting dilutions were either 1:8 or 1:16 (1:2 for positive control animals), and 10 serial half dilutions of each sample were analyzed. Results of the sRBC ELISA were analyzed using multipoint analysis as previously described (Kawabata et al. 1995).

To determine serum anti-KLH IgM antibody levels, mice were immunized with 2 mg KLH by *iv* injection on Day 24 of the study. Blood was obtained via cardiac puncture under CO₂ anesthesia on Day 29. Serum IgM anti-KLH antibody levels were determined, using multipoint analysis comparing serially diluted samples to the linear portion of the KLH standard curve, using a commercially available mouse IgM anti-KLH antibody standard (BD Pharmingen) (White et al. 2007). The data were expressed in terms of µg/ml.

2.4.4 MLR to DBA/2 mouse spleen cells—The one-way MLR was conducted as described previously (Guo et al. 2000). The incorporation of [³H]-thymidine into

proliferating cells was used as the assay endpoint, and the data were expressed as counts per minute (CPM)/ 10^5 cells.

2.4.5 Anti-CD3 mediated spleen cell proliferation—The proliferation of T-lymphocytes following stimulation with anti-CD3 antibody was evaluated as previously described by Smith et al. (2010) using BD Biocoat T cell activation plates (BD Biosciences). The incorporation of [^3H]-thymidine by proliferating cells was used as the endpoint of the assay, and the data were expressed as CPM/ 2×10^5 cells.

2.4.6 CTL Activity—The cytotoxic activity of T_{CTL} cells against P815 mastocytoma cells was assessed in two phases as described previously (Bradley et al. 1994). Briefly, splenocytes from BCE-treated mice were co-cultured with P815 cells to induce the formation of P815-specific T_{CTL} . After 5 days, the cells were harvested and incubated in 96 well plates in the presence of [^{51}Cr]-labeled P815 cells for 4 hours to assess the ability of the generated T_{CTL} to lyse the mastocytoma cells. One hundred microliters of supernatant from each well was counted on a Wallac 1480 Wizard 3" gamma counter (PerkinElmer, Turku, Finland). Release of [^{51}Cr] into the supernatant was used as the endpoint of the assay, and results were expressed as percent cytotoxicity, calculated as (Experimental release CPM – Spontaneous release CPM)/(Maximum release CPM – Spontaneous release CPM). The maximum release and spontaneous release of [^{51}Cr] into the media were determined by adding 0.1 ml of 0.1% Triton X-100 and complete RPMI media, respectively, to each of 12 replicate cultures containing 0.1 ml of target cells.

2.4.7 Delayed-type hypersensitivity (DTH) response to *C. albicans*—The DTH response to *C. albicans* was conducted as described by Smith and White (2010). Mice were immunized by subcutaneous (*sc*) injection in the right flank with formalin fixed *C. albicans* organisms on Day 21 and challenged with the *C. albicans* antigen chitosan in the right footpad on Day 29. Footpad swelling was measured prior to and 24 ± 2 hours after challenge. Footpad swelling results were calculated as [(Post measurement – Pre measurement) $\times$ 100] and reported in terms of $\text{mm} \times 100$. A challenge only group, which received the chitosan injection on Day 29 without prior immunization with *C. albicans*, was included to control for background footpad swelling.

2.4.8 NK Cell Activity—NK cell activity was assessed as previously described (Wilson et al., 2001) with modification (Auttachoat et al. 2009). Briefly, YAC-1 cells (target cells; ATCC, Manassas, VA) were radiolabeled with [^{51}Cr], adjusted to a final concentration of 5×10^4 viable cells/ml, and added to wells of a 96-well plate in a volume of 100 μl . Spleen cells from BCE-treated mice (effector cells) were added to duplicate wells in a volume of 100 μl at appropriate concentrations to obtain effector:target (E:T) ratios of 200:1, 100:1, 50:1, 25:1, 12.5:1, and 6.25:1. Following a 4 hr incubation, 100 μl of supernatant from each well was counted using a Wallac 1480 Wizard 3" gamma counter. Results were expressed as percent cytotoxicity, calculated as described above for the CTL assay.

2.4.9 MPS Activity—The functional activity of the MPS was evaluated by measuring the vascular clearance and uptake of [^{51}Cr]-labeled sRBC ([^{51}Cr]-sRBC) by the fixed tissue macrophages of the liver, spleen, lung, and thymus. The assay was conducted as previously described (White et al. 1985). Briefly, on Day 29, mice received [^{51}Cr]-sRBC by *iv* injection, and blood was collected from the tail vein at multiple time points over a 30 minute period (60 minutes for positive control animals) to determine the vascular half-life of the radioactive sRBC. Sixty minutes after the injection of the [^{51}Cr]-sRBC, animals were euthanized, exsanguinated, and the liver, spleen, lungs, thymus, and kidneys were removed,

weighed, and counted in a Wallac 1480 Wizard 3" gamma counter. Results were expressed in terms of percent uptake and in terms of CPM/mg of tissue ("specific activity").

2.4.10 Bone Marrow DNA Synthesis, Colony Formation, and Differentials—

Bone marrow cells were obtained by flushing the medullary cavities of the left femur with RPMI containing 2% FBS. Single cell suspensions were prepared by vortex and agitation, and nucleated cells were enumerated and adjusted to a concentration of 3×10^6 cells/ml. For measurement of DNA synthesis, 200 μ l aliquots of each sample were removed, added in triplicate to wells of a 96-well plate, labeled with 1 μ Ci [3 H]-thymidine per well, and incubated for 3 hours at 37°C. Following incubation, cells were harvested using a cell harvester and then counted using a PerkinElmer Microbeta 1480 Trilux Luminescence and Scintillation Counter. DNA synthesis was assessed by quantifying the incorporation of [3 H]-thymidine into the cells. Results are presented in terms of CPM/ 6×10^5 cells.

For the colony formation assays, commercially available MethoCult® kits (STEMCELL Technologies, Vancouver, British Columbia, Canada) were used according to kit instructions. Cells were cultured in a methylcellulose medium containing one of three different colony-stimulating factors (CSF). Recombinant CSF-M (40 ng/ml; R&D Systems, Minneapolis, MN, USA) was used to determine the number of macrophage (M) progenitors, CSF-GM (20 ng/ml; R&D Systems) was used to determine the number of granulocyte-macrophage (GM) progenitors; and erythropoietin (CSF-E; 3 units/ml; STEMCELL Technologies) was used to determine the number of erythrocyte progenitors. The numbers of colony-forming units (CFU) and burst-forming units (BFU) were determined at the end of a 2–5 day incubation period for cultures containing erythropoietin (CFU-E and BFU-E colony counts), and the number of CFU for cultures containing CSF-GM or CSF-M were determined after a 12–13 day incubation period (to obtain CFU-GM and CFU-M colony counts). Data were expressed in terms of CFU/femur and CFU/ 10^5 bone marrow cells for the CFU-GM and CFU-M endpoints, and as both (CFU or BFU)/femur and (CFU or BFU)/ 2×10^5 bone marrow cells for the CFU-E and BFU-E endpoints.

