Effects of Methylmercury Exposure on the Immune and Neurological Responses of Mice to *Toxoplasma gondii* Infection

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i. Abstract

Toxoplasma gondii is a protozoan parasite that causes life-threatening disease in congenitally infected infants and immunocompromised patients, such as those inflicted with AIDS. Toxoplasmic encephalitis (TE) is a common presenting condition in an AIDS infection. People become infected with *T. gondii* by ingesting tissue cysts in undercooked meats or by ingesting oocysts excreted by cats. Methylmercury (MeHg) is a well-documented neurotoxicant that accumulates in the brain and causes severe mental and visual dysfunction, including chronic encephalopathy. Consumption of contaminated fish, grains, and seeds are common sources of human exposure to methylmercury.

Studies from our laboratory suggest that oral exposure to a single high dose of 20 mg/kg MeHg does not increase the susceptibility to acute toxoplasmosis in CBA/J mice. Therefore, we further investigated endpoints associated with immunotoxicity and neurotoxicity in 6-week old, female CBA/J mice exposed to both MeHg and *T. gondii* during a chronic *T. gondii* infection. We examined both single and multiple doses of MeHg exposure in a chronic parasitic infection model. In the single high dose study, four groups of six-week-old, female CBA/J mice were either fed 25 *T. gondii* tissue cysts of the ME-49 strain or given vehicle. Six weeks later, two out of the four groups (*T. gondii* and

vehicle control) were orally gavaged with a single dose of 20 mg/kg body weight of MeHg and sacrificed seven days post exposure. Experiments from the multiple MeHg dose study were performed under similar conditions with the same number of groups and dosed by oral gavage with 8 mg/kg body weight of MeHg on days 0, 2,4,7,10,13. These mice were sacrificed on day 17 or 18 after initiating MeHg exposure.

Flow cytometry following exposure to a single dose of MeHg in mice with a chronic *T. gondii* infection revealed significant changes ($P < 0.05$) within the T cell subpopulation percentages caused by exposure to MeHg. For example, the thymic $CD4^+CD8^+$ T cell subpopulations were increased ($P < 0.05$). However, MeHg had no significant effect on the $CD4^+CD8^-$, $CD4^-CD8^+$, or non-T cell subpopulations in the spleen. Furthermore, MeHg increased splenic cellularity and spleen-to-body-weight ratios with or without a concurrent *T. gondii* infection. MeHg also caused a significant decrease in mouse body weight. There was a significant ($P < 0.05$) increase in brain tissue cyst counts within the group exposed to both MeHg and *T. gondii* (16 ± 4, mean $\pm$ SE, n=7) versus *T. gondii* alone (4 ± 1, n=8). Histopathological examination demonstrated that the brain was affected, as lesions, gliosis, and meningitis were notable in mice given *T. gondii*.

Exposure of mice to multiple doses of MeHg also resulted in effects on the immune system of CBA/J mice with and without chronic toxoplasmosis. Total cellularity and numbers of $CD4^+CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ T-cell subpopulations show a marked decrease in number in the thymus, while total

cellularity was also decreased in the spleen following concurrent exposure to *T. gondii* and MeHg. Flow cytometric examination of lymphocyte populations ($CD4^+$ and $CD8^+$ lymphocytes) in the spleen and thymus demonstrated differences from control in the groups exposed to *T. gondii* and MeHg. Histopathological examination did not reveal any significant lesions.

The data from experiments in which single or multiple doses of MeHg were given to mice with a chronic *T. gondii* infection indicate that concurrent exposure, to both MeHg and *T. gondii*, dependent on dose and time of exposure had notable effects, especially on the immune system (Supported by NIH Grant F36GM20301).

ii. Acknowledgements

I would like to dedicate this work of art to my life lines … these extraordinary individuals include my parents, Mrs. Linda Reed (Mama Bear) and Mr. Robert J. Reed (Greedie Reedie, deceased). Mom and Dad, you knew my potential for as long as I can remember and it was your knowledge that strengthened me to go where no other member of our family had gone before – the realm of higher education. I am forever grateful to you both for all that you have instilled in me and for the many more smiles and joyous events that we will have together (Daddy, I know you know that I know you know). I love you both. My trio on the home front … my 3 biological sisters, Shannon (Nannonbird), Zamika (Mika), and Sadena (Dena). You were the reasons I stayed and kept my pace, to be the inspiration that you sometimes felt you didn't have – it's real, if I can do it (as I have) then hell, so can you. My futuristic folks … my 2 nieces and nephew, Amber (Ammy), Justen Allen, and NayNay Nyara. The most active and motivating children I could possibly have been related to, rest assured that bright and beaming futures are ahead for each of you.

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I must include my CMMID family as well…I could not have survived the enduring early mornings and all night lab hours without Alexa Rosypal and Kay Carlson, affectionately known as Keebs – I will always hold you close to my heart. Thank you to Sharonda Meade, Selen Olgun, and Melinda Pomeroy for always having my back, I know what a task that is! I would like to express my gratitude to my very close and dear friends from years past and present, I look forward to the bright future we have ahead of us. I would like to express my appreciation to Sophia Bichotte, Iris Camacho, Shonna Crisden, Neil Daniel, Beverly Harrington, Cory Lanier, Ayodeji Oladunjoye, Jaye T. Oliver, Joey Prevost, Gyasi Quince, Eric Sanders, Marlette Spencer, Sheryl Tolbert, and of course all of those acquaintances that have made such a great impact on my life. I want to give a big kiss to my little girl Nykey, you've been there through the good, the bad, and the ugly and I love you for it always. Lastly, I would like to thank the administrators and faculty members that I have had the pleasure of learning from and working with over the past 4 ½ years while at Virginia Tech. Surely, there are too many to name, but I would like for those individuals who have had both personal and professional contact with me to know that it was not

in vain and that my future has been impacted greatly by the skills you've taught me.

iii. Declaration of Work Performed

I, Marquea D. King, attest that I performed all work described therein with the exception of the following.

Joan Kalnitsky in the Flow Cytometry Laboratory of the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) performed all flow cytometric analyses.

Barbara Wise of the Toxicology Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine performed the methylmercury quantification by atomic absorption.

All histopathology was performed under the direct supervision of Dr. Bernard Jortner of the Laboratory for Neurotoxicity Studies, VMRCVM.

Immune data was analyzed under the partial supervision of Dr. Robert Gogal of the Center for Molecular Medicine and Infectious Diseases, VMRCVM.

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vii. Abbreviations

Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of Variance
AP	Alkaline Phosphatase
CD	Cluster of Differentiation
CMI	Cell – Mediated Immunity
CNS	Central Nervous System
CTL	Cytotoxic T Lymphocyte
DMSO	Dimethyl Sulfoxide
DN	Double Negative
DP	Double Positive
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme-Linked Immunoabsorbent Assay
FITC	Fluorescein Isothiocyanate
GFAP	Glial Fibrillary Acidic Protein
GI	Gastrointestinal tract
GLM	General Linear Model
HAH	Halogenated Aromatic Hydrocarbon
HBSS	Hanks Balanced Salt Solution
H&E	Hematoxylin & Eosin
Hg	Mercury
$HgCl_2$	Mercuric Chloride
HIV	Human Immunodeficiency Virus

ICR	International Cancer Research
IFN	Interferon Gamma
IgG	Immunoglobulin-G
IgM	Immunoglobulin-M
IL-10	Interlukin-10
MeHg	Methylmercury
NK	Natural Killer cell
PBS	Phosphate Buffered Saline
PKC	Protein Kinase C
POD	Peroxidase
ppm	Parts per million
rpm	Revolutions per minute
SAS	Statistical Analysis Software
SE	Standard Error
PE	Phycoerythrin
TCDD	2,3,7,8-Tetrachloro-di-benzo-p-dioxin
TcR	T Cell Receptor
TE	Toxoplasmic Encephalitis
T. gondii	*Toxoplasma gondii*
TNF	Tumor Necrosis Factor
TUNEