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FINAL REPORT

EFFECTS OF AMMONIUM PERCHLORATE ON IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID PARAMETERS IN B6C3F1 FEMALE MICE

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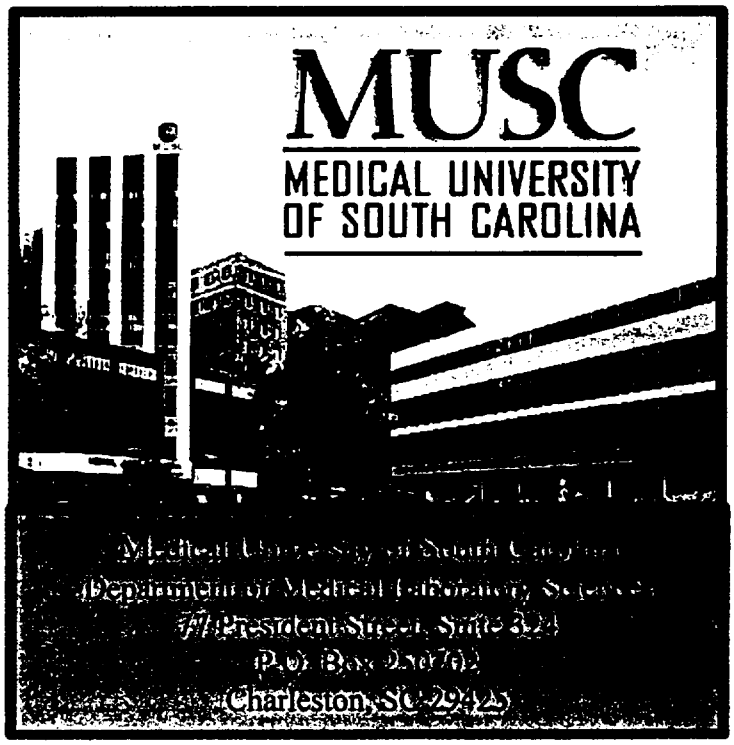

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COMPLIANCE STATEMENT

The analyses in the report entitled "Evaluation of Immunotoxicological, Hematological and Thyroid Parameters after Exposure to Ammonium Perchlorate for 90 Days in Mice" were conducted to be in compliance with the Environmental Protection Agency's Good Laboratory Practices Standards, 40 CFR 792. An audit was conducted by Dr Dave Mattie of AFRL/HEST on 15-16 July 98. No discrepancies were noted.

INTRODUCTION

Ammonium perchlorate (AP, NH_4ClO_4) (AP) is a white, crystalline solid anion that is used as an oxidant in solid propellants for rockets and missiles and in munitions. Its production and storage has resulted in the contamination of soil and water resources on government and contractor installations (TERA, 1996). Since the toxicity of AP ultimately determines the extent to which contaminated sites must be remediated, it is of interest to investigate AP's toxicity.

This study has evaluated a battery of thyroid, immunotoxicological, and hematological parameters to determine the potential effects of AP. Thyroid assessment included quantitation of T3, T4, and TSH in serum, and histopathology of thyroid tissue. Hematological evaluation of the mice included a complete blood count (CBC) with a white blood cell (WBC) differential and the assessment of bone marrow cellularity and stem cell proliferative function. Thus, apparent deficiencies in blood cell maturation or effects on mature blood cell populations could be detected. Immunological parameters include descriptive and functional assays, and two host challenge models. Immune parameters include thymus and spleen weight and cellularity, CD4/CD8 splenocyte and thymocyte subpopulations, natural killer cell (NK) activity, delayed type hypersensitivity (DTH), cytotoxic T cell (CTL) activity, peritoneal macrophage phagocytosis and nitrite production, specific IgM and IgG response to T cell dependent sheep red blood cell (SRBC) challenge, liver and kidney weight. Host challenge models include a challenge with *Listeria* monocytogenes and a cancer challenge using B16F10 melanoma tumors. Immunological parameters assessed specific portions of the immune system, while host challenge models assessed the *in vivo* cooperation of several immunological parameters in resistance to bacterial and tumor cell challenge.

OBJECTIVE

A battery of immunological, hematological and thyroid endpoints were measured in B6C3F1 female mice exposed to AP in drinking water for 14 or 90 days, or 30 days post cessation of a 90-day exposure to AP (120 day). This information will facilitate the determination of a NOAEL or LOAEL for AP.

MATERIALS AND METHODS

Experimental Protocol

B6C3F1 female mice were exposed to AP (0, 0.1, 1.0, 3.0, or 30 mg/kg/day) via drinking water for 14 or 90 days. Immunological, hematological and thyroid parameters were assessed on day 14 or 90, or 30 days post-exposure to 90 days of AP (120-day experiment). Typically, each experiment consisted 6 mice per treatment group for a total 30 mice per experiment. Experiments were repeated at least twice unless indicated otherwise.

Test Article and Vehicle Control Material

Test Article Acquisition: Ammonium perchlorate (AP) (Aldrich catalog #20, 850-7, lot 03907LF) was provided by Dr. David R. Mattie of AFRL/HEST (Operational Toxicology Branch) at Wright Patterson Air Force Base (WPAFB). The ammonium perchlorate is of the same lot used in previous studies at WPAFB. Refer to Appendix C for additional information regarding stability and concentration verification of ammonium perchlorate dosing solutions.

Test Article Storage: AP was stored in a sealed glass container in a dry and explosive proof cabinet.

Test Article Preparation: A primary stock solution of AP was prepared approximately every 1-2 months and a working solution was prepared weekly and stored in sealed glass containers at 6°C. AP (10.0000 g) was weighed on the Mettler balance model AB54 (Serial Number 1116481499, calibration due 1/2000) in a plastic weigh boat. Using a funnel, the AP was transferred to a 200.0 mL volumetric flask. Water obtained from a Barnstead Ultrapure Water System with a 0.2μ final filter was added to the flask. Water was used to rinse both the funnel and weigh boat into the volumetric flask. The water was added to the 200.0 mL mark on the volumetric flask. A teflon stir bar was placed in the flask and the solution was stirred for a minimum of 30 minutes. This (10g/200mL) results in a 50 mg/mL stock solution. Two 2-mL aliquots were sent to Wright Patterson AFB for analysis, and one 2-mL portion was frozen as a retain. The stock solution was poured into a glass bottle, labeled, and placed in a refrigerator. The stock solution was not used until the results of the analysis were verified.

Analysis of Dosing Preparations: Samples of the primary and working stock solutions were sent to Dr. David R. Mattie of AFRL/HEST (Operational Toxicology Branch) WPAFB, for verification of ammonium perchlorate levels. In addition, random water samples from animal water bottles were sent periodically to verify levels of AP. During the past year, there was only one discrepancy with a water sample obtained from an individual water bottle in a low dose treatment group (0.1 mg/kg/day). Calculations to determine AP levels were reviewed and resampling indicated that the level of AP exposure was correct. Refer to Appendix C for additional information regarding stability and concentration verification of ammonium perchlorate dosing solutions.

Animals and Animal Husbandry

Animal Housing: B6C3F1 female mice were purchased from Charles River Breeding Laboratories, Raleigh, NC. Mice used in all experiments were 8-10 weeks of age at the start of each study. The mice were housed in an AAALAC approved facility. During the exposure periods to AP via drinking water, animals were housed individually and bedding, food and water were changed weekly. Mice were observed daily. Sentinel mice were maintained in each animal room and tested quarterly for the following: Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse polio virus, reovirus type 3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, hantaan virus, ectromelia virus, mouse adenovirus FL/K87, polyoma virus, mouse cytomegalovirus, *Encephalitozoon cuniculi*, epizootic diarrhea of infant mice virus, mouse thymic virus, cilia-associated respiratory bacillus, mouse parvovirus, PCR and histopathology for *Helicobacter* species.

Acclimation: Mice were acclimated for at least 7 days prior to the initiation of the studies.

Food Consumption: Mouse chow (Tek Lab Sterilizable Rodent Diet, formula no. 8656) was provided ad libidum. This mouse chow contains approximately 1.89 ppm of iodine.

Water Consumption: Water (with or without AP) was provided ad libidum. Water consumption was measured weekly for each individual mice. In this study, mice consistently consumed an average of 4 mL of water per day.

Environmental Conditions: The mice were maintained in an AAALAC approved facility at 21-24°C (70-75°F), 40-60% relative humidity, and with a 12 hour light/dark cycle. They were housed in cages containing sawdust bedding and covered with filtering bonnets.

Experimental Procedures

Study Group Design: Each experiment consisted of four treatment groups (0.1, 1.0, 3.0, or 30 mg/kg/day of AP) and one control (no AP). Six mice were randomly assigned per group. Various hematological, immunological, and thyroid parameters were measured in mice after exposure to AP for 14 or 90 consecutive days, or 30 days post-exposure to 90 days of AP (120-day experiment). To identify results from each experimental mouse, a number and a unique letter corresponding to each experiment was assigned. All experiments have been repeated at least twice unless otherwise designated.

Justification of Dose Level Selection: The dose levels used in this study were based on previous studies in rats performed at WBAFB. The lower doses (0.1, 1.0, 3.0 mg/kg/day) were incorporated to assist in the determination of a low or no adverse effect level for AP. These lower level exposures were also comparable to expected levels in human exposures.

Randomization and Group Assignment: Mice were randomly assigned to each control or treatment group and cages were labeled accordingly.

Treatment: In all experiments, AP was administered via drinking water to mice housed individually. Control mice received water only. To adjust for the appropriate exposure, initial experiments were performed to determine the amount of water consumed daily by individual mice. Typical daily consumption of water for individual mice was on the average of 4 mL. This information was used to calculate the level of ammonium perchlorate in drinking water to achieve the following exposures: 0, 0.1, 1.0, 3.0, & 30 mg/kg/day. Water bottles were prepared weekly and adjusted to weekly changes in the average body weight of mice for each treatment group. Additionally, control water bottles set in empty cages on the same cage holding racks as the test mice were prepared weekly to determine water loss due to evaporation or random dripping.

Parameters Evaluated

Body weights. Body weight was measured before the beginning of every experiment and weekly thereafter. This information was to adjust for the appropriate level of AP in the drinking water and also to detect any possibility of overt toxicity that may occur as a result of AP exposure. The balance was calibrated daily before each use.

Thyroid histopathology. Mice were sacrificed on day 14, 90, or 120. On these assay days, the thyroid was carefully removed with the aid of a dissecting microscope and immediately placed in 10% buffered formalin. Thyroid slides were prepared, sectioned, and stained with hematoxylin and eosin by the Department of Histology at the Medical University of South Carolina. Slides were forwarded to a board certified veterinary pathologist, Dr. John Latendresse, D.V.M., Ph.D. Thyroid weight was not determined due to the difficulty associated with the dissection of the thyroid gland in mice. For additional information, refer to the report prepared by Dr. John Latendresse, D.V.M., Ph.D. in Appendix B.

Thyroid hormones. Serum hormone levels were measured at 14, 90 and 120 days post exposure to AP. TSH was measured using a radioimmunoassay kit (RIA) (Amersham, NJ) containing an antibody for rat TSH. Anti-rat TSH antibodies have been used to measure mouse TSH in several studies (Goya, R.G., *et al.,* 1995; Enomoto, T., *et al.,* 1990; Kruger, T.E., *et al.,* 1989; Khairallah, M., *et al.,* 1987). Total serum T3 and T4 was measured by means of RIA commercial kits developed for canine T3 and T4 measurement (Diagnostic Products Incorporated, Los Angeles, CA). Levels of serum thyroglobulin have not been determined in this study due to the minimal amount of serum obtainable from mice. Standard operating protocols for the TSH, T3, and T4 radioimmunoassays have been included in Appendix A. Additional mice were gavaged with 400 mg/kg/day for 7 days with propylthiouracil (PTU) suspended in an olive oil vehicle. Serum from these mice was collected 24 hours after the last exposure and this provided positive control serum for the RIAs. PTU induces hypothyroidism with corresponding increases in TSH and decreases in T4 and T3. In addition, select serum samples were sent to a reference laboratory, Antech Diagnostics, to verify serum levels of T4 that were achieved in-house. Antech Diagnostics also used the canine T4 kit provided by DPI.

Organ weights and total cellularity. Kidney, liver, thymus, and spleen weight were measured on day 14, 90 and 120 using a Mettler balance (calibrated before each use). Fatty tissue was trimmed

from all organs prior to weighing. Total cellularity was determined for bone marrow, spleen, thymus, and macrophages using a Coulter Model ZM particle counter (Coulter Electronics, Hileah, FL). To obtain cell suspensions for thymus and spleen, the organs were maintained in 3 mL of sterile RPMI 1640 with 10% fetal calf serum and "mashed" using two sterile frosted slides. The Coulter counter was completely serviced in the spring of 1998 prior to beginning these studies. Prior to use, the Coulter counter was adjusted using cell suspensions that were verified using a hemacytometer. Initially, technical issues related to the Coulter counter were experienced when determining cellularities, however, this has since been corrected.

CD4/CD8 thymic and splenic subpopulations. Spleen or thymus cells were labeled with fluorescent (phycoerythrin or fluorescein isothiocyanate) rat IgG_2 monoclonal antibodies specific for murine CD4 or CD8 (Caltag, Burlingame, CA). In this procedure, single cell suspensions of thymocytes and splenocytes were washed and resuspended in phosphate buffered saline, pH 7.4 containing 0.1% sodium azide and 1% bovine serum albumin. Monoclonal antibodies incubated with cells for at least 30 minutes at 6°C in the dark. Red blood cells were lysed and removed by several washings with phosphate buffered saline, pH 7.4 containing 0.1% sodium azide and 1% bovine serum albumin. Lastly, the cells were fixed with 1% paraformaldehyde and stored at 6°C in the dark. Analysis was performed on a flow cytometer available at the MUSC Medical Laboratory. Non-stained cells and isotypic antibody controls were used to establish gates for the CD4/CD8 subpopulations in thymic and splenic cells. The same gates were maintained for spleen or thymus in all experiments due to the consistency in the labeling and preparation of the cells.

Hematology. Mice were anesthetized with methoxyflurane (Metaphane©, Shering-Plow Inc.) while blood was collected retroorbitally into EDTA coated microtainers and mixed immediately to avoid clot formation. The blood was stored in the refrigerator and sent by courier on the same day of collection to Antech Diagnostics (Farmington, NY) for complete blood count (CBC) analysis with white blood cell (WBC) differential. CBC analysis included erythrocyte and leukocyte number, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Hematology was assessed on day 14 or 90 of AP exposure. In addition, reticulocytes were evaluated after a 90-day exposure to AP and platelet counts were evaluated after 14-day exposure to AP. Blood that was collected from mice was used both for hematology and also serum hormone analysis. Therefore, very little blood was available for additional platelet and reticulocyte analyses.

Stem cell assay. This procedure was based on previous studies assessing granulocyte-macrophage progenitors and erythrocyte progenitors (Bradley, *et al.*, 1966; Burns, L.A., *et al.*, 1994:17:271-315; Phillips, K., *et al.*, 1997). Bone marrow cells were aseptically washed and collected from the medullary cavity of the femur from each mouse. Cells counts were determined using the Coulter counter and 4.5×10^4 total cells were added to Methocult™ medium (GF M3434, Stem Cell Technologies, Inc., Vancouver, BC) for the colony assay. Methocult™ is a methylcellulose medium that contains 30% fetal bovine serum, 1% pokeweed mitogen-stimulated murine spleen cell conditioned medium (contains colony stimulating factors: CFU-GM + CFU-M + CFU-G), 1% bovine serum albumin, 0.9% methylcellulose prepared in Alpha MEM, 10^{-4} M 2-mercaptoethanol and 3 units/ml erythropoietin (CFU-E). Cultures were performed in duplicate and incubated at 37°C and 5% CO_2 for 10 days. Colonies were counted using an inverted microscope and were

defined as 50 cells or more. Identification of the types of colonies was not performed, rather total colony forming units (CFU) per 10^5 bone marrow cells was reported. This was assessed on day 14 or 90 of AP exposure.

Natural killer (NK) activity. NK cells are lymphocytes that possess anti-tumor capability or kill virally infected host cells without prior exposure to the foreign cell. An *in vitro* cytotoxicity assay using ^{51}Cr-labeled Yac-1 cells was used as described previously (Holsapple, M.P., *et al.*, 1988). Spleens were removed on the assay day for each experiment and cytotoxicity testing was performed using a standard 4-hour ^{51}Cr release assay with labeled Yac-1 target cells (ATCC, TIB 160) (Duke, S., *et. al.*, 1985). Splenocyte cell densities were adjusted in complete media (RPMI, 10% fetal calf serum, 50IU penicillin and 50μg streptomycin) using counts obtained from the Coulter counter. Three replicates were prepared of the following splenocyte to Yac-1 ratios, 100:1, 50:1 and 25:1. After the 4-hour incubation at 37°C and 5% CO_2, 25 μL of supernatant was transferred to a 96-well plate containing solid scintillate (LumaPlate, Packard, Meriden, CT). The plate air dried overnight and was counted for 5 minutes after a 10-minute dark delay on the Packard Top Count. Maximum release was determined by adding ^{51}Cr-labeled Yac-1 cells to 0.1N sodium hydroxide to lyse tumor cells. Spontaneous release was determined by incubating only Yac-1 tumor cells in complete media. The results were expressed in lytic units per 10^7 splenocytes using 10% lysis as the reference point (Bryant, J., *et al* 1992). The effector to target (ET) ratios chosen for the NK assay (100:1, 50:1, and 25:1) lie on the linear portion of the curve generated by plotting log ET ratio vs. percent specific lysis for a control experiment which employed 5 different ratios ranging from 200:1 to 10:1. This method is useful in terms of interpretation because it computes lytic units which represent an "average" activity across differing ET ratios and is more robust in its resistance to occasional data aberrations. In addition, preliminary studies indicated correlating results between the TopCount and gamma counter procedures. NK activity was assessed on day 14 or 90 of AP exposure.

Cytotoxic T cell (CTL) activity. Generation of cytotoxic T cells was determined using a standard 4-hour ^{51}Cr-release assay using P815 target cells (Murray, *et al.*, 1985). Mice were immunized via intraperitoneal injection with P815 cells (freshly passaged in DBA-2 mice) on day 3 in the 14-day AP study or day 79 in the 90-day AP study. Eleven days later, the mice were sacrificed and their splenocytes were incubated at 37°C for 4 hours with ^{51}Cr-labeled P815 tumor cells at ET ratios of 100:1, 30:1, 10:1, and 3:1. The supernatant (25 μL) was transferred to a 96-well plate containing solid scintillate (LumaPlate, Packard, Meriden, CT). The plate air dried overnight and was counted for 5 minutes after a 10-minute dark delay on the Packard Top Count. Maximum release was determined by adding ^{51}Cr-labeled P815 cells to 0.1N sodium hydroxide to lyse tumor cells. Spontaneous release was determined by incubating P815 tumor cells only in complete media. The results were expressed in lytic units per 10^7 splenocytes using 10% lysis as the reference point (Bryant, J., *et al.*, 1992). This method is useful in terms of interpretation because it computes lytic units which represent an "average" activity across differing ET ratios and is more robust in its resistance to occasional data aberrations. Preliminary studies indicated correlating results between the TopCount and gamma counter procedure.

Nitrite production by peritoneal macrophages. Peritoneal macrophages were aseptically isolated by peritoneal lavage and incubated 24 hours with 10 μg/mL of lipopolysaccharide (LPS) (Sigma,

St. Louis, MO), 500 Units/mL of interferon-gamma (IFN-y) (Sigma, St. Louis, MO) or both LPS and IFN-y (Keil, D., *et al.*, 1995). Before adding stimulants, macrophages were added to sterile flat bottom 96-well tissue culture plates and incubated for 1 hour in a humidified incubator set at 37°C and 5% CO_2. Cells were washed with prewarmed medium to remove non-adhered leukocytes. Control cells contained macrophages only without any stimulant and background was always less than 0.5 μM. Nitrite was spectrophotometrically measured after the addition of Griess reagent to supernatants from each well. A standard nitrite curve was established for each experiment and data were expressed in μM of nitrite production. Peritoneal macrophage nitrite production was assessed on day 14 or 90 of AP exposure.

Phagocytosis. Peritoneal macrophages were aseptically isolated by peritoneal lavage. The cell density was determined and adjusted to $2x10^5$/well. Macrophages were added to sterile Lab-Tek tissue culture chamber/slides and incubated for 1 hour in a humidified incubator set at 37°C and 5% CO_2. Cells were washed with prewarmed medium to remove non-adhered leukocytes. Washed *Listeria monocytogenes* was added at an approximate ratio of 1 macrophage per 10 bacterial cells. Sterile normal mouse serum (10%) was added to permit opsonization of bacteria. After a 4-hour incubation, the macrophages were aseptically washed 10 times to remove non-phagocytized bacteria. The remaining cells were fixed, stained with Wright's stain, and then a coverslip was applied. The number of bacteria associated per macrophage was determined using a light microscope. To reduce subjectivity in the reading process, at least 2-3 independent reviewers enumerated slides. Phagocytosis was assessed on day 14, 90, or 120 of AP exposure.

Spleen IgM and IgG antibody response to the T-dependent antigen sRBC. Serum titers of the primary IgM and IgG response to sRBC was determined using an ELISA assay system (Temple, L., *et al.*, 1993). The ELISA is a semi-quantitative method to evaluate the amount of total specific antibody secreted in the serum, *in vivo*. In the 14-day AP study, mice were immunized intravenously with sRBC (1×10^8 total cells) on day 9 and serum was collected on day 14 (5 days post challenge) to determine IgM sRBC antibody levels. In the 90-day AP study, mice were immunized intravenously with sRBC (1×10^8 total cells) on day 75 and serum was collected on day 79 (4 days post challenge) and day 90 (15 days post challenge) to determine specific IgM or IgG sRBC antibody levels, respectively. Optimization of the ELISA was performed using pooled normal serum to establish the appropriate titer of sRBC membrane coating antigen (1μg/ml) and the secondary antibody dilution (1: 5,000 for IgM and 1:7,500 for IgG). Controls for non-specific binding were included and were less than 0.070 O.D. (405 nm). Antibody levels were not expressed in terms of protein concentration or activity since a standard for specific antibody was not available. Rather, the antibody titer levels were expressed as the absorbance at nm.

Delayed type hypersensitivity. In the 14-day study, mice were injected intravenously on day 7 with a sublethal challenge of *Listeria monocytogenes*. In the 90-day study, mice were challenged on day 83. A stock of *Listeria monocytogenes* (serotype 4 of strain 19303) was obtained from J. Ann McCay at MCV and maintained at –70°C. Listeria challenges were 2300 and 2700 CFU for the 14 and 90 day studies, respectively. These challenges were close to the reported LD_7 of 3100 CFU (Holsapple, *et al.*, 1985). Seven days after each challenge, mice were euthanized and the spleens were aseptically removed. Splenocyte cell suspensions were prepared and incubated at 37°C and 5% CO_2 with various titers of soluble listeria antigen (SLA) (0.1-8 μg/ml) for 48 hours. After 48

hours, cells were pulsed with ^{3}H-thymidine (0.5 μCi/well). Eight hours later, cells were harvested and ^{3}H-thymidine uptake was measured using the Packard TopCount. The SLA was prepared by initially sonicating a washed pellet of *L. monocytogenes* in sterile saline. Sonicated samples were microcentrifuged and the supernatant was collected and sterile filtered (0.2 microns). A protein assay on the supernatant was done to quantify the amount of soluble *Listeria* antigen. SLA was stored at –70°C.

B16F10 melanoma tumor challenge model. On day 76, mice were injected intravenously with B16F10 melanoma cells (1 x 10^5 total cells/mouse) (purchased from ATCC) (Holsapple, M.P., *et al.*, 1988). Fourteen days following tumor challenge (day 90), mice were sacrificed and the lungs were removed and placed in labeled vials containing Bouin's fixative. The number of nodules was enumerated using a dissecting microscope by at least two independent readers.

Listeria monocytogenes challenge model. A stock of *Listeria monocytogenes* (serotype 4 of strain 19303) was obtained from J. Ann McCay at MCV and maintained at –70°C. Typically, death is used as an endpoint after sublethal challenge ($\approx LD_{10\text{-}20}$) with listeria challenge. In this study, however, death was not used as an endpoint due to the possibility of prolonged suffering in mice. Alternatively, bacterial colony counts were performed on the liver and spleen of mice sacrificed on day 90, 4 days post-intravenous challenge with listeria. The liver and spleen are major sites for listeria replication and bacteria can be detected as soon as 24-72 hours after challenge. In previous studies with mice, an LD_8 was reported at 2000 CFU (Bleavins, M.R., *et al.*, 1995) and an LD_7 was reported at 3100 CFU (Holsapple, *et al*, 1985). Therefore, a target challenge range of 3000 CFU/mouse was determined using the information from previsous studies in addition to range studies performed at MUSC. This challenge level was also consistent with levels of septicemia in range-finding studies 4 days post-challenge. To determine levels of septicemia, liver and spleen were aseptically removed and weighed. Pre-measured portions of each organ were diluted in sterile saline, 1:10, 1:10^2, 1:10^3, 1:10^4, and 1:10^5. One ml of each dilution was added to prewarmed brain heart infusion agar and poured into a sterile petri dish. These plates were incubated overnight at 37°C and enumerated after a 24 hour incubation. Preliminary experiments to determine if AP was directly cytotoxic to *Listeria monocytogenes in vitro* were also performed.

Antinuclear antibody screening. Antinuclear antigens were a gift from ImmunoVision (Springdale, AR) and were employed to screen for autoantibodies in serum from the 14 and 90 day treatment groups *via* ELISA. The following modification of the manufacturer's protocol was utilized. Affinity purified human autoantigens (Sm/RNP, Jo, SCL-70, Ro and La) as well as purified human thyroglobulin were diluted to 10 U/ml in 0.05 M carbonate buffer, pH 9.6. (Note: one unit is defined to be the amount of antigen which generates an absorbance of 1.2 when assayed by ImmunoVision's standard protocol.) Diluted antigen (100 μl) was coated separately onto Nunc Immunoplate 96 well plates and incubated overnight at 4°C. Wells were then blocked for 1 h at room temperature (RT) with phosphate buffered saline containing 1.0% bovine serum albumin (PBSB) Following triplicate washing with PBSB + 0.05 % Tween 20 (PBSBT), 100 μl of serum, diluted 1/25 in PBSBT, was added to the appropriate well(s) an incubated for 1 h at RT. Positive control serum from autoimmune mice was obtained from the laboratory of Dr. Gary Gilkeson (Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC.) Following triplicate washing with PBSBT, horseradish peroxidase-conjugated goat anti-

mouse IgG (Sigma A 3673) was diluted 1: 7500 in PBSBT and 100 µl was added to each well and allowed to incubate for 1 h at RT. Freshly prepared substrate solution (1.0 mM 2,2'-azino-bis(3 ethylbenzthiazoline-6-sulfonic acid in 100 mM citrate buffer, pH 4.2 + 1.0 M urea-H_2O_2) was added to each well (100 µl) and incubated for 10 min. Absorbance at 405 nm was measured on a Packard Instrument (Meridian, CT) plate reader.

STATISTICAL ANALYSES

Where appropriate, data were combined from two or three experiments and evaluated by the Kolmogorov-Smirnov test for normality and Bartlett's test for homogeneity of variance. When the data were characteristic of a normal distribution and equal variance, a two-way analysis of variance (ANOVA) was performed with experiments and treatments as factors. If no interaction was identified due to combining multiple experiments, Tukey's pairwise comparison was performed to determine if significant differences were observed in treatments as compared to the control group ($p<0.05$). If a significant interaction was identified in the two-way ANOVA, data from each experiment were analyzed independently using a one-way ANOVA and Tukey's pairwise comparison ($p<0.05$). When the data were not normally distributed or the variances were not equal, data were analyzed using Kruskal-Wallis test. If the Kruskal-Wallis test was significant, the Mann-Whitney test was used to determine which of the treatments were significantly different as compared to the control group ($p<0.05$). Significant differences have been indicated in graphs and tables with an asterisk symbol ($p<0.05$). It is important to note that the statistical analysis approach applied in this report has been updated from previous reports submitted to the EPA.

MAINTENANCE OF RAW DATA, RECORDS AND SPECIMENS

Bound and chemical resistant lab notebooks were maintained by each individual involved in the project. In addition, at least two tape or disk back-ups of data on the computers were made periodically and stored offsite.

RESULTS

Body weights. No significant differences in body weight were observed between any of the treatment groups as compared to the controls in the 14, 90, or 120 day experiments (Graph 1. p29).

Water consumption and AP exposure. In these studies, actual exposure to AP via drinking water was on the average, within 10% of the target AP exposure levels. It is important to note that mice exposed to 30 mg/kg/day consumed slightly less water on a weekly basis (≈3mL/week less than control animals) and this effect was more evident in the 14-day studies. Consequently, exposure in the high treatment group was on the average for the 14-day studies, 27 mg/kg/day, whereas the 90-day or 120-day studies had a mean exposure of 28 and 29 mg/kg/day of AP respectively (refer to Table 2, p31). Although there is some slight variability, the exposures are still within 10% of the target exposure level.

Thyroid histology. Refer to report prepared by Dr. John Latendresse, D.V.M., Ph.D. in Appendix C.

T4. T4 hormone measurements obtained for each time point were combined for statistical analyses. Data sets for the 14, 90, or 120-day studies were normally distributed and variance was homogeneous. Therefore, ANOVA with Tukey's multicomparison test was used ($p<0.05$). In the 14-day study, significant decreases in T4 levels were observed after exposure to 3.0 and 30 mg/kg/day of AP. In the 90-day study, significant decreases in T4 levels were observed after exposure to 1.0, 3.0 and 30 mg/kg/day of AP. Furthermore, the T4 levels returned to normal (not significantly different from control) 30 days post-cessation of a 90-day AP exposure (120-day) (refer to Tables 3-5, p.32-34). A positive control, propylthiouracil, was included in the T4 radioimmunoassay procedure. As expected, substantially decreased levels of T4 (0.4 ± 0.1 μg/dL) were detected with this kit.

The T4 levels measured in house were compared with those measured at a reference laboratory (Antech Diagnostics, Farmington, NY). Using a paired t-test, the samples measured at MUSC were not significantly different ($p<0.05$) to those measured by Antech Diagnostics (refer to Tables 3-5, p.32-34). The commercial RIA canine T4 kit provided by DPI was also used by Antech Diagnostics to determine mouse serum T4 levels.

T3. T3 hormone measurements for each time point were combined for statistical analyses. Only the 120-day experiment was not considered normal by the Kolmogrov-Smirnov normality test. Consequently, the non-parametric Kruskal-Wallis test was performed on the 120-day data, while ANOVA with Tukey's multicomparison was used for the 14 and 90-day studies. No significant changes in T3 levels were observed after exposure to 0.1, 1.0, 3.0 or 30 mg/kg/day of AP ($p<0.05$) (refer to Tables 3-5, p.32-34). A positive control, propylthiouracil, was included in the T3 radioimmunoassay procedure. As expected, substantially decreased levels of T3 (75 ± 0.0 ng/dL) were detected with this kit.

TSH. TSH hormone measurements obtained for each time point were combined for statistical analyses. Data sets for the 14, 90, or 120-day studies were normally distributed and possessed homogeneity of variance. Consequently, the data were analyzed by ANOVA and Tukey's multiple comparisons ($p<0.05$). No significant changes in TSH levels were observed after exposure to 0.1, 1.0, 3.0 or 30 mg/kg/day of AP as compared to controls (refer to Tables 3-5, p.32-34). A positive control, propylthiouracil, was included in the TSH radioimmunoassay procedure. As expected, substantially increased levels of TSH (11.85 ± 1.85 ng/mL) were detected using this kit.

Organ weights. Organ weights obtained at 14 or 90 days were compiled for statistical analyses. No significant differences as compared to controls were observed in spleen, kidney, and liver weights after exposure to AP for 14 or 90 days. With regard to thymus weight, a significant decrease at 30 mg/kg/day during the 14-day study was detected ($p<0.05$), yet no significant differences were observed at 90 days. These data were expressed as percent of organ to final body gram weight (Tables 6-8, p.35-37).

Organ Cellularity. As indicated in previous interim reports, substantial variability was noted in early experiments regarding inconsistent and erroneous changes in cellularity. However, this was attributed to technical issues that have been since corrected. Where necessary, these experiments

were repeated on at least two or more occasions and no significant changes in thymic, splenic, peritoneal, or bone marrow cellularity were observed after exposure to AP for 14 or 90 days as compared to controls (Tables 9-11, p.38-40). In addition, extra data were included indicating splenocyte and peritoneal cellularity were normal on day 120.

CD4/CD8 thymic and splenic subpopulations. In the 14-day study, no significant changes were observed in splenic lymphocyte CD4/CD8 subpopulations, yet a significant increase was noted in the percent of CD4-/CD8+ thymic lymphocytic subpopulation after exposure to 0.1 and 1.0 mg/kg/day of AP ($p<0.05$) (Tables12-15, p.41-44). In the 90-day study, no significant differences were noted for any of the CD4/CD8 lymphocytic subpopulations both in the spleen and thymus (Table 12-15, p. 41-44). For this reasons, these subpopulations were not assessed on day 120.

Hematology. No significant changes were detected in peripheral blood WBC and RBC counts, RBC indices, hemoglobin, and hematocrit assessed at 14 or 90 days of AP exposure (Tables 16-17, p. 45-46). Therefore, these parameters were not assessed at 120 days. However, some significant differences were observed in the WBC differentials after exposure to AP for 14 days (Tables 18, p. 47). The percent of lymphocytes in the peripheral blood was increased significantly in 3.0 mg/kg/day AP treatment group. Conversely, blood monocytes were decreased significantly in the 1.0, 3.0, and 30 mg/kg/day AP treatment groups. In the 90-day study, the peripheral blood cell populations were normal as compared to control. Thus, differentials were not assessed on day 120.

There is an increasing trend in the percentage of reticulocytes in the peripheral blood in each of the AP treatment groups exposed for 90 days (Table 19, p. 48). Only 3.0 mg/kg/day of AP for 90-days significantly increased the percentage of peripheral reticulocytes. Due to minimal availability of blood obtained from each mouse, reticulocytes have not been evaluated in any additional studies.

Platelet counts were evaluated after a 14-day exposure to AP (Tables 18, p. 47). In the 0, 0.1, 1.0, and 30 mg/kg/day AP treatment groups, five whole blood samples were submitted for platelet count determination. However, in the 3.0 mg/kg/day AP treatment group, only four samples were submitted and one of these was clotted. This resulted in only three specimens for the 3.0 mg/kg/day AP treatment group and a large within group variability as indicated by the standard deviation. Using these data, no significant differences were observed in any of the AP treatment groups as compared to controls. Due to minimal availability of blood obtained from each mouse, platelet counts have not been evaluated in any additional studies.

Stem cell assay. The stem cell assay was performed in one 90-day study and no significant differences were observed as compared to controls (Table 20, p.49). This is consistent with the hematogical profile obtained in this study since several of these parameters were also not affected significantly.

Natural Killer Cell (NK) Activity. Variability in this assay was reported in previous preliminary reports. However, these technical issues have been addressed and these data reported were obtained from at least two or more consistent experiments. In one of the 14-day experiments, NK activity was significantly enhanced in the 1.0 mg/kg/day AP treatment group as compared to controls ($p<0.05$) (Tables 21-22, p.54-55). However, this observation was not consistent in the

second 14-day study. In the 90-day studies, a significant increase in the 30 mg/kg/day treatment group was observed and this effect was persistent after 30 days cessation of AP (day 120).

Cytotoxic T Cell (CTL) Activity. As in the case of the NK assay, some variability in early assays was reported in preliminary reports. The technical issues have since been corrected. As a result, no significant change in CTL activity was observed in mice treated with 0.1, 1.0, 3.0, or 30 mg/kg/day AP for 14 or 90 days (Tables 23-24, p.52-53).

Macrophage Nitrite Production. Nitrite production by peritoneal macrophages was assessed after 14 and 90-day exposure to AP (Tables 25-26, p.54-55). No significant differences in nitrite production of peritoneal macrophages stimulated with either LPS, IFN-γ, or both LPS and IFN-γ were observed in the 14 or 90-day AP studies.

Macrophage Phagocytosis. Phagocytosis was significantly decreased in the 1.0 and 30 mg/kg/day AP treatment groups in the 14-day study (Tables 27-29, p. 56-58). Additonally, phagocytosis was also decreased in the 3.0 mg/kg/day AP treatment group, however this was not statistically significant. Furthermore, significant decreases in phagocytosis were identified in the 0.1, 1.0, 3.0, and 30 mg/kg/day AP treatment groups after exposure to AP for 90 days. Phagocytic function was restored to normal at day 120 in all treatment groups when compared to control.

SRBC specific IgM and IgG antibody responses. No significant changes to *in vivo* IgM or IgG antibody production to specific sRBC antigen was detected using analysis of variance and Tukey's multicomparison. It is important to note that in the 14 day study, IgM was assessed on day 5 after challenge rather than day 4 as in the 90 day study. IgM is reported to peak *in vivo* on day 4 after challenge and this would be a more appropriate time to evaluate these levels. However, the information gained from the 14 day study, eventhough assessed on day 5, is consistent with observations in the 90-day study.

Delayed Type Hypersensitivity. As reported in the methods section, a titer of 0.1-8μg/mL of listeria antigen was used to stimulate lymphocytes *in vitro*. A representative titer of 2 μg/mL of listeria antigen caused optimal lymphocyte proliferative effects in control cells and has been shown in this report (Tables 31-32, p.60-61).. Significant enhancement in splenocyte proliferation were observed in the splenocytes from the 30 mg/kg/day treatment group only ($p<0.05$). This was observed in both of the 14 and 90-day studies.

B16F10 Tumor Resistance Model. Data obtained from two 90-day tumor challenge experiments were combined for statistical analyses (Table 33, p.62). These data were normal and possessed homogeneity of variance. A two-way analysis of variance was performed and no interaction was indicated between experiments. No significant differences were observed in number of tumors present on the lungs in the AP treatment groups as compared to controls ($p=0.281$). Lung tumors were counted independently by at least two persons and the results were similar.

***Listeria monocytogenes* Challenge Model.** Two experiments were performed that identified susceptibility to *Listeria monocytogenes* challenges at a low (2700 CFU) and high (5360 CFU)

immunization levels (Table 34, p.63). Colony counts were expressed as CFU per gram of liver and these data were transformed by log base 10 and expressed as log listeria.

In the experiment using the high challenge level, 6 mice were assigned to each treatment or control group for a total of 30 mice in the experiment. Only CFU per gram of liver weight was determined on day 4 after listeria challenge. Spleens were also cultured during this experiment; however, the titer of the bacteria was too numerous to count (TNTC) in most of the dilutions plated. ANOVA followed by Tukey's multiple comparison test indicated that a significant decrease in CFU/gram of liver was measured in the 3.0 mg/kg/day AP treatment group ($p<0.05$). This was the only treatment group that was significantly different from the control, but it is interesting to note that there is a dose-responsive trend in these data suggesting increased resistance to a high challenge of listeria.

In the experiment using the low listeria challenge level, 8 mice were assigned to each treatment or control group for a total of 40 mice in the experiment. Both spleen and liver from each individual mouse were aseptically cultured and no significant differences in any of the AP treatment groups as compared to controls was identified using a one-way ANOVA and non-parametric Kruskal-Wallis test ($p=0.074$ for liver data and $p=0.438$ for spleen data).

In addition, AP does not have direct bactericidal activity on *Listeria monocytogenes* as determined in preliminary *in vitro* studies. This was performed by adding various concentrations of AP that were similar to calculated expected concentrations *in vivo* directly to brain heart infusion broth and assessing survival of listeria. As a result of this study, it is not likely that AP would have direct cytotoxic effects to listeria *in vivo*.

Antinuclear Antibody Screening. Serum from 14 and 90 day treatment groups were screened for autoantibodies by ELISA using commercially available purified human autoantigen. These antigenic molecules are highly conserved between species and are known to cross-react with autoantibodies from different species (Agris, P.F. *et al.*, 1990; Bullard-Dillard, R., *et al.*, 1992; Balfi, S., *et al.*, 1989; Tollervey, D., *et al.*, 1987; Kuppers, R.C., *et al.*, 1996) Although serum from autoimmune MRL mice was positive for all antigens, no positive signal was seen with serum from either non-AP treated control mice or AP dosed animals.

DISCUSSION AND CONCLUSIONS

The experimental design for this study included a total sample size of 30 mice per experiment with 6 animals per treatment or control group. This sample size was selected because on a typical sacrifice day, it is technically difficult to perform the battery of immunotoxicological tests on much more than 30 mice. In addition, it is not unusual to utilize 6 animals per treatment group and this has been done in several immunotoxicological studies (Dean, J., *et al.*, 1983; Ercal, N., 1996; Smialowicz, R. J., *et al.*, 1994). To increase the power of the study, repeat experiments were often combined to increase the number of animals per treatment group to 12. In these cases, a two-way analysis of variance was applied and typically, no interactions were reported between experiments.

In this study, female B6C3F1 mice were used because they are commonly utilized in immunotoxicology studies. Using the same strain and sex provides standardization in toxicology testing. However, this is also a limiting factor as the reported chemical effects in B6C3F1 mice may not always be representative of other strains of mice. In addition, the use of female B6C3F1 mice may not serve as the most appropriate model for extrapolating to sensitive human populations. Also, incorporating male mice may have strengthened this study, but this would have doubled the expense and time required for the project.

To strengthen this study, positive controls were included in a variety of assays performed in this study. Dexamethasone served as a positive control for many of the functional immune assays because it is known to universally to suppress many immunological parameters. In addition, propylthiouracil served as a positive control in the RIA thyroid assays since this chemical is know to induce a state of hypothyroidism.

Serum thyroid hormone assessment included kits not specifically designed for murine hormones. However, this is not expected to be in issue in the assessment of T3 and T4 serum hormones. Several other studies have utilized the canine kit from DPI to measure murine T3 and T4 (Goya, *et al.*, 1995; Wagle, N.M., *et al.*, 1994; Hotz, K.J., *et al.*, 1997; Sagartz, J.E., *et al.*, 1997). Through personal communication with DPI, it was learned that they do not sell a specific mouse T3 or T4 kit, but in fact, they advocate the use of the canine kit for murine samples. DPI indicated that there is sufficient cross reactivity between murine and canine T3 and T4 to obtain an accurate assessment of murine T3 and T4. Control levels of T3 and T4 obtained from mice in our study were consistent with those reported in the literature (Goya, *et al.*, 1995; Wagle, N.M., *et al.*, 1994; Hotz, K.J., *et al.*, 1997; Sagartz, J.E., *et al.*, 1997). In addition, a comparative study determined that samples tested for T4 at MUSC were not significantly different from samples tested for T4 at a reference laboratory as analyzed by a paired t-test.

Alternatively, assessment of TSH using an antibody specific for rat TSH may not be the ideal approach to measuring mouse TSH levels. However, a monoclonal antibody specific for mouse TSH is not readily available. Anti-rat TSH has been reported to cross-react with mouse TSH and consequently, several studies have reported the application of anti-rat TSH antibodies to measure mouse TSH (Goya, R.G., *et al.*, 1995; Enomoto, T., *et al.*, 1990; Kruger, T.E., *et al.*, 1989; Khairallah, M., *et al.*, 1987). In some cases, the use of a rat antibody to TSH required the use of a crude mouse TSH standard obtainable from the National Pituitary Institute (Los Angeles, CA). However, personal communication with a reference laboratory that performs this testing on a routine basis, indicated that the incorporation of this mouse TSH standard was indeed crude and not the most accurate approach to assessing mouse TSH. In another study, an enzyme-linked immunoassay kit from Sigma Chemical Company was reported to measure TSH levels in mice (Griffen, R.J., *et al*, 1996). However, personal communication with Sigma indicated that they do not offer such a product. Thus, the underlying notion is that measurement of mouse TSH is not a straightforward process and limitations do exist. However, it is encouraging to know that even if with the use of the rat specific TSH antibody, detection of increased levels of murine TSH were measured in serum from positive control mice treated with propylthiouracil. Thus, it is believable that this kit may be efficient in measuring substantial changes in murine TSH levels, yet perhaps

not sensitive enough to detect minimal changes in murine TSH. Therefore, if perchlorate caused substantial changes in TSH in mice, it would be expected that this kit could detect these changes.

The thyroid profile observed in mice of this study is inconsistent to hormone patterns observed in classical hypothyroidism. AP is known to cause hypothyroidism with subsequent increases in TSH and decreases in both circulating T3 and T4. However, the profile obtained in mice exposed to AP was one of no change in TSH and T3, with significant decreases in T4. In addition, histology indicated positive pathological effects in the thyroid (refer to Appendix C). Factors known to affect thyroid hormones include thyroid hormone binding to serum proteins, sex, age, circadian changes, female estrus cycle, environmental temperature, animal housing conditions, animal cage transport and handling (Dohler, *et al*, 1979). Many of these factors were standardized in this study as animals were transported similarly, single housed in controlled conditions, and all mice tested were female of the same age. To control for differences in thyroid hormones due to circadian rhythm, mice were sacrificed in a sequence that avoided the sacrifice of all control mice early in the morning and all high dose groups sacrificed 3 hours later. Due to the variability in hormones and the number of factors that influence these levels, it may be difficult to explain this reported thyroid profile achieved in mice exposed to AP. Interestingly, another study that assessed the effects of humic acids on thyroid hormone levels in mice also obtained a similar thyroid profile as reported in this study (Tien-Shang, *et al.*, 1994, J. Endocrinol. Invest. 17:787-791).

The primary effect observed in mice treated with AP was indicated in decreased phagocytosis function of peritoneal macrophages. Upon cessation of the AP exposure, peritoneal macrophage phagocytic ability was restored. Except for decreased percentages of blood monocytes, other parameters related to macrophages were unaffected such as nitrite production and total peritoneal cellularity. Remarkably, where phagocytosis was decreased almost 50% in some treatment groups, corresponding resistance to *Listeria monocytogenes* was normal. Several immunological parameters have been identified to play a role in resistance to listeria infection including phagocytosis. Thus, this observation may suggest that compensation from other immunological parameters may prevent impaired resistance to infection when phagocytosis is substantially decreased.

It may be argued that the method used to review macrophage phagocytic slides in this study was subjective. To address this issue, additional analyses were included to reduce potential subjectivity in this assay. Briefly, phagocytosis was evaluated by incubating macrophages with listeria on specific slides that were washed, stained, and reviewed. This approach required a reviewer to enumerate bacteria associated with macrophages on prepared slides. To address potential subjectivity associated with this process, two to three reviewers were assigned the same slides and they performed their counts independently. In addition, greater than 100 counts were performed by each reviewer for each sample well. When these data were combined from multiple experiments, no interaction between experiments or reviewers were determined in the two-way analysis of variance while significant and consistent suppression of phagocytosis occurred.

Excluding phagocytosis, few immunological parameters were affected after exposure to AP. Enhanced immune response was detected in the high treatment group only (30 mg/kg/day) in NK assay and this effect was persistant at 120 days. In the DTH assay, enhanced proliferative response

was observed in the high dose treatment group, however, it is not known if this effect is persistent as this parameter was not assessed after a recovery period on day 120. In the WBC differential data, a dose-responsive decrease in blood monocytes was observed. In one treatment group, blood lymphocytes were also decreased. These changes in the WBC differential were detected after a 14-day exposure to AP, however, they did not persist after exposure to AP for 90 days. After 90 days of AP, the percent of reticulocytes were increased in the 3.0 mg/kg/day treatment group only. However, this data is not consistent with the lack of change in bone marrow parameters, RBC numbers and indices. Thus, it is not likely that the results observed in the reticulocyte percentages would be representative of any detrimental effect on hematological parameters. Furthermore, the changes in the parameters described above are overall not suggestive of deleterious changes in immunological function.

Several immunological and hematological parameters were not affected after exposure to AP for 14 or 90 days. These included total WBC and RBC number, hematocrit, hemoglobin, RBC indices, CTL activity, spleen and thymus weight, cellularity and CD4/8 lymphocyte subpopulations, kidney, liver, and body weight, *in vivo* antibody responses to sRBC, macrophage nitrite production, bone marrow cellularity and proliferative function and autoantibody production. It must be noted that a significant change in thymus weight was observed in only one treatment group in the 14-day study, however, this change was not persistent and was normal at 90 days. The reason(s) for this change is not yet known.

As noted in the results section of this report, unusual variability in organ cellularities, NK, and CTL assays were reported previously. This was attributed to technical errors associated with the Coulter cell counter and these problems have been since corrected. The experiments that were affected by this technical issue were repeated on alternate occasions to obtain consistent and reliable data regarding these parameters.

Resistance to tumor challenge was normal in mice treated with AP for 90 days. Overall, this observation is consistent with results obtained from the NK and CTL assays, and lymphocyte CD4/8 subpopulation data from the thymus and spleen. Except for a significant and persistent increase in NK activity in the high treatment group only (30 mg/kg/day), and an increase in thymic CD8+ cells at in the 14-day study only, these parameters were normal as compared to controls. Generally, the lymphocyte subpopulation evaluation indicated that adequate numbers of CD4 and CD8 cells were available and the NK and CTL function was essentially normal. This study suggests these parameters that are integral in eliminating tumors, were not drastically affected to cause changes in resistance to tumor challenge.

Generally, host resistance assays employ a challenge level in the range of an $LD_{10\text{-}20}$. According to previous studies employing this strain of listeria, the challenge level of 2700 CFU would likely be in this range. It has been shown that the challenge level in host resistance assays can affect the profile of resistance or susceptibility to a challenge agent. Thus, a second experiment utilizing a high challenge level of listeria (5360 CFU) provides some indication of the susceptibility to infection during exposure to AP. Although data regarding splenocyte bacterial counts were not available in this study, a dose-responsive trend of increased resistance to listeria was evident in the data obtained from the liver. Furthermore, this effect was significant in the 3.0 mg/kg/day AP

treatment group. Due to differences in sample size, these data obtained in the high challenge experiment (6 animals per treatment) may not be as robust as the low challenge experiment (8 animals per treatment). However, it is interesting to note that even with a smaller sample size, the high challenge level of bacteria did not indicate any evidence of profound immunosuppression and was not remarkably different as that achieved in the low challenge listeria model.

AP does not alter susceptibility to infection after immunization with *Listeria monocytogenes* after a low challenge (2700 CFU). Primary immunological parameters employed in early resistance to listeria include cell-mediated parameters such as macrophage phagocytosis and nitrite production, neutrophils, and interferon-gamma production from T cells. It is interesting to note that of the parameters that would be involved in listeria resistance, AP primarily affected peritoneal macrophage phagocytosis. Macrophage nitrite production, peritoneal macrophage cellularity, the percent of CD4/CD8 thymocytes and splenocytes were not affected. This study suggests that with an almost 50% decrease in phagocytosis function, resistance to listeria infection is normal. This would support redundancy and compensation in the immune system, as other immune parameters integral to resistance to listeria seem to compensate for the deficit in phagocytosis.

Quantitating the bacterial levels on day 4 after challenge with listeria offers a cross-section in time of the progression of listeria infection. Thus, it may be argued that using death as an endpoint would offer more information regarding susceptibility to listeria after exposure to AP. However, day 4 includes the time when peak levels of bacteremia occur in the spleen and liver. In addition, cell-mediated immune functions are involved in combating this infection during the early progression of this disease. Thus, the ability of macrophages to phagocytize and produce nitric oxide to kill listeria is important during this time. In light of this fact, it is interesting that macrophage phagocytic ability is significantly decreased after exposure to AP, yet resistance to listeria infection is not impaired. Consequently, the approach employed in this study to assess host resistance to listeria, complements the information gained from macrophage immune parameters.

To date, this is the first comprehensive immunotoxicological and hematological evaluation for AP. In light of the normal host responses to tumor and listeria challenges after exposure to AP for 90 days, it is not likely that the few alterations in the above immunological or hematological parameters is sufficient to cause detrimental immunosuppression after AP exposure for 90 days in B6C3F1 female mice. As expected, AP targeted the thyroid gland and affected thyroid hormone levels in serum. However, the thyroid effects observed after AP exposure in mice were not similar to the profile demonstrated in classical hypothyroidism and it is possible that this may be attributed to species differences.

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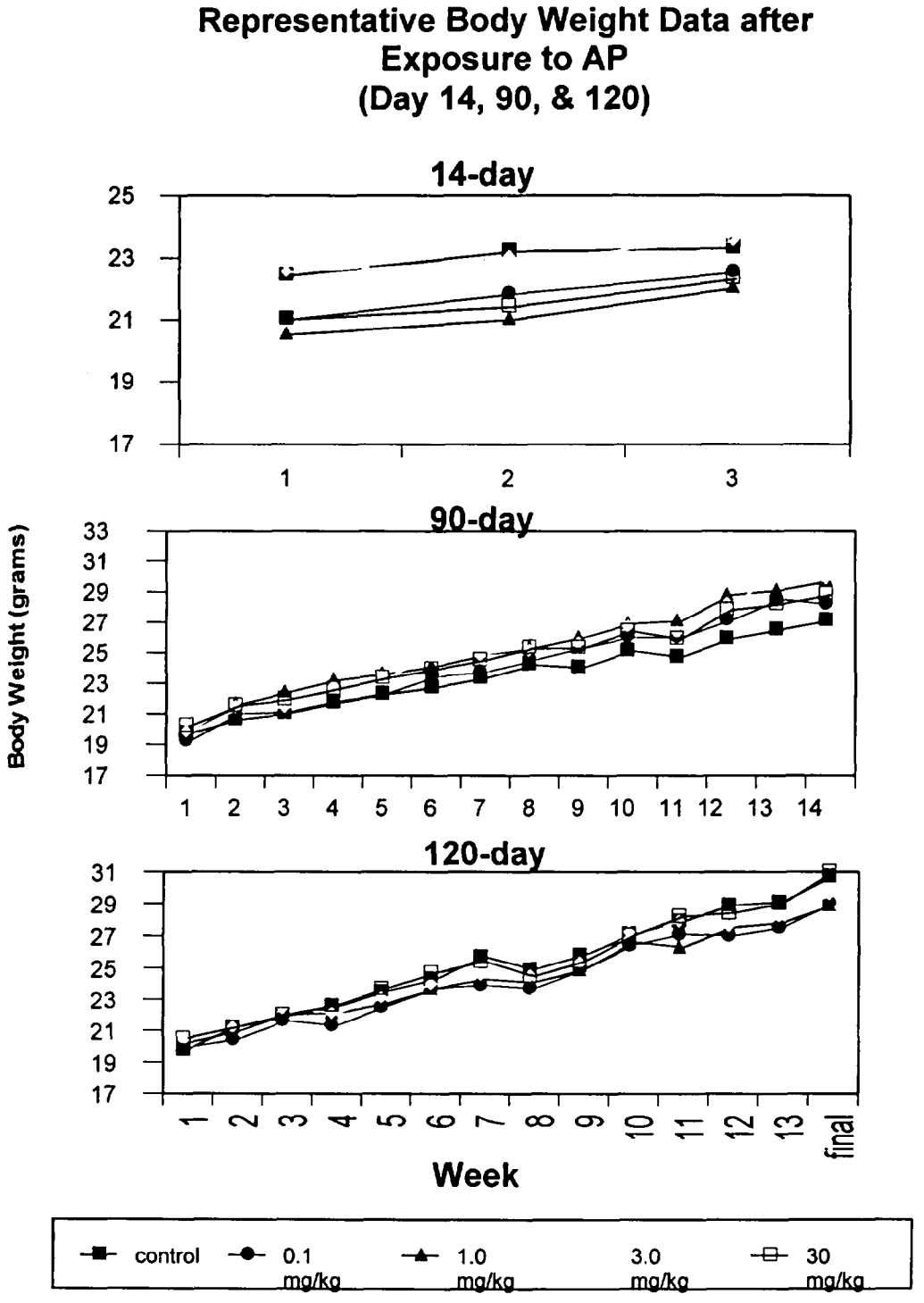

GRAPH 1. Body weight during exposure to AP in drinking water. Body weight was measured weekly and these data were used to calculate ammonium perchlorate drinking water exposures. Analysis of variance using Tukey's multicomparison ($p<0.05$) indicated that there were no significant differences in body weight between any treatments as compared to controls at weekly time points in all 14, 90, and 120-day studies.

TABLE 1.
Comparison of T4 serum samples measured by Antech Diagnostics and MUSC

Serum samples obtained from mice in 3 different studies	Antech Diagnostics Results for Serum T4 (ug/mL)	In-House MUSC Lab Results for Serum T4 (ug/mL)
Mouse 1G	3.9	2.7
Mouse 7G	2.2	2.7
Mouse 14G	2.7	2.2
Mouse 21G	3.3	3.0
Mouse 27G	2.8	3.0
Mouse 1K	3.5	3.7
Mouse 7K	2.9	3.1
Mouse 13K	4.1	4.5
Mouse 19K	2.9	4.1
Mouse 25K	2.0	2.5
Mouse 4C	5.6	3.8
Mouse 8C	3.3	3.2
Mouse 17C	3.1	2.8
Mouse 23C	2.6	2.2
Mouse 27C	2.0	2.5

Table 1. Comparison of T4 levels measured at a reference laboratory as compared to in-house (MUSC) measurements. A paired t-test indicated no significant differences between T4 levels measured at Antech Diagnostics as compared to T4 levels measured at MUSC ($p<0.05$).

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TABLE 2
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF AMMONIUM PERCHLORATE EXPOSURE VIA DRINKING WATER

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
14-DAY	MEAN	NA	0.09133	0.9280	2.7297	27.74
	S.E.M.		0.00329	0.0350	0.0908	1.27
	Number of Experiments		15	15	15	15
90-DAY	MEAN	NA	0.09646	0.9548	2.8461	29.066
	S.E.M.		0.00210	0.0212	0.0862	0.697
	Number of Experiments		16	16	16	16
120-DAY	MEAN	NA	0.09531	0.9715	2.8144	28.517
	S.E.M.		0.0069	0.0285	0.0936	0.388
	Number of Experiments		2	2	2	2

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TABLE 3
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF SERUM THYROID HORMONE LEVELS
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
T4 (μg/dL)	MEAN	3.418	3.3227	3.1773	**2.9545***	**2.6727***
	S.E.M.	0.11	0.0914	0.0935	0.0802	0.0754
	N	22	22	22	22	22
T3 (ng/dL)	MEAN	160.1	142.5	162.14	145.83	135.19
	S.E.M.	11.3	7.28	8.87	5.32	6.26
	N	17	18	18	18	18
TSH (ng/mL)	MEAN	3.972	4.039	3.178	3.793	3.138
	S.E.M.	0.219	0.343	0.393	0.26	0.321
	N	9	7	12	10	12

Positive Control – 400 mg/kg/day for 7 days of propylthiouracil (0.4 ± 0.1 μg/dL of T4, 75 ± 0.0 ng/dL of T3, and 11.85 ± 1.85 ng/mL of TSH)
*Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05)

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TABLE 4
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF SERUM THYROID HORMONE LEVELS
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
T4 (μg/dL)	MEAN	3.4	3.0944	**2.788***	**2.811***	**2.383***
	S.E.M.	0.143	0.0985	0.106	0.0893	0.11
	N	18	18	18	18	18
T3 (ng/dL)	MEAN	132.67	120.6	125.00	128.9	127.91
	S.E.M.	6.73	10.1	7.68	12.2	8.83
	N	12	12	12	11	11
TSH (ng/mL)	MEAN	4.32	3.765	3.719	3.893	4.102
	S.E.M.	0.269	0.17	0.142	0.276	0.206
	N	22	22	22	20	16

Positive Control – 400 mg/kg/day for 7 days of propylthiouracil (T4 = 0.4 ± 0.1 μg/dL; T3 = 75 ± 0.0 ng/dL; and TSH = 11.85 ± 1.85 ng/mL)
* Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05)

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TABLE 5
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF SERUM THYROID HORMONE LEVELS
(SCHEDULED EUTHANASIA – **DAY 120**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
T4 (μg/dL)	MEAN	3.275	3.267	3.242	2.983	3.392
	S.E.M.	0.158	0.141	0.105	0.159	0.152
	N	12	12	12	12	12
T3 (ng/dL)	MEAN	170.9	161.5	167.9	149.9	154.8
	S.E.M.	15.5	18.8	19.4	17.1	13.9
	N	12	11	11	12	11
TSH (ng/mL)	MEAN	3.407	3.856	3.733	3.675	3.63
	S.E.M.	0.175	0.251	0.269	0.248	0.3
	N	10	12	12	12	11

Positive Control – 400 mg/kg/day for 7 days of propylthiouracil (T4 = 0.4 ± 0.1 μg/dL; T3 = 75 ± 0.0 ng/dL; and TSH = 11.85 ± 1.85 ng/mL)
No significant differences were observed in treatment compared to control after exposure to AP (0.0 mg/kg/day)

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TABLE 6
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF ORGAN WEIGHT DATA
(PERCENT OF GRAM BODY WEIGHT)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
THYMUS	MEAN	0.3244	0.30372	0.30539	0.30483	**0.28939***
	S.E.M.	0.0113	0.00856	0.00972	0.00513	0.00775
	N	18	18	18	18	18
SPLEEN	MEAN	0.3790	0.3471	0.3863	0.37679	0.3625
	S.E.M.	0.0101	0.0101	0.0131	0.00987	0.0134
	N	24	24	24	24	24
KIDNEY	MEAN	1.3301	1.3257	1.3535	1.3561	1.3743
	S.E.M.	0.022	0.0217	0.0213	0.0196	0.0249
	N	12	12	12	12	12
LIVER	MEAN	5.427	5.397	5.5054	5.319	5.1119
	S.E.M.	0.110	0.103	0.0976	0.122	0.0974
	N	12	12	12	12	12

* Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05).

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TABLE 7
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF ORGAN WEIGHT DATA
(PERCENT OF GRAM BODY WEIGHT)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
THYMUS	MEAN	0.16054	0.15775	0.16112	0.15479	0.16279
	S.E.M.	0.00754	0.00501	0.00336	0.00540	0.00421
	N	24	24	24	24	24
SPLEEN	MEAN	0.33175	0.3368	0.34221	0.34942	0.35867
	S.E.M.	0.00881	0.0117	0.00712	0.00726	0.00812
	N	24	24	24	24	24
KIDNEY	MEAN	1.1853	1.1759	1.2107	1.2487	1.2601
	S.E.M.	0.0525	0.0230	0.0333	0.0278	0.0293
	N	12	12	12	12	12
LIVER	MEAN	4.966	5.0275	4.8333	4.8682	4.8730
	S.E.M.	0.152	0.0728	0.0707	0.0932	0.0989
	N	12	12	12	12	12

No significant differences observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 8
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF ORGAN WEIGHT DATA
(PERCENT OF GRAM BODY WEIGHT)
(SCHEDULED EUTHANASIA – **DAY 120**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
THYMUS	MEAN	0.14892	0.14417	0.13867	0.13725	0.14583
	S.E.M.	0.00587	0.00845	0.00406	0.00535	0.00498
	N	12	12	12	12	12
SPLEEN	MEAN	0.3289	0.3243	0.03324	0.03292	0.3231
	S.E.M.	0.0148	0.0124	0.0213	0.0125	0.0110
	N	12	12	12	12	12
KIDNEY	MEAN	1.2139	1.2203	1.2038	1.2231	1.1940
	S.E.M.	0.0302	0.0250	0.0299	0.0307	0.0355
	N	12	12	12	12	12
LIVER	MEAN	4.753	4.718	4.7144	4.6610	4.593
	S.E.M.	0.106	0.104	0.0946	0.0877	0.110
	N	12	12	12	12	12

No significant differences observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 9
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF ORGAN TOTAL CELLULARITY DATA
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
THYMUS	MEAN	1.36×10^8	1.12×10^8	1.30×10^8	1.37×10^8	1.45×10^8
	S.E.M.	9.99×10^6	9.12×10^6	1.01×10^7	8.68×10^6	1.29×10^7
	N	12	12	12	12	12
SPLEEN	MEAN	1.02×10^8	1.10×10^8	9.43×10^7	8.67×10^7	8.87×10^7
	S.E.M.	7.08×10^6	4.43×10^6	4.94×10^6	4.73×10^6	3.94×10^6
	N	12	12	12	12	12
MACROPHAGE (PERITONEAL)	MEAN	1.56×10^7	1.43×10^7	1.46×10^7	1.44×10^7	1.32×10^7
	S.E.M.	1.76×10^6	1.69×10^6	2.17×10^6	1.80×10^6	2.32×10^6
	N	12	12	12	12	12
BONE MARROW	MEAN	5.45×10^6	5.32×10^6	5.57×10^6	5.77×10^6	6.75×10^6
	S.E.M.	9.31×10^5	8.43×10^5	9.56×10^5	1.12×10^6	1.28×10^6
	N	12	12	12	12	12

No significant differences observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 10
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF TOTAL ORGAN CELLULARITY
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
THYMUS	MEAN	5.565×10^7	4.825×10^7	4.904×10^7	5.212×10^7	5.514×10^7
	S.E.M.	3.313×10^6	2.172×10^6	2.994×10^6	3.054×10^6	2.892×10^6
	N	24	24	24	24	
SPLEEN	MEAN	1.462×10^8	1.401×10^8	1.353×10^8	1.390×10^8	1.269×10^8
	S.E.M.	8.45×10^6	8.365×10^6	8.058×10^6	1.14×10^7	1.152×10^7
	N	24	24	24	24	24
PERITONEAL MACROPHAGE	MEAN	7.377×10^6	7.428×10^6	7.806×10^6	7.768×10^6	7.105×10^6
	S.E.M.	3.897×10^5	3.503×10^5	4.406×10^5	6.442×10^5	5.283×10^5
	N	24	24	24	24	24
BONE MARROW	MEAN	8.411×10^6	8.666×10^6	8.693×10^6	8.746×10^6	9.24×10^6
	S.E.M.	4.97×10^5	5.752×10^5	6.262×10^5	5.632×10^5	1.064×10^6
	N	24	24	24	24	24

No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 11
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF ORGAN TOTAL CELLULARITY DATA
(SCHEDULED EUTHANASIA – **DAY 120**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
THYMUS	MEAN	ND	ND	ND	ND	ND
	S.E.M.					
	N					
SPLEEN	MEAN	1.245×10^8	1.340×10^8	1.282×10^8	1.356×10^8	1.414×10^8
	S.E.M.	5.775×10^6	9.472×10^6	7.635×10^6	6.142×10^6	7.160×10^6
	N	12	12	12	12	12
MACROPHAGE (PERITONEAL)	MEAN	8.404×10^6	7.803×10^6	7.945×10^6	8.732×10^6	7.591×10^6
	S.E.M.	6.463×10^5	6.198×10^5	6.712×10^5	1.105×10^7	7.020×10^5
	N	12	12	12	12	12
BONE MARROW	MEAN	ND	ND	ND	ND	ND
	S.E.M.					
	N					

ND = Not Done because no significant change observed after 90 day exposure to AP
No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 12
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID
PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF THYMIC LYMPHOCYTE
CD4/8 SUBPOPULATIONS DATA
(PERCENT OF TOTAL CELLS)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
CD4-/CD8+	MEAN	2.783	**3.625***	**3.650***	2.992	3.417
	S.E.M.	0.151	0.226	0.155	0.161	0.180
	N	12	12	12	12	12
CD4+/CD8+	MEAN	80.78	81.98	81.66	81.60	82.483
	S.E.M.	1.13	1.52	1.21	0.723	0.860
	N	12	12	12	12	12
CD4-/CD8-	MEAN	3.367	3.117	3.075	3.292	3.033
	S.E.M.	0.184	0.280	0.229	0.207	0.155
	N	12	12	12	12	12
CD4+/CD8-	MEAN	13.08	11.27	11.60	12.117	11.050
	S.E.M.	1.01	1.16	0.933	0.645	0.740
	N	12	12	12	12	12

*Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05)
No significant change in total splenocyte cellularity occurred in these experiments. Therefore percent of total cells was expressed.

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TABLE 13
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID
PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF THYMIC LYMPHOCYTE
CD4/8 SUBPOPULATIONS DATA
(PERCENT OF TOTAL CELLS)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
CD4-/CD8+	MEAN	2.978	2.856	2.867	2.939	2.783
	S.E.M.	0.149	0.196	0.203	0.158	0.136
	N	18	18	18	18	18
CD4+/CD8+	MEAN	83.683	82.11	84.739	82.51	85.417
	S.E.M.	0.619	1.81	0.568	1.16	0.489
	N	18	18	18	18	18
CD4-/CD8-	MEAN	4.283	4.044	3.867	4.283	3.667
	S.E.M.	0.329	0.281	0.211	0.316	0.182
	N	18	18	18	18	18
CD4+/CD8-	MEAN	9.072	10.99	8.506	10.272	8.128
	S.E.M.	0.458	1.65	0.385	0.915	0.384
	N	18	18	18	18	18

No significant differences were observed in treatments compared to control (0.0 mg/kg/day)
No significant change in total splenocyte cellularity occurred in these experiments. Therefore, percent of total cells was expressed.

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TABLE 14
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID
PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF SPLENIC LYMPHOCYTE
CD4/8 SUBPOPULATIONS DATA
(PERCENT OF TOTAL CELLS)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
CD4-/CD8+	MEAN	11.742	12.417	11.342	11.833	12.550
	S.E.M.	0.441	0.433	0.260	0.414	0.507
	N	12	12	12	12	12
CD4+/CD8+	MEAN	0.1333	0.1500	0.1167	0.1333	0.1417
	S.E.M.	0.0142	0.0230	0.0112	0.0256	0.0229
	N	12	12	12	12	
CD4-/CD8-	MEAN	66.80	64.92	67.733	67.07	64.77
	S.E.M.	0.943	1.04	0.796	1.13	1.38
	N	12	12	12	12	12
CD4+/CD8-	MEAN	21.333	22.492	20.825	20.958	22.550
	S.E.M.	0.596	0.655	0.596	0.775	0.928
	N	12	12	12	12	12

No significant differences were observed in treatments compared to control (0.0 mg/kg/day)
No significant change in total splenocyte cellularity occurred in these experiments. Therefore, percent of total cells was expressed.

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TABLE 15
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID
PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF SPLENIC LYMPHOCYTE
CD4/8 SUBPOPULATIONS DATA
(PERCENT OF TOTAL CELLS)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
CD4-/CD8+	MEAN	11.194	11.789	10.950	11.356	11.300
	S.E.M.	0.262	0.429	0.243	0.245	0.295
	N	18	18	18	18	18
CD4+/CD8+	MEAN	0.3294	0.3944	0.511	0.3222	0.4167
	S.E.M.	0.0498	0.0734	0.154	00540	00776
	N	17	18	18	18	18
CD4-/CD8-	MEAN	67.539	66.88	68.206	67.428	67.67
	S.E.M.	0.876	1.10	0.751	0.741	1.04
	N	18	18	18	18	18
CD4+/CD8-	MEAN	20.244	20.956	20.328	20.900	20.633
	S.E.M.	0.502	0.671	0.609	0.525	0.735
	N	18	18	18	18	18

No significant differences were observed in treatment compared to control (0.0 mg/kg/day)
No significant change in total splenocyte cellularity occurred in these experiments. Therefore percent of total cells was expressed.

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TABLE 16
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF HEMATOLOGY PARAMETERS
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
WHITE BLOOD CELLS (cells x $10^3/\mu L$)	MEAN	2.300	3.133	2.667	2.533	2.920
	S.E.M.	0.619	0.374	0.145	0.291	0.282
	N	6	6	6	6	5
RED BLOOD CELLS (cells x $10^6/\mu L$)	MEAN	8.218	8.498	8.728	8.403	8.838
	S.E.M.	0.272	0.109	0.115	0.396	0.0629
	N	6	6	6	6	5
HEMATOCRIT (Percent)	MEAN	43.27	44.20	50.98	43.68	45.76
	S.E.M.	1.64	0.489	6.47	1.80	0.367
	N	6	6	6	6	5
HEMOGLOBIN (grams/dL)	MEAN	13.45	13.70	13.95	13.917	14.22
	S.E.M.	0.316	0.148	0.141	0.183	0.092
	N	6	6	6	6	5
MCV (fL)	MEAN	52.7	52.2	51.6	52.2	51.8
	S.D.	1.4	0.8	1.1	2.2	1.1
	N	6	6	6	6	5
MCH (pg)	MEAN	16.4	16.1	16.0	16.7	16.1
	S.D.	0.5	0.1	0.3	1.9	0.1
	N	6	6	6	6	5
MCHC (Percent)	MEAN	31.2	31.0	31.1	32.1	31.1
	S.D.	1.5	0.4	0.6	3.1	0.6
	N	6	6	6	6	5
PLATELETS (cells x $10^6/\mu L$)	MEAN	1.04	1.02	1.13	1.25	1.06
	S.D.	0.08	0.07	0.06	.34	0.13
	N	6	6	6	6	5

No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 17
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF HEMATOLOGY PARAMETERS
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
WHITE BLOOD	MEAN	2.892	2.467	2.483	2.683	3.283
CELLS	S.E.M.	0.207	0.258	0.324	0.215	0.359
(cells x $10^3/\mu L$)	N	12	12	12	12	12
RED BLOOD	MEAN	9.026	8.962	8.895	9.3308	8.977
CELLS	S.E.M.	0.142	0.113	0.126	0.0758	0.207
(cells x $10^6/\mu L$)	N	12	12	12	12	12
HEMATOCRIT	MEAN	47.85	48.267	47.742	48.80	46.90
(Percent)	S.E.M.	0.561	0.489	0.642	0.426	0.575
	N	12	12	12	12	12
HEMOGLOBIN	MEAN	14.592	14.425	14.442	15.067	14.658
(grams/dL)	S.E.M.	0.154	0.090	0.140	0.153	0.180
	N	12	12	12	12	12
MCV	MEAN	53.3	54.1	54.0	52.4	52.4
(fL)	S.D.	2.85	2.85	2.25	2.0	2.6
	N	12	12	12	12	12
MCH	MEAN	16.2	16.1	16.3	16.2	16.5
(pg)	S.D.	0.5	0.4	0.6	0.4	0.9
	N	12	12	12	12	12
MCHC	MEAN	30.3	30.0	30.2	30.9	31.3
(Percent)	S.D.	1.2	1.4	0.8	1.2	1.1
	N	12	12	12	12	12
RETICULOCYTES	MEAN	0.7	3.1	3.5	**4.3***	3.3
(Percent)	S.D.	0.7	1.5	2.5	3.2	1.3
	N	6	6	6	6	5

* Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05)

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TABLE 18
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF PERIPHERAL BLOOD WBC DIFFERENTIAL DATA
(PERCENT OF TOTAL CELLS)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
NEUTROPHILS	MEAN	14.95	14.29	13.629	11.938	14.45
	S.E.M.	1.16	1.86	0.916	0.954	1.60
	N	12	12	12	12	10
LYMPHOCYTES	MEAN	75.80	79.08	81.18	**83.10***	79.41
	S.E.M.	1.54	2.77	1.24	1.52	2.11
	N	12	12	12	12	12
MONOCYTES	MEAN	6.879	4.67	**4.00✣**	**3.167✣**	**3.385✣**
	S.E.M.	0.866	1.10	0.544	0.494	0.475
	N	12	12	12	12	10

* Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05)
✣ Significantly different from control (0.0 mg/kg/day) using Mann-Whitney Test (p-value < 0.05)
No significant change in total WBC number occurred in these experiments. Therefore percent of total cells was expressed.