For bone marrow differentials, cell preparations were seeded at 1×10^6 cells per well into 96-well microtiter plates. Cell populations evaluated included: B-cell lineage (CD45R/B220⁺), T-cell lineage (CD3⁺), erythroid lineage (TER-119⁺), granulocytes/monocytes (CD11b⁺), and neutrophils (Gr-1⁺). High expression of Gr-1 indicated a predominantly neutrophil population, whereas intermediate expression of Gr-1 was more indicative of a mixed population containing neutrophils, myelocytes, and other immature progenitors (Hestdal et al. 1991).

Each bone marrow preparation was stained with antibodies as follows: one well contained bone marrow cells, rat anti-mouse CD45R/B220 conjugated to PE and rat anti-mouse CD11b conjugated to FITC. A second well contained bone marrow cells and hamster anti-mouse CD3 conjugated to FITC. A third well contained rat anti-mouse Gr-1 conjugated to FITC and rat anti-mouse TER-119 conjugated to PE. Following the initial staining with antibody and washing with staining buffer, PI was added to each well as a viability stain, and cells were enumerated on a Becton Dickinson FACScan flow cytometer. Five thousand cells were counted for each sample.

2.5 Statistical Analysis

Values presented represent the mean $\pm$ standard error (SE) for 8 mice per group. All statistical analysis was conducted using Data Entry, Modification, and Statistics Programs Version 6.0 (proprietary Apple program). Bartlett's Test for homogeneity (Bartlett 1937) was used to select the type of analysis to be conducted. Homogeneous data were analyzed using a one-way analysis of variance (ANOVA), while non-homogeneous data were

analyzed using a non-parametric ANOVA (Kruskal and Wallis 1952; Wilson 1956). When significant differences occurred, ad hoc analysis to determine differences between the vehicle control and the experimental groups was conducted using Dunnett's Test (1955) for homogeneous data or the Wilcoxon Rank Test (1945) for non-homogeneous data. All positive control groups and the DTH challenge only group were compared individually to the vehicle control using Student's t Test (Sokal and Rohlf 1981). The Jonckheere's Test (1954) was used to test for dose-related trends. In all evaluations, $p < 0.05$ indicated statistically significant differences.

3. Results

3.1 General Toxicological Endpoints

In conducting these studies, one animal in each of three treatment groups (62.5, 500, and 1000 mg/kg) did not survive to study termination. Gross pathological evaluation indicated that each of these animals died as a result of gavage error. All animals receiving BCH treatment tolerated exposure well.

The combined body weight data from multiple studies are shown in Figure 1. No differences were observed in body weights during the course of the studies (Figure 1, left panel). However, in terms of overall weight gain (i.e. Day 29 weight – Day 1 weight), animals exposed to BCE at all doses except for 125 mg/kg gained significantly more weight than vehicle control mice (Figure 1, right panel).

Terminal body weight and selected organ weight data were collected in three studies. The first study was a dedicated organ weight study. The second set of organ weights were obtained when collecting organs for histopathology. In these two studies, animals received no injections of sRBC. The third set of organ weights collected was from the MPS study, in which animals received an injection of radiolabeled sRBC 60 min prior to euthanasia and weighing of the organs. In the majority of the studies, treatment with BCE had no effects on organ weights (data not shown), with the exception of the liver weight. Shown in Figure 2, left panel, are the liver weight results from the dedicated organ weight study, where no effects were observed on absolute liver weights. The center panel of Figure 2 shows the liver weights from the histopathology study. An increasing trend in absolute liver weights was observed, with the increases reaching the level of statistical significance at the high BCE dose level. In the liver weights obtained from the MPS study (right panel of Figure 2), an increasing trend was observed, with the increases again reaching the level of statistical significance at the high dose. In addition to the effects observed on the liver, absolute spleen weights were increased at 500 and 1000 mg/kg in one study (data not shown), however these effects were not observed in any of the other studies conducted.

Table 1 gives the hematology results, while Table 2 gives the leukocyte differential results. Hematological analysis indicated that BCE treatment for 28 days resulted in a dose-dependent increase in MCV, reaching the level of statistical significance at BCE doses greater than or equal to 125 mg/kg. These increases ranged from 5% to 8% as compared to the vehicle control group. In addition, a significant dose-related increasing trend was observed in MCH, although the increases reached the level of statistical significance only at the 500 mg/kg dose (10% increase). The remaining hematology parameters were not significantly affected by BCE exposure. Blood leukocyte differentials were also unaffected when evaluated in terms of absolute values. When evaluated as percent values, increases in the percentage of neutrophils ranging from 27% to 32% were observed at the 62.5, 125, and 1000 mg/kg doses. In addition, the percentage of lymphocytes in the blood was decreased (19%) at the 125 mg/kg dose level only.

3.2 Immunological Endpoints

The results from the thymus cell phenotyping study are presented in Table 3. While a significant decreasing trend in thymus cell numbers was observed, the decreases only reached the level of statistical significance at the 250 mg/kg BCE dose, where cell numbers were decreased 27% when compared to the vehicle control group. In similar fashion, decreasing trends with significant decreases at the 250 mg/kg dose were observed for each of the T-lymphocyte populations in the thymus when evaluated as absolute values. These decreases ranged from 30% to 36%. In addition, a significant decrease (24%) was observed in double positive immature T-lymphocytes at the high dose (1000 mg/kg). When evaluated as percent values, significant decreases were observed in CD3⁺ cells at 125mg/kg and 250 mg/kg BCE. Furthermore, a dose-related decrease in the percentage of immature CD4⁺CD8⁺ T cells was observed, reaching the level of statistical significance at the 500 and 1000 mg/kg doses. No other significant differences in thymocyte phenotypes were observed.

Spleen cell phenotyping results are reported in Table 4. No statistically significant differences were observed in spleen cell numbers, although a decreasing trend was observed. Dose-related decreases were observed in the absolute numbers of B cells, total T cells, T_H cells, T_{CTL} cells, and macrophages, each reaching statistical significance at the 1000 mg/kg BCE dose. In addition, NK cell numbers were significantly decreased at all doses, in a dose-dependent manner, with the exception of the 125 mg/kg dose. Decreases in absolute NK cell numbers ranged from 35% to 56% when compared to the vehicle control. In terms of percent values, each of the spleen cell populations evaluated exhibited dose-related decreases, reaching the level of statistical significance at 500 mg/kg for B cells and T_H cells, and at the 1000 mg/kg dose for total T cells, T_{CTL} cells, and macrophages. The percentage of NK cells was dose-responsively and significantly decreased at all doses; these decreases ranged from 26% to 41%.

BCE treatment for 28 days did not significantly affect the humoral immune component of the immune system. No significant effect was observed on the AFC response when evaluated as either Specific Activity or Total Spleen Activity (Figure 3, panels A and B). Similarly, no effects were observed on serum IgM antibody levels to either sRBC or the soluble KLH antigen (Figure 3, panels C and D, respectively). In contrast to the absence of effects on humoral immunity, mixed results were observed in assays evaluating cell-mediated immunity. As shown in Figure 4, treatment with BCE resulted in a dose-related and statistically significant increase in cytotoxic activity of T_{CTL} cells. Increases in cytotoxic activity were observed for most of the E:T ratios in the animals treated with 125, 250, and 1000 mg/kg of BCE (Figure 4). Consistent with these findings, significant increases were also observed in the proliferative response against allogeneic leukocytes at the 250 and 1000 mg/kg dose levels in one of the two MLR studies conducted (Figure 5B), although these effects were not dose-responsive. In another MLR study (Figure 5A), no significant effects were observed. Similarly, no significant effects were observed in anti-CD3 mediated proliferation of spleen cells (Figure 5C) or on the holistic DTH response to *C. albicans* (Figure 6), which, unlike the CTL assay, evaluates primarily a CD4⁺ T cell response.

BCE treatment did not impact innate immune function, as measured by an overall lack of effect on NK cell activity (Table 5). In addition, treatment with BCE did not affect the functional ability of the fixed macrophages of the MPS to phagocytize sRBC and remove them from the blood in the same manner that the cells of the MPS clear pathogens (Table 6). While a significant decrease was observed in the specific uptake of the lung macrophages at the 250 mg/kg dose, the effect was not dose-responsive. Furthermore, a decrease in activity was not observed when the data were evaluated in terms of percent uptake and appeared to be due to increased lung weight at this dose level.

BCE treatment for 28 days also had little overall effect on the bone marrow of female B₆C₃F₁ mice. Nucleated bone marrow cell numbers were unaffected (Table 7), as was the DNA synthesis of bone marrow cells over a three-hour culture period (Table 8). Furthermore, the numbers of CFU-M, CFU-GM, and CFU-E were also unaffected (Figure 7), with the exception of a decrease (29%) in the number of CFU-E per 2×10⁵ cells in mice treated with 62.5 mg/kg BCE (Figure 7, right panel). This decrease was not dose-responsive or statistically significant when the data were evaluated on a CFU per femur basis. Furthermore, a decrease was not observed in either of the BFU-E parameters, which are precursors to CFU-E (data not shown).

Table 7 gives the absolute and percent values of the various bone marrow cell phenotypes. There were no effects on the absolute numbers of any of the various bone marrow cell populations, with the exception of CD11b⁺ cells, where decreases of 32% and 29% were observed at the 125 and 250 mg/kg BCE exposure levels, respectively. When evaluated in terms of percent values, a significant increase (30%) in the percentage of B-220⁺ cells was observed at the 62.5 mg/kg dose level, but was not observed at higher doses. The percentage of cells expressing intermediate levels of Gr-1 (i.e. Gr-1⁺[Int]) was significantly decreased (15%–19%) at the 62.5, 500, and 1000 mg/kg doses, while significant decreases in percent Gr-1⁺[High] cells, percent total Gr-1⁺ cells, and the percentage of CD11b⁺ cells were observed at all dose levels. The decreases in percent Gr-1⁺[High] cells ranged from 12% to 15%, while decreases in percent total Gr-1⁺ cells ranged from 12% to 16%, and decreases in CD11b⁺ cells ranged from 11% to 17%. Finally, the percentage of TER-119⁺ cells was significantly increased (19% to 33%) at all BCE dose levels.

4. Discussion

An absence of estrogenic effects as measured by the *in vivo* mouse uterotrophic assay has recently been demonstrated in studies using the same formulation of BCE employed herein (Mercado-Feliciano et al. 2012), while the presence of a phytoestrogen (formononetin) in black cohosh rhizomes has been disputed (Avula et al. 2009; Jarry et al. 1985; Jiang et al. 2006; Jiang et al. 2011; Kennelly et al. 2002; Panossian et al. 2004). Interestingly, however, the effects of BCE in the present studies are very similar to the effects of the phytoestrogen genistein (a soy isoflavone) on the immune system of female B₆C₃F₁ mice (the same sex and strain used in these studies). Specifically, genistein treatment resulted in a dose-related increase in T_{CTL} activity and a concurrent lack of effect on the AFC response, anti-CD3-mediated T cell proliferation, the MLR, NK cell activity, and MPS activity (Guo et al. 2001).

Ninety-day toxicity studies conducted in Wistar Han rats have demonstrated that BCE treatment resulted in decreased thymus weight manifesting primarily as lymphocyte apoptosis, however, no effects were observed in female B₆C₃F₁/N mice in that same report (Mercado-Feliciano et al. 2012). The results of the present studies are consistent with the latter findings. Specifically, no effects on thymus weights were observed in B₆C₃F₁/N mice, although we do report dose-related changes in the absolute and percent values of thymus cell phenotypes. The observed dose-related decreases in splenic NK1.1⁺ cells and lack of effect on *ex vivo* NK cell cytotoxic activity suggest a possible enhancement in the activity of the remaining NK cells in the spleens of BCE-exposed mice. This hypothesis is consistent with other reports of effects on NK cell activity in mice following exposure to various plant extracts (Guruvayoorappan and Kuttan 2007; Sheeja and Kuttan 2007; Thejass and Kuttan 2006).

Two *ex vivo* proliferative assays that assess cell-mediated immunity, i.e. anti-CD3 mediated proliferation (in which proliferation occurs as a result of the direct activation of the T cell

receptor complex) and the MLR (which requires appropriate recognition of allogeneic stimulator cells prior to proliferation of responder cells), were unaffected following BCE exposure, with the exception of a non-dose-responsive enhancement in one of the MLR studies. Furthermore, the DTH response to *C. albicans*, which is predominantly a CD4⁺ mediated response, was also unaffected. The *in vivo* DTH, being a holistic assay, requires antigen recognition and processing, T cell proliferation, and the development of effector CD4⁺ T cells capable of releasing appropriate cytokines and immunological factors that culminate in increased vascular permeability and footpad swelling. In contrast, the CTL response is predominantly CD8⁺ mediated, requiring the ultimate development of effector CD8⁺ T cells that are capable of cytolysis of target cells such as the P815 mastocytoma. Previous studies have demonstrated that functional effects are more sensitive than observational parameters in predicting immunotoxicity (Luster et al. 1992), as effects on observational parameters, including spleen cell numbers and/or phenotypes, do not always correlate with changes in immune function. Indeed, the enhancement of the T_{CTL} response following BCH treatment is of particular note in light of the decreased absolute numbers and percentages of T_{CTL} in the spleen, suggesting the possibility that the fewer T_{CTL} in the BCE-treated groups may be primed such that they are capable of greater cytotoxic activity than the T_{CTL} of the vehicle control group.

There are also other reports of the immunoenhancing properties of plant extracts. Extracts of dok din daeng (*Aeginetia indica* Roxb.), a medicinal herb used for centuries in Thailand, have shown the ability to enhance T_{CTL} activity and cell mediated immune responses (Auttachoat et al. 2004b), with a general absence of effects on other components of the immune system (Auttachoat et al. 2004a). In addition, exposure to an ethanolic extract of *Aerva lanata*, a woody perennial native to parts of Africa, increased T_{CTL} activity in BALB/c mice (Siveen and Kuttan 2012). Interestingly, black cohosh rhizomes and dietary supplements have been shown to contain more than 50 distinct triterpene glycosides (Cicek et al. 2011; Jiang et al. 2011). Such plant-derived terpenes and terpenoids have documented immunomodulating and immunoenhancing effects and have been implicated as the primary pharmacologically active constituents of many herbal medicines (Rios 2010). This suggests the possibility that these constituents may be responsible in part for the enhanced T_{CTL} effects observed following treatment with BCE and other plant extracts.

In the present studies, absolute and relative liver weights were increased following BCE treatment for 28 days at 1000 mg/kg. Similarly, the 90-day study conducted by the NTP also reported increases in liver weights, but at BCE dose levels of both 500 mg/kg and 1000 mg/kg. BCE exposure in humans has been linked to the development of hepatitis/autoimmune hepatitis in several case reports (Cohen et al. 2004; Guzman et al. 2009; Lynch et al. 2006; Nisbet and O'Connor 2007; Pierard et al. 2009). Furthermore, increased T_{CTL} activity has been implicated in the destruction of liver parenchymal cells in the pathogenesis of autoimmune hepatitis (Longhi et al. 2010). These reports, along with the increases in T_{CTL} activity reported here, suggest that BCE-mediated effects on CD8⁺ T cells may be one mechanism by which black cohosh exposure contributes to the development of autoimmune hepatitis in humans.

Hematological effects observed following 28 days of exposure to BCE in the female B₆C₃F₁/N mouse are also consistent with effects reported in the 90-day BCE toxicity studies (Mercado-Feliciano et al. 2012). Specifically, the significant dose-responsive increases in MCV and MCH in the present studies foreshadow the effects observed in the longer duration studies, where statistically significant increases were also observed at lower BCE doses. Furthermore, although no significant differences were observed, the decreasing trend in erythrocyte numbers in the present studies is also consistent with the erythropoietic dysfunction observed in the 90-day studies.

To further examine the potential myelotoxic effects of BCE, several evaluations of the bone marrow were conducted, including bone marrow cell DNA synthesis, bone marrow cell phenotyping, and CFU formation following stimulation with various recombinant CSFs. While few significant effects were observed in bone marrow cell phenotypes when evaluated as absolute values, significant decreases in the Gr-1⁺ (i.e. neutrophil and myelocyte) and CD11b⁺ (i.e. granulocyte and monocyte) populations were observed. In addition, a corresponding increase in the percentage of erythroid lineage cells (i.e. TER-119⁺) was observed, which is consistent with the slight, albeit not statistically significant, increase in reticulocyte percentages in the peripheral blood (Table 1). The absence of other remarkable effects on the bone marrow, including the general lack of effect on erythropoietin-stimulated CFU and BFU formation, paired with the macrocytosis and increased MCV (in the absence of a reticulocytosis) observed in this and the 90-day studies conducted by the NTP (Mercado-Feliciano et al. 2012), suggests that BCE has an indirect effect on erythropoiesis that the addition of erythropoietin to bone marrow cell cultures may have masked.

5. Conclusion

These results in female B₆C₃F₁/N mice indicated that 28 days of exposure to BCE at doses up to 1000 mg/kg had little effect on the various components of innate and humoral immunity, as well as cell-mediated immunity, with the exception of an enhancement of the T_{CTL} response. Furthermore, hematological indications of erythropoietic dysfunction were observed that were consistent with and predictive of effects observed in 90-day toxicity studies in the female B₆C₃F₁/N mouse. The NTP is currently investigating the mechanisms underlying the effects of BCE on erythropoiesis and is considering clinical studies to evaluate whether similar effects may occur in women taking BCE.

Acknowledgments

Special thanks to Ronnetta Brown, Jackson Mitchell, Anthony Rapisardi, Dr. Wimolnut Auttachoat, and Dr. Tai Guo for outstanding technical assistance in conducting this research. The authors would also like to acknowledge the outstanding technical assistance of Deborah Musgrove, who was responsible for the conducting the technically challenging bone marrow colony assays. The authors also thank Drs. Minerva Mercado-Feliciano and Michelle C. Cora for their critical review of the manuscript. This work was supported by NTP Contract N01-ES-55538.

Abbreviations

AAGM1	anti-asialo GM1
AFC	antibody-forming cell
BCE	black cohosh extract
BFU	burst-forming unit
CD	cluster of differentiation
CFU	colony-forming unit
CFU-E	erythroid progenitor
CFU-GM	granulocyte-macrophage progenitor
CFU-M	macrophage progenitor
CPS	cyclophosphamide
CSF	colony-stimulating factor
CSF-E	erythropoietin

CSF-GM	granulocyte-macrophage CSF
CSF-M	macrophage CSF
CTL	cytotoxic T lymphocyte
DTH	delayed-type hypersensitivity
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
Ig	immunoglobulin
KLH	keyhole limpet hemocyanin
LH	luteinizing hormone
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MLR	mixed leukocyte response
MPS	mononuclear phagocytic system
MVE	maleic vinyl ether
NK	natural killer
PE	phycoerythrin
PI	propidium iodide
sRBC	sheep red blood cells
T_{CTL}	cytotoxic T cells
T_H	helper T cells
VH	vehicle control

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Figure 1. Body weights and change in body weights in female B₆C₃F₁/N mice treated with BCE for 28 days

Left panel: periodic weights; Right panel: overall body weight gain. Animals were weighed at the start of the study (Day 1) and on Days 8, 15, 22, and 29. Results are expressed in grams (g) and are from 15 combined studies (N = 116–120 animals per group). Asterisks indicate statistically significant differences from VH control; *p < 0.05; **p < 0.01.

Figure 2. Absolute liver weights in female B₆C₃F₁/N mice treated with BCE for 28 days
Results are presented in mg (wet weight) and are from three sets of organ weights obtained (N = 23–24 animals per group). Asterisks indicate statistically significant differences from VH control; **p < 0.01.

Figure 3. Evaluation of the TDAR in female B₆C₃F₁/N mice treated with BCE for 28 days
Mice were immunized with either 7.5×10^7 sRBC or 2 mg KLH by *iv* injection on either day 25 (sRBC) or day 24 (KLH) of the study. Positive control mice received 50 mg/kg CPS daily beginning on the day of immunization. Panel A: AFC Specific Activity (IgM AFC/10⁶ splenocytes); Panel B: AFC Total Spleen Activity (IgM AFC/Spleen); Panel C: serum IgM anti-sRBC antibody titers (Log₂[Titer]); Panel D: serum IgM anti-KLH antibody levels (μg/ml). Asterisks indicate statistically significant differences from VH control; **p < 0.01; N = 7–8 animals per group.

Figure 4. Cytotoxic T cell activity of female B₆C₃F₁/N mice treated with BCE for 28 days
 Mice were treated with BCE for 28 days. Splenocytes were co-cultured with P815 mastocytoma cells in upright cell culture flasks for 5 days, harvested, and then cultured in 96-well plates with [⁵¹Cr]-labeled P815 cells for 4 hr. An aliquot (100 μ l) of supernatant was collected from each well and counted on a gamma counter to determine the release of [⁵¹Cr] into the supernatant following the lysis of the target cells by splenic NK cells. Results are expressed as percent (%) cytotoxicity (N = 8 animals per group). Asterisks indicate statistically significant differences from VH control; *p < 0.05; **p < 0.01.

Figure 5. Mixed leukocyte and anti-CD3 mediated splenic T cell proliferative responses in female B₆C₃F₁/N mice treated with BCE for 28 days

Panels A and B: MLR studies; splenocytes were cultured for 5 days in the presence of mitomycin C-treated DBA/2 allogenic stimulator cells. Panel C: Anti-CD3 mediated proliferation; splenocytes were cultured in the presence or absence of anti-CD3 antibody in 96-well plates for 3 days. All MLR and anti-CD3 cultures were labeled with [³H]-thymidine 18–24 hr prior to harvest. Incorporation of [³H]-thymidine into proliferating cells was used as the endpoint of the assay. Results are expressed as CPM/10⁵ splenocytes for the MLR (N = 7–8 animals per group) and as CPM/2x10⁵ splenocytes for the anti-CD3 assay (N = 8 animals per group). Asterisks indicate statistically significant differences from VH control; *p < 0.05; **p < 0.01.

Figure 6. Delayed-type hypersensitivity response of female B₆C₃F₁/N mice treated with BCE for 28 days

Mice were sensitized on day 21 with formalin-fixed *C. albicans*, and challenged in the right footpad on day 29 with the *C. albicans* antigen, chitosan. Footpad swelling was determined 24 hours post-challenge, and results are reported as mm × 100 (N = 8 animals per group). Asterisks indicate statistically significant differences from VH control; **p < 0.01.

Figure 7. Bone marrow colony forming units in female B₆C₃F₁/N mice treated with BCE for 28 days

The bone marrow from the left femur of BCE-treated mice was collected, teased into individual single-cell suspensions, and cell numbers were adjusted to $1 \times 10^6/\text{ml}$ (CFU-M and CFU-GM) or $2 \times 10^6/\text{ml}$ (CFU-E). An aliquot of cells was cultured with one of three colony-stimulating factors. After culturing for several days, the numbers of CFU-M (left panel), CFU-GM (middle panel), and CFU-E were determined ($N = 7-8$ animals per group). Asterisks indicate statistically significant differences from VH control; * $p < 0.05$; ** $p < 0.01$.

Table 1

Hematology in female B₆C₃F₁/N mice treated with BCE for 28 days

Parameter	Vehicle	BCE (mg/kg)					CPS 50mg/kg	Trend Analysis
		62.5	125	250	500	1000		
Erythrocyte (10 ⁶ /mm ³)	9.09 ± 0.36	9.44 ± 0.27	9.63 ± 0.07	8.91 ± 0.39	8.55 ± 0.27	8.78 ± 0.15	7.72 ± 0.47 *	p 0.01
Reticulocytes (%)	3.86 ± 0.19	3.77 ± 0.16	3.64 ± 0.11	3.72 ± 0.23	3.78 ± 0.13	4.04 ± 0.20	1.36 ± 0.14 **	NS
Hemoglobin (g/dl)	13.8 ± 0.8	14.7 ± 0.4	15.1 ± 0.2	14.2 ± 0.6	14.2 ± 0.2	14.2 ± 0.3	11.8 ± 0.2 *	p 0.05
Hematocrit (%)	44.1 ± 1.9	46.5 ± 1.4	48.9 ± 0.6	45.5 ± 2.0	44.4 ± 1.5	46.1 ± 0.9	40.6 ± 0.7	NS
MCV (fl)	48.5 ± 0.4	49.3 ± 0.2	50.8 ± 0.3 **	51.1 ± 0.2 **	51.9 ± 0.3 **	52.5 ± 0.2 **	49.6 ± 0.3	p 0.01
MCH (pg)	15.1 ± 0.4	15.6 ± 0.2	15.7 ± 0.2	16.0 ± 0.2	16.6 ± 0.5 **	16.2 ± 0.2	15.8 ± 1.1	p 0.01
MCHC (g/dl)	31.2 ± 0.9	31.6 ± 0.4	30.9 ± 0.5	31.2 ± 0.4	32.1 ± 1.1	30.8 ± 0.4	26.1 ± 3.6	NS
Platelets (10 ³ /μl)	620.13 ± 82.18	609.50 ± 51.38	531.75 ± 31.13	684.88 ± 57.64	678.29 ± 121.28	722.63 ± 118.26	1122.00 ± 37.30 **	NS
Leukocytes (10 ³ /mm ³)	6.71 ± 1.02	7.16 ± 0.93	6.15 ± 0.73	4.97 ± 0.88	5.05 ± 0.76	7.01 ± 0.86	2.39 ± 0.31 **	NS

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration Values represent the mean (± SE) from 7 or 8 animals per group;

* p < 0.05;

** p < 0.01

Table 2

Blood leukocyte differentials in female B₆C₃F₁/N mice treated with BCE for 28 days

Parameter	Vehicle	BCE (mg/kg)					CPS 50 mg/kg	Trend Analysis
		62.5	125	250	500	1000		
Leukocyte Differential (Percent Values)								
%Lymphocytes	67.68 ± 1.69	59.74 ± 2.53	54.68 ± 2.01**	58.79 ± 3.08	61.54 ± 3.35	59.96 ± 2.21	72.28 ± 2.65	NS
%Neutrophils	22.44 ± 1.61	28.54 ± 1.79*	28.93 ± 1.28*	28.12 ± 1.86	26.34 ± 1.77	29.56 ± 0.94*	16.09 ± 1.69*	p 0.05
%Monocytes	5.99 ± 0.80	6.11 ± 0.31	8.20 ± 0.69	7.47 ± 0.56	5.81 ± 0.47	6.28 ± 0.77	6.75 ± 0.83	NS
%Eosinophils	2.96 ± 0.49	4.11 ± 0.67	5.75 ± 1.08	3.92 ± 0.93	4.59 ± 1.04	2.99 ± 0.76	3.84 ± 0.76	NS
%Basophils	0.93 ± 0.16	1.50 ± 0.34	2.44 ± 0.45	1.70 ± 0.44	1.71 ± 0.46	1.21 ± 0.30	1.04 ± 0.32	NS
Leukocyte Differential (Absolute Values; 10 ³ /mm ³)								
Lymphocytes	4.44 ± 0.62	4.23 ± 0.56	3.41 ± 0.48	2.79 ± 0.40	2.98 ± 0.35	4.20 ± 0.57	1.69 ± 0.19**	NS
Neutrophils	1.61 ± 0.30	2.08 ± 0.32	1.74 ± 0.17	1.48 ± 0.34	1.39 ± 0.26	2.05 ± 0.22	0.41 ± 0.09**	NS
Monocytes	0.38 ± 0.05	0.45 ± 0.07	0.50 ± 0.07	0.37 ± 0.07	0.30 ± 0.05	0.45 ± 0.07	0.16 ± 0.03**	NS
Eosinophils	0.22 ± 0.06	0.29 ± 0.05	0.36 ± 0.07	0.23 ± 0.07	0.27 ± 0.08	0.22 ± 0.06	0.10 ± 0.02	NS
Basophils	0.07 ± 0.02	0.10 ± 0.02	0.14 ± 0.03	0.10 ± 0.03	0.11 ± 0.04	0.09 ± 0.02	0.03 ± 0.01	NS

Values represent the mean (± SE) from 7 or 8 animals per group;

* p < 0.05;

** p < 0.01

Table 3Thymocyte phenotypes in female B₆C₃F₁/N mice treated with BCE for 28 days

Exposure	Thymus Cells	CD3 ⁺	^a CD4 ⁺ CD8 ⁻	^b CD4 ⁻ CD8 ⁺	^c CD4 ⁺ CD8 ⁺
Absolute Values (×10⁶)					
Vehicle	95.9 ± 4.2	10.7 ± 0.5	9.7 ± 0.4	2.2 ± 0.1	78.4 ± 3.8
BCE					
62.5 mg/kg	98.0 ± 5.3	10.2 ± 0.5	10.2 ± 1.2	2.2 ± 0.2	77.9 ± 4.0
125 mg/kg	104.3 ± 8.7	10.5 ± 1.2	9.8 ± 0.9	2.0 ± 0.2	84.7 ± 6.9
250 mg/kg	69.7 ± 5.3 [*]	6.8 ± 0.6 ^{**}	6.6 ± 0.6 [*]	1.5 ± 0.2 [*]	54.9 ± 4.2 ^{**}
500 mg/kg	85.2 ± 6.8	8.7 ± 0.7	8.3 ± 0.7	1.9 ± 0.2	64.6 ± 5.0
1000 mg/kg	81.4 ± 5.9	9.5 ± 0.7	8.0 ± 1.0	1.8 ± 0.2	59.6 ± 4.2 [*]
CPS (50 mg/kg)	10.5 ± 1.5 ^{**}	4.9 ± 0.8 ^{**}	3.8 ± 0.5 ^{**}	0.9 ± 0.1 ^{**}	1.2 ± 0.6 ^{**}
Trend Analysis	p 0.05	p 0.05	p 0.01	p 0.01	p 0.01
Percent Values					
Vehicle		11.1 ± 0.3	10.2 ± 0.4	2.3 ± 0.1	81.6 ± 0.6
BCE					
62.5 mg/kg		10.5 ± 0.4	10.2 ± 0.7	2.3 ± 0.1	79.6 ± 0.5
125 mg/kg		9.9 ± 0.5 [*]	9.4 ± 0.3	2.0 ± 0.1	81.3 ± 0.7
250 mg/kg		9.7 ± 0.2 ^{**}	9.4 ± 0.2	2.1 ± 0.1	78.7 ± 1.2
500 mg/kg		10.3 ± 0.5	9.8 ± 0.3	2.2 ± 0.1	76.0 ± 1.0 ^{**}
1000 mg/kg		12.0 ± 1.3	9.8 ± 0.9	2.2 ± 0.2	73.3 ± 1.4 ^{**}
CPS (50 mg/kg)		47.5 ± 6.2 ^{**}	38.0 ± 4.5 ^{**}	9.3 ± 0.9 ^{**}	12.0 ± 6.3 ^{**}
Trend Analysis		NS	p 0.05	p 0.05	p 0.01

^a T_H cell;^b T_{CTL} cell;^c Double-positive immature T cell

Values represent the mean (± SE) from 8 animals per group;

^{*} p < 0.05;

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Table 4

Splenocyte phenotypes in female B₆C₃F₁/N mice treated with BCE for 28 days.

Treatment	Spleen Cells	^a Ig ⁺	^b CD3 ⁺	^c CD4 ⁺ CD8 ⁺	^d CD4 ⁺ CD8 ⁺	^e CD4 ⁺ CD8 ⁺	^f NK1.1 ⁺ CD3	^g MAC-3 ⁺
Absolute Values (×10⁶)								
Vehicle	125.0 ± 10.9	64.5 ± 6.5	35.4 ± 3.4	25.6 ± 2.1	11.9 ± 1.3	0.1 ± 0.1	5.7 ± 0.6	6.7 ± 0.7
BCE								
62.5 mg/kg	120.2 ± 9.9	61.5 ± 4.5	35.4 ± 3.5	23.8 ± 2.4	10.5 ± 1.5	0.1 ± 0.1	3.7 ± 0.4 [*]	6.0 ± 0.7
125 mg/kg	123.9 ± 11.4	61.9 ± 4.6	35.3 ± 3.6	24.8 ± 2.3	11.8 ± 1.3	0.1 ± 0.1	4.2 ± 0.5	6.3 ± 0.6
250 mg/kg	127.7 ± 12.8	62.0 ± 5.9	33.8 ± 3.8	22.4 ± 2.4	10.1 ± 1.5	0.1 ± 0.1	3.6 ± 0.3 ^{**}	6.9 ± 0.6
500 mg/kg	110.7 ± 8.1	51.7 ± 3.5	27.9 ± 3.2	19.1 ± 1.9	8.4 ± 1.1	0.1 ± 0.1	3.6 ± 0.4 ^{**}	5.3 ± 0.5
1000 mg/kg	89.3 ± 9.2	40.4 ± 5.0 ^{**}	19.7 ± 3.0 [*]	13.1 ± 2.0 ^{**}	5.5 ± 1.0 ^{**}	0.1 ± 0.1	2.5 ± 0.4 ^{**}	3.6 ± 0.3 ^{**}
CPS (50 mg/kg)	47.0 ± 3.7 ^{**}	20.4 ± 2.2 ^{**}	15.2 ± 1.2 ^{**}	11.9 ± 0.9 ^{**}	4.7 ± 0.5 ^{**}	0.0 ± 0.0	NA	2.3 ± 0.3 ^{**}
AAGMI (1:10)	136.4 ± 11.5	NA	NA	NA	NA	NA	0.7 ± 0.1 ^{**}	NA
Trend Analysis	p 0.05	p 0.01	p 0.01	p 0.01	p 0.01	p 0.01	p 0.01	p 0.01
Percent Values								
Vehicle		51.3 ± 1.3	28.4 ± 1.4	20.7 ± 1.1	9.6 ± 0.7	0.1 ± 0.1	4.6 ± 0.3	5.3 ± 0.2
BCE								
62.5 mg/kg		51.4 ± 0.6	29.3 ± 1.0	19.7 ± 0.8	8.4 ± 0.7	0.1 ± 0.1	3.0 ± 0.2 ^{**}	4.9 ± 0.2
125 mg/kg		50.5 ± 1.2	28.5 ± 1.1	20.2 ± 0.8	9.4 ± 0.5	0.1 ± 0.1	3.4 ± 0.1 ^{**}	5.1 ± 0.2
250 mg/kg		48.7 ± 1.1	26.5 ± 1.5	17.6 ± 0.7	7.7 ± 0.6	0.1 ± 0.1	2.9 ± 0.3 ^{**}	5.5 ± 0.3
500 mg/kg		46.9 ± 0.6 ^{**}	24.6 ± 1.8	17.0 ± 0.7 [*]	7.4 ± 0.6	0.1 ± 0.1	3.2 ± 0.2 ^{**}	4.8 ± 0.2
1000 mg/kg		44.5 ± 2.0 [*]	21.3 ± 1.6 ^{**}	14.0 ± 1.2 ^{**}	5.8 ± 0.6 ^{**}	0.1 ± 0.1	2.7 ± 0.2 ^{**}	4.0 ± 0.2 ^{**}
CPS (50 mg/kg)		42.9 ± 2.0 ^{**}	32.5 ± 1.7	26.0 ± 2.0 [*]	10.5 ± 1.2	0.1 ± 0.1	NA	4.9 ± 0.2
AAGMI (1:10)		NA	NA	NA	NA	NA	0.5 ± 0.1 ^{**}	NA
Trend Analysis		p 0.01	p 0.01	p 0.01	p 0.01	p 0.01	p 0.01	p 0.01

^aB₂-lymphocyte;^bT_H-lymphocyte;^cHelper T-lymphocyte;

^d Cytotoxic T-lymphocyte;
^e Immature T-lymphocyte;
^f Natural Killer cell;
^g Macrophage
Values represent the mean ($\pm$ SE) from 8 animals per group;
* $p < 0.05$;
** $p < 0.01$
NA = Not Applicable

Table 5

Natural killer cell activity female B₆C₃F₁/N mice treated with BCE for 28 days.

Parameter	Vehicle	BCE (mg/kg)					AAGMI 1:10 dilution	Trend Analysis
		62.5	125	250	500	1000		
<u>Effector:Target Ratio</u>								
200:1	14.9 ± 0.9	13.7 ± 1.2	15.8 ± 0.6	18.5 ± 0.9 *	16.0 ± 0.6	15.4 ± 1.1	<1 **	NS
100:1	10.0 ± 0.8	7.9 ± 0.3	10.1 ± 0.4	11.7 ± 0.4	10.5 ± 0.5	9.9 ± 0.8	<1 **	NS
50:1	6.1 ± 0.5	4.6 ± 0.4 *	6.3 ± 0.4	7.2 ± 0.4	5.8 ± 0.3	5.5 ± 0.3	<1 **	NS
25:1	3.4 ± 0.4	2.0 ± 0.3 *	3.2 ± 0.2	3.8 ± 0.3	3.0 ± 0.4	2.7 ± 0.2	<1 **	NS
12.5:1	1.4 ± 0.4	<1	1.1 ± 0.3	1.6 ± 0.3	<1	<1	<1 **	NS
6.25:1	<1	<1	<1	<1	<1	<1	<1 *	NS

Values represent the mean (± SE) from 8 animals per group;

* p < 0.05;

** p < 0.01

NS = Not Significant

Table 6

Functional activity of the mononuclear phagocytic system in female B₆C₃F₁/N mice treated with BCE for 28 days.

Parameter	Vehicle	BCE (mg/kg)					MVE 50 mg/kg	Trend Analysis
		62.5	125	250	500	1000		
Vascular Half-Life (min)	14.0 ± 3.2	11.9 ± 1.5	11.8 ± 1.1	11.0 ± 1.1	10.0 ± 1.0	16.2 ± 4.5	184.6 ± 69.5*	NS
Body Wgt (g)	22.3 ± 0.4	22.8 ± 0.4	23.1 ± 0.7	22.5 ± 0.5	22.2 ± 0.5	22.9 ± 0.5	22.0 ± 0.3	NS
Liver Wgt (mg)	802 ± 34	847 ± 33	877 ± 30	835 ± 33	871 ± 28	1062 ± 47**	1041 ± 35**	p 0.01
% Uptake	26.0 ± 3.0	34.8 ± 1.6	26.4 ± 2.2	30.3 ± 1.6	32.0 ± 2.3	29.8 ± 4.1	5.0 ± 0.7**	NS
cpm/mg	165 ± 20	210 ± 7	155 ± 12	183 ± 8	183 ± 13	144 ± 20	23 ± 3**	NS
Spleen Wgt (mg)	63 ± 3	68 ± 3	76 ± 4	67 ± 4	75 ± 3	72 ± 4	73 ± 2*	p 0.05
% Uptake	11.4 ± 1.1	11.8 ± 0.6	12.4 ± 0.7	11.9 ± 1.2	12.8 ± 0.5	12.6 ± 1.5	8.5 ± 0.9	NS
cpm/mg	928 ± 109	893 ± 60	870 ± 82	902 ± 95	857 ± 45	893 ± 76	574 ± 63*	NS
Lung Wgt (mg)	177 ± 7	185 ± 5	204 ± 10	210 ± 10*	197 ± 5	189 ± 8	172 ± 8	NS
% Uptake	1.7 ± 0.5	1.0 ± 0.1	1.6 ± 0.3	0.9 ± 0.1	1.0 ± 0.1	1.9 ± 0.6	4.8 ± 0.7**	NS
cpm/mg	48 ± 13	29 ± 3	40 ± 8	21 ± 3*	26 ± 3*	52 ± 16	143 ± 27**	NS
Thymus Wgt (mg)	54 ± 2	64 ± 5	62 ± 2	54 ± 3	58 ± 3	49 ± 3	44 ± 2**	NS
% Uptake	0.052 ± 0.013	0.034 ± 0.006	0.041 ± 0.009	0.029 ± 0.007	0.029 ± 0.007	0.039 ± 0.012	0.23 ± 0.03**	NS
cpm/mg	5 ± 1	3 ± 1	3 ± 1	3 ± 1	2 ± 1	4 ± 1	26 ± 3**	NS
Kidney Wgt (mg)	301 ± 9	319 ± 9	318 ± 9	318 ± 7	314 ± 8	329 ± 10	318 ± 7	NS
% Uptake	1.7 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.3 ± 0.2	p 0.05
cpm/mg	28 ± 2	28 ± 4	28 ± 1	32 ± 2	28 ± 1	29 ± 2	21 ± 3	NS

Values represent the mean (± SE) from 8 animals per group (N = 6 for the 1000 mg/kg group);

* p < 0.05;

** p < 0.01

NS = Not Significant

Table 7

Bone marrow cell phenotypes in female B₆C₃F₁/N mice treated with BCE for 28 days.

Treatment	Cells/Femur	^a B220 ⁺	^b CD3 ⁺	^c Gr-1 ⁺ (Int)	^d Gr-1 ⁺ (High)	^e Gr-1 ⁺ (Total)	^f CD11b ⁺	^g TER-119 ⁺
Absolute Values (×10⁶)								
Vehicle	11.91 ± 0.76	1.7 ± 0.2	0.2 ± 0.1	1.6 ± 0.1	4.0 ± 0.3	5.6 ± 0.5	5.9 ± 0.5	3.6 ± 0.3
BCE								
62.5 mg/kg	11.69 ± 1.33	2.2 ± 0.3	0.1 ± 0.1	1.3 ± 0.2	3.4 ± 0.4	4.7 ± 0.6	5.1 ± 0.6	4.0 ± 0.4
125 mg/kg	9.40 ± 0.77	1.6 ± 0.1	0.2 ± 0.1	1.1 ± 0.1	2.7 ± 0.2	3.8 ± 0.3	4.0 ± 0.3 [*]	3.4 ± 0.3
250 mg/kg	9.61 ± 0.72	1.4 ± 0.1	0.1 ± 0.1	1.1 ± 0.1	2.8 ± 0.3	3.9 ± 0.3	4.2 ± 0.3 [*]	3.7 ± 0.3
500 mg/kg	10.56 ± 0.73	1.5 ± 0.2	0.2 ± 0.1	1.1 ± 0.1	3.0 ± 0.3	4.1 ± 0.4	4.3 ± 0.4	4.2 ± 0.3
1000 mg/kg	10.45 ± 1.05	1.4 ± 0.1	0.2 ± 0.1	1.2 ± 0.1	3.1 ± 0.3	4.2 ± 0.5	4.5 ± 0.5	4.0 ± 0.4
CPS (50 mg/kg)	5.51 ± 0.56 ^{**}	0.1 ± 0.1 ^{**}	0.1 ± 0.1 [*]	0.2 ± 0.1 ^{**}	0.2 ± 0.1 ^{**}	0.5 ± 0.1 ^{**}	0.6 ± 0.1 ^{**}	4.5 ± 0.4
Trend Analysis	NS	p 0.01	p 0.01	p 0.01	p 0.05	p 0.05	p 0.05	NS
Percent Values								
Vehicle	14.3 ± 1.4	1.5 ± 0.2	13.2 ± 0.5	33.4 ± 1.1	46.5 ± 1.4	49.2 ± 1.2	30.4 ± 1.5	
BCE								
62.5 mg/kg	18.6 ± 1.1 [*]	1.3 ± 0.1	11.2 ± 0.3 ^{**}	28.8 ± 0.6 ^{**}	39.8 ± 0.7 ^{**}	43.3 ± 0.9 ^{**}	34.6 ± 0.9	
125 mg/kg	16.6 ± 1.1	1.6 ± 0.1	11.9 ± 0.3	28.3 ± 0.5 ^{**}	40.0 ± 0.7 ^{**}	42.9 ± 0.6 ^{**}	36.2 ± 1.1 ^{**}	
250 mg/kg	14.2 ± 0.8	1.4 ± 0.1	11.7 ± 0.3	29.1 ± 1.1 ^{**}	40.7 ± 1.4 ^{**}	43.7 ± 1.3 ^{**}	37.9 ± 1.3 ^{**}	
500 mg/kg	13.7 ± 1.2	1.7 ± 0.1	10.7 ± 0.6 ^{**}	28.3 ± 1.0 ^{**}	39.0 ± 1.3 ^{**}	40.8 ± 1.1 ^{**}	40.3 ± 1.6 ^{**}	
1000 mg/kg	13.4 ± 0.9	1.6 ± 0.1	11.0 ± 0.3 ^{**}	29.4 ± 1.0 [*]	40.2 ± 1.2 ^{**}	42.6 ± 0.8 ^{**}	38.7 ± 0.9 ^{**}	
CPS (50 mg)	1.3 ± 0.1 ^{**}	2.0 ± 0.1	4.2 ± 0.3 ^{**}	4.0 ± 0.6 ^{**}	8.2 ± 0.8 ^{**}	10.1 ± 0.7 ^{**}	82.5 ± 1.2 ^{**}	
Trend Analysis	p 0.05	NS	p 0.01	NS	p 0.05	p 0.01	p 0.01	p 0.01

^aB-lymphocyte lineage;

^bT-lymphocyte lineage;

^cNeutrophils, myelocytes and other immature progenitors;

^dNeutrophils;

^eTotal Gr-1⁺ cells;

^fGranulocytes and monocytes;

^gErythroid lineage

Values represent the mean ($\pm$ SE) from 8 animals per group;

* $p < 0.05$;

** $p < 0.01$

NS = Not Significant

Table 8

Bone marrow DNA synthesis in female B₆C₃F₁/N mice treated with BCE for 28 days.

Parameter	Vehicle	BCE (mg/kg)				CPS 50 mg/kg	Trend Analysis
		62.5	125	250	500	1000	
Cells/Femur ($\times 10^6$)	10.54 $\pm$ 0.80	9.68 $\pm$ 0.78	10.12 $\pm$ 1.02	10.53 $\pm$ 1.21	11.20 $\pm$ 0.83	10.39 $\pm$ 0.48	NS
DNA Synthesis (CPM)	82434 $\pm$ 7149	76498 $\pm$ 4938	72964 $\pm$ 7083	63681 $\pm$ 5353	69615 $\pm$ 4353	69968 $\pm$ 9636	NS

Values represent the mean ($\pm$ SE) from 7 or 8 animals per group;

**
p < 0.01