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TABLE 19
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID
PARAMETERS IN B6C3F1 FEMALE MICE

SUMMARY OF PERIPHERAL BLOOD
WBC DIFFERENTIAL DATA
(PERCENT OF TOTAL CELLS)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
NEUTROPHILS	MEAN	13.83	14.50	15.75	13.625	12.92
	S.E.M.	1.08	0.935	0.867	0.983	1.66
	N	12	12	12	12	12
LYMPHOCYTES	MEAN	80.96	79.79	79.29	81.48	83.23
	S.E.M.	1.61	1.18	1.45	1.15	1.69
	N	12	12	12	12	12
MONOCYTES	MEAN	4.667	5.333	5.208	4.567	3.308
	S.E.M.	0.689	0.655	0.836	0.581	0.380
	N	12	12	12	12	12

No significant differences were observed in treatment compared to control (0.0 mg/kg/day)
No significant change in total WBC number occurred in these experiments. Therefore percent of total cells was expressed.

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TABLE 20
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

BONE MARROW CELL PROLIFERATION ASSAY
(EXPRESSED AS COLONY FORMING UNITS/10^5 CELLS)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
BONE MARROW ASSAY	MEAN	126.8	142.43	173.7	142.9	133.34
	S.E.M.	11.8	8.06	41.6	13.7	8.32
	N	6	6	6	6	6

Positive Control = 50 mg/kg/day for 2 days of Dexamethasone (87.88 ± 3.03 CFU/10^5 cells)
No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 21
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

NATURAL KILLER CELL ASSAY
(EXPRESSED AS LYTIC UNITS/10^7 CELLS)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
ASSAY #1	MEAN	20.00	16.917	21.15	26.42	23.583
	S.E.M.	1.87	0.898	2.58	1.62	0.905
	N	6	6	6	6	6
ASSAY #2	MEAN	30.68	35.47	**46.62***	30.88	39.07
	S.E.M.	2.28	3.57	3.05	1.95	1.87
	N	6	6	6	6	6

Positive Control = 50 mg/kg/day for 2 days of Dexamethasone (6.4 ± 0.0 lytic units/10^7 cells)
*Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05)

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TABLE 22
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

NATURAL KILLER CELL ASSAY
(EXPRESSED AS LYTIC UNITS/10^7 CELLS)
(SCHEDULED EUTHANASIA – **DAY 90 and 120**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
90-DAY ASSAY	MEAN	26.22	28.98	27.76	35.29	**40.99***
	S.E.M.	4.76	3.06	4.06	4.90	3.46
	N	12	12	12	12	12
120-DAY ASSAY	MEAN	17.68	23.20	21.77	24.93	**32.98***
	S.E.M.	2.27	2.14	2.74	2.79	2.22
	N	6	6	6	6	6

90-Day Positive Control = 50 mg/kg/day for 2 days of dexamethasone (1.90 ± 1.20 lytic units/10^7 cells)
120-Day Positive Control = 50 mg/kg/day for 2 days of dexamethasone (3.450 ± 0.150 lytic units/10^7 cells)
* Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05)

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TABLE 23
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

CYTOTOXIC T LYMPHOCYTE ASSAY
(EXPRESSED AS LYTIC UNITS/10^7 CELLS)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
CTL ASSAY	MEAN	90.42	113.4	83.0	118.3	90.30
	S.E.M.	7.83	19.4	10.7	16.4	9.73
	N	6	6	6	6	6

No significant differences were observed in treatments compared to controls (0.0 mg/kg/day)

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TABLE 24
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

CYTOTOXIC T LYMPHOCYTE ASSAY
(EXPRESSED AS LYTIC UNITS/10^7 CELLS)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
CTL ASSAY	MEAN	129.9	127.5	141.5	137.2	140.2
	S.E.M.	14.6	17.9	17.2	19.7	19.6
	N	12	12	12	12	12

Positive Control = 50 mg/kg/day for 2 days of Dexamethasone (27.30 ± 5.80 lytic units/10^7 cells)
No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 25
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

NITRITE PRODUCTION BY PERITONEAL MACROPHAGES
(μM OF NITRITE PER 2 x 10^5 CELLS)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
IFN-γ	MEAN	11.10	9.829	10.66	8.71	13.18
(500 units/mL)	S.E.M.	1.43	0.754	1.52	1.01	1.31
	N	18	18	17	18	16
LPS	MEAN	15.37	15.09	13.67	14.85	17.82
(10 μg/mL)	S.E.M.	1.28	1.07	1.09	1.23	1.16
	N	18	18	18	18	15
IFN-γ & LPS	MEAN	24.78	24.39	22.07	23.00	27.91
(500 units/mL &	S.E.M.	2.23	1.39	1.62	1.46	1.94
10 μg/mL)	N	18	18	18	18	16

No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 26
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

NITRITE PRODUCTION BY PERITONEAL MACROPHAGES
(μM OF NITRITE PER 2 x 10^5 CELLS)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
IFN-γ	MEAN	9.05	8.72	7.90	8.519	10.97
(500 units/mL)	S.E.M.	1.23	1.19	1.06	0.660	1.29
	N	11	12	12	11	12
LPS	MEAN	36.00	37.09	30.22	23.09	30.37
(10 μg/mL)	S.E.M.	6.31	3.19	3.51	2.47	5.19
	N	6	6	6	5	6
IFN-γ & LPS	MEAN	34.08	32.40	33.27	30.95	36.05
(500 units/mL &	S.E.M.	7.06	6.54	6.54	5.38	7.87
10 μg/mL)	N	12	12	12	11	12

No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 27
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID
PARAMETERS IN B6C3F1 FEMALE MICE

MEAN NUMBER OF LISTERIA MONOCYTOGENES ASSOCIATED WITH PERITONEAL MACROPHAGES (PHAGOCYTOSIS)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
PHAGOCYTOSIS ASSAY	MEAN	18.47	16.57	**13.81***	14.44	**11.40****
	S.E.M.	1.29	1.33	0.894	1.38	0.701
	N	12	12	12	12	12

Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (*p-value < 0.05, **p-value < 0.01)

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TABLE 28
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID
PARAMETERS IN B6C3F1 FEMALE MICE

MEAN NUMBER OF LISTERIA MONOCYTOGENES ASSOCIATED WITH PERITONEAL MACROPHAGES (PHAGOCYTOSIS)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
PHAGOCYTOSIS ASSAY	MEAN	19.70	14.75**	**12.08****	**13.46****	**11.14****
	S.E.M.	1.19	1.18	1.26	1.52	1.32
	N	18	17	17	18	20

** Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.01)

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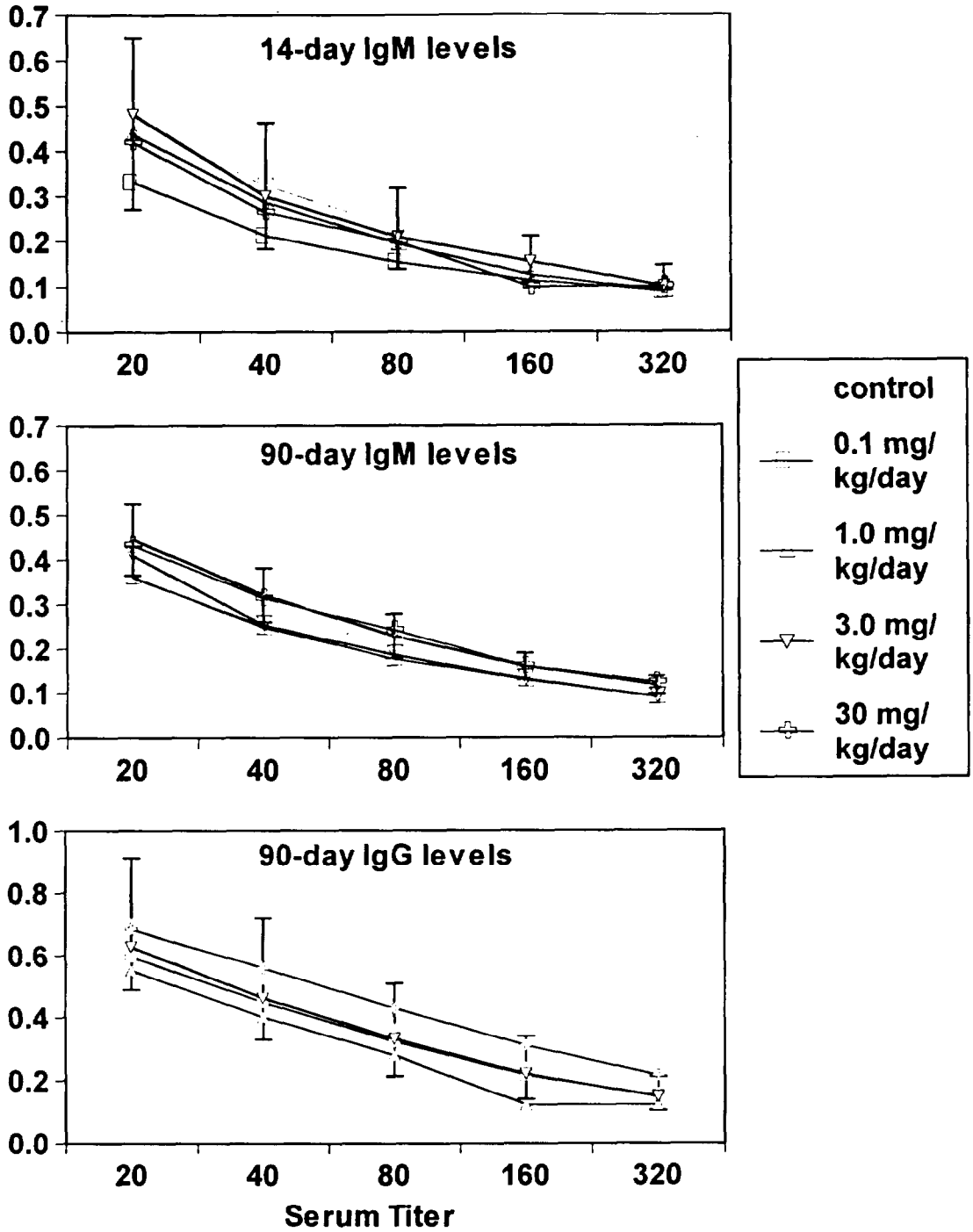
TABLE 29
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND THYROID
PARAMETERS IN B6C3F1 FEMALE MICE

MEAN NUMBER OF LISTERIA MONOCYTOGENES
ASSOCIATED WITH PERITONEAL MACROPHAGES
(PHAGOCYTOSIS)
(SCHEDULED EUTHANASIA – **DAY 120**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
PHAGOCYTOSIS ASSAY	MEAN	24.95	22.95	21.02	25.53	21.98
	S.E.M.	2.91	2.37	2.05	1.75	1.48
	N	12	12	10	12	12

No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

The Effects of 14 and 90 Day Exposure to AP on *In Vivo* IgM and IgG Levels after sRBC Immunization



Adult B6C3F1 female mice were exposed to ammonium perchlorate (0, 0.1, 1.0, 3.0, or 30 mg/kg/day) via drinking water for 14 or 90 days. Mice were challenged with sRBC and detection of specific IgM or IgG was performed using an ELISA based on a protocol provided by L. Temple, *et al.* The aborbance at 405 nm is graphed with the corresponding serum titer. No significant differences were observed in any of the treatment groups as compared to controls using analysis of variance and Tukey's pairwise comparisons ($p<0.05$). For clarity of the graph, standard deviation is shown in controls only.

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TABLE 31
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

DELAYED TYPE HYPERSENSITIVITY ASSAY
(CPM H^3)
(SCHEDULED EUTHANASIA – **DAY 14**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
NO STIMULATION (Background)	MEAN	4098	4404	4074	3918	4841
	S.E.M.	484	453	267	379	673
	N	12	12	12	11	12
SLA 2µg/mL	MEAN	29159	35863	48446	43284	**48149***
	S.E.M.	4740	5317	7992	6499	7214
	N	12	12	12	11	12

*Significantly different from control (0.0 mg/kg/day) using Tukey's pairwise comparison (p-value < 0.05)

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TABLE 32
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

DELAYED TYPE HYPERSENSITIVITY ASSAY
(CPM H^3)
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
NO STIMULATION (Background)	MEAN	5191	5858	4390	4336	4158
	S.E.M.	665	534	530	529	340
	N	10	11	12	11	12
SLA 2μg/mL	MEAN	30421	43734	34754	38974	**58297***
	S.E.M.	7620	7933	6341	10006	9948
	N	10	11	12	11	12

*Significantly different from control (0.0 mg/kg/day) using Tukey's Multiple Comparison (p-value < 0.05)

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TABLE 33
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

HOST RESISTANCE - B16F10 TUMOR CHALLENGE
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
LUNG	MEAN	131.3	159.1	106.9	128.3	125.4
(Mean number of	S.E.M.	13.4	10.5	20.3	16.1	19.8
tumors per lung)	N	11	11	12	12	12

No significant differences were observed in treatments compared to control (0.0 mg/kg/day)

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TABLE 34
EFFECTS OF AMMONIUM PERCHLORATE ON
IMMUNOTOXICOLOGICAL, HEMATOLOGICAL, AND
THYROID PARAMETERS IN B6C3F1 FEMALE MICE

HOST RESISTANCE - LISTERIA CHALLENGE
2700 or 5360 CFU CHALLENGE
(SCHEDULED EUTHANASIA – **DAY 90**)

LEVEL (MG/KG/DAY)		0.0	0.1	1.0	3.0	30.0
LIVER	MEAN	5.427	4.399	5.228	5.209	4.631
2700 CFU Challenge	S.E.M.	0.533	0.219	0.380	0.141	0.186
(Log10 of Listeria	N	7	8	8	8	8
per gram of Liver)						
SPLEEN	MEAN	5.851	5.824	6.557	6.411	6.361
2700 CFU Challenge	S.E.M.	0.551	0.208	0.325	0.178	0.279
(Log10 of Listeria	N	8	8	8	8	8
per gram of Spleen)						
LIVER	MEAN	6.403	5.047	5.943	3.947	4.877
5360 CFU Challenge	S.E.M.	0.338	0.416	0.181	0.322	0.424
(Log10 of Listeria	N	6	6	6	6	6
per gram of Liver)						

No significant differences observed in treatments compared to control (0.0 mg/kg/day)

APPENDICE A. THYROID HORMONE SOPS

IMMUNOTOXICOLOGY STANDARD OPERATING PROCEDURE NUMBER	
TITLE: TSH Quantitation (BIOTRAK ratTSH Assay Kit)	page 1 of 3
Prepared: 04/20/99	Location: Strom Thurmond Room 334
Revised by: Date:	Approved by: Date:

Principle: The Biotrak rat thyroid stimulating hormone ^{125}I assay system utilizes a high specific activity ^{125}I tracer, together with a highly specific and sensitive antiserum. Separation of the antibody bound from free fraction is achieved with an Amerlex-M second antibody preparation, thus allowing a simple magnetic separation. The assay is based on the competition between unlabeled rTSH and a fixed quantity of ^{125}I-labeled rTSH for a limited number of binding sites on a rTSH specific antibody. With fixed amount of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand.

Reference: BIOTRAK Rat Thyroid Stimulating Hormone assay system.

Materials:
Assay buffer
Rat TSH lyophilized standard
Antiserum – Rabbit anti-rTSH Serum, lyophilized
^{125}I rTSH tracer
Amerlex-M second antibody reagent
Pipets with disposable polypropylene tips
Vortex mixer
Refrigerator
Disposable polypropylene tubes – 12x75mm
100 mL glass cylinder
Distilled or deionized water
Gamma Counter
Magnetic separators

Procedure:

1. Reagent Preparation: Warm the bottle containing assay buffer concentrate to 40°C or until gel-like material melts. Avoid temperatures above 60°C. Transfer contents of the bottle, with washings, to a 100mL cylinder and dilute to 100mL with distilled or deionized water. Mix well. Assay buffer is used to reconstitute all other components – standard, antiserum, tracer – in prescribed amounts on each bottle.

2. Preparation of working standards: Label 6 polypropylene tubes 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2. Pipette 500µL of assay buffer into all tubes. Into 3.2 tube, pipette 500µL of stock standard and mix thoroughly. Transfer 500µL from the 3.2 tube to the 1.6 tube and vortex thoroughly. Repeat this doubling dilution successively with the remaining tubes. 100µL aliquots from each serial dilution give rise to 6 standard levels of rTSH ranging from 0.1ng to 3.2ng/tube.

3. Equilibrate all reagents to room temperature.

4. Label polypropylene tubes in duplicate for total counts (TC) , non-specific binding (NSB), zero standard, standards and samples.

5. Pipette 200µL assay buffer into the non-specific binding (NSB) tubes

6. Starting with the most dilute, pipette 100µL of each standard into the appropriately labeled tubes.

7. Pipette 100µL unknown samples into appropriately labeled tubes.

8. Pipette 100µL antiserum into all tubes except NSB and TC.

9. Pipette 100µL of tracer into all tubes. The TC tubes should be stoppered and put aside for counting.

10. Vortex mix all tubes thoroughly. Cover the tubes, and **incubate overnight** (16-24 hours) at room temperature.

11. Allow Amerlex-M second antibody to come to room temperature. Gently shake and swirl to ensure a homogenous suspension. Add 400µL into each tube except the TC. Vortex mix all tubes thoroughly and incubate at room temperature for 10 minutes.

12. Separate the antibody bound fraction using the magnetic separation rack. Place tubes in contact with magnetic separator and leave for 15 minutes. After separation, do not remove tube from rack. Decant the supernatant and invert the tubes on an absorbent pad and allow to drain for five mintues.

13. On completion of magnetic separation, firmly blot the rims of the inverted tubes on the absorbent pad. Re-invert the tubes and remove form the magnetic separator.

14. Determine the radioactivity present in each tube by counting for 1 minute on a gamma counter.

Calculation of results:

1. Calculate the average counts per minute (CPM) for each set of replicate tubes.

2. Calculate the percent NSB/TC: %NSB/TC = (NSB/TC) x 100

3. Users may wish to subtract NSB from all specific binding tubes.

4. Calculate the percent Bo/TC: % Bo /TC = (Bo – NSB) x 100/TC

5. Calculate the percent bound/Bo for each standard and sample:
   %B/Bo = (Standard or sample CPM – NSB) x 100/(Bo-NSB)

6. A standard curve may be generated by plotting the percent B/Bo as a function of the log rTSH concentration.

7. Plot %B/Bo (y-axis) against ng standard per tube (x-axis). The ng per tube can be read directly from the graph.

IMMUNOTOXICOLOGY STANDARD OPERATING PROCEDURE **NUMBER**	
TITLE: Thyroid Total T4 Quantitation (Coat-A-Count Canine T4)	page 1 of 2
Prepared: 04/20/99	Location: Strom Thurmond Room 334
Revised by: Date:	Approved by: Date:

Principle: The Coat-A-Count T4 procedure is a solid-phase radioimmunoassay, in which ^{125}I labeled T4 competes for a fixed time with T4 in the serum sample for sites on T4-specific antibody, in the presence of blocking agents for thyroid hormone-binding proteins. The antibody is immobilized to the wall of the polypropylene tube. Simple decantation of the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled T4. Counting the tube in a gamma counter then yields a number, which converts by way of a calibration curve to a measure of the T4 present in the canine sample.

Reference: Coat-A-Count Canine T4 assay kit.

Materials:
Polypropylene tubes coated with antibodies to T4
Polypropylene tubes without antibody coating
Iodinated tetraiodothyronine (^{125}I tracer)
T4 Calibrators – one set of six vials, labeled A thru F, of lyophilized processed canine serum
Gamma Counter
Vortex mixer
Serum sample
Micropipets

Procedure:

1. At least 30 minutes before use, reconstitute the zero calibrator A with 2.0 mL of distilled or deionized water, and each of the remaining calibrators B through F with 1.0 mL of distilled or deionized water. Use volumetric pipets and mix by gentle swirling. Store refrigerated: stable at 2-8 °C for 30 days after reconstitution. The reconstituted calibrators contain, respectively, 0, 0.5, 1.5, 3, 6 and 15 micrograms of T4 per deciliter in processed canine serum. The calibrator concentrations are subject to slight changes depending on the Lot #.

2. Plain tubes: Label four plain (uncoated) 12x75mm polypropylene tubes TC (total counts) and NSB (nonspecific binding) in duplicate.

3. Label twelve T4 Ab-coated tubes A (maximum binding) and B through F in duplicate. Label additional Ab-coated tubes for controls and serum samples.

4. Pipet 25 μL of the zero calibrator A into the NSB and A tubes, and 25 μL of each remaining calibrator, control and serum sample into the tubes prepared. Pipet directly to the bottom.

5. Add 1.0 mL of ^{125}I canine T4 to every tube. Vortex briefly and gently.

6. Incubate for 2 hours at room temperature.

7. Decant thoroughly. Decant the contents of all tubes **except the TC (total count) tubes.** Remove all visible moisture by inverting tubes onto an absorbent pad and allowing them to drain for five minutes. Tap tubes on absorbent pad to remove any excess moisture around the end.

8. Count for 1 minute in a gamma counter.

Calculation of Results:

First calculate the average NSB-corrected counts per minute for each calibrator and sample:
Net Counts = Average CPM – Average NSB Counts

Next, determine the binding of each tube as a percent of the maximum binding (MB), with the NSB-corrected counts of the A tubes taken as 100%:

Percent Bound = (Net Counts/ Net MB Counts) x 100

Using logit-log graph paper, plot Percent Bound on the vertical (probability) axis against Concentration on the horizontal (logarithmic) axis for each of the nonzero calibrators, and draw a straight line approximating the path of these points. Results for the unknown may then be read from the line by interpolation.

IMMUNOTOXICOLOGY STANDARD OPERATING PROCEDURE **NUMBER**	
TITLE: Thyroid Total T3 Quantitation (Coat-A-Count Canine T3)	page 1 of 2
Prepared: 04/20/99	Location: Strom Thurmond Room 334
Revised by: Date:	Approved by: Date:

Principle: The Coat-A-Count T3 procedure is a solid-phase radioimmunoassay, in which ^{125}I labeled T3 competes for a fixed time with T3 in the serum sample for sites on t3-specific antibody. This reaction takes place in the presence of blocking agents which serve to liberate the bound triiodothyronine from carrier proteins; hence the assay measures total T3, since both free and protein-bound T3 from the sample are able to compete with radiolabeled T3 for antibody sites. The antibody is immobilized to the wall of the polypropylene tube. Simple decantation of the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabeled T3. Counting the tube in a gamma counter then yields a number, which converts by way of a calibration curve to a measure of the T3 present in the canine sample.

Reference: Coat-A-Count Canine T3 assay kit.

Materials:
Polypropylene tubes coated with antibodies to T3
Polypropylene tubes without antibody coating
Iodinated triiodothyronine (^{125}I tracer)
T3 Calibrators – one set of six vials, labeled A thru F, of lyophilized processed canine serum
Gamma Counter
Vortex mixer
Serum sample
Micropipets
Incubator or waterbath (37°C)

Procedure:

1. At least 30 minutes before use, reconstitute the zero calibrator A with 2.0 mL of distilled or deionized water, and each of the remaining calibrators B through F with 1.0 mL of distilled or deionized water. Use volumetric pipets and mix by gentle swirling. Store refrigerated: stable at 2-8 °C for 30 days after reconstitution. The reconstituted calibrators contain, respectively, 0, 20, 50, 100, 200 and 600 nanograms of T3 per deciliter in processed canine serum. The calibrator concentrations are subject to slight changes depending on the Lot #.

9. Plain tubes: Label four plain (uncoated) 12x75mm polypropylene tubes TC (total counts) and NSB (nonspecific binding) in duplicate.

10. Label twelve T3 Ab-coated tubes A (maximum binding) and B through F in duplicate. Label additional Ab-coated tubes for controls and serum samples.

11. Pipet 100 μL of the zero calibrator A into the NSB and A tubes, and 100 μL of each remaining calibrator, control and serum sample into the tubes prepared. Pipet directly to the bottom.

12. Add 1.0 mL of ^{125}I canine T3 to every tube. Vortex briefly and gently.

13. Incubate for 2 hours at 37°C.

14. Decant thoroughly. Decant the contents of all tubes **except the TC (total count) tubes.** Remove all visible moisture by inverting tubes onto an absorbent pad and allowing them to drain for five minutes. Tap tubes on absorbent pad to remove any excess moisture around the end.

15. Count for 1 minute in a gamma counter.

Calculation of Results:

First calculate the average NSB-corrected counts per minute for each calibrator and sample:
Net Counts = Average CPM – Average NSB Counts

Next, determine the binding of each tube as a percent of the maximum binding (MB), with the NSB-corrected counts of the A tubes taken as 100%:

Percent Bound = (Net Counts/ Net MB Counts) x 100

Using logit-log graph paper, plot Percent Bound on the vertical (probability) axis against Concentration on the horizontal (logarithmic) axis for each of the nonzero calibrators, and draw a straight line approximating the path of these points. Results for the unknown may then be read from the line by interpolation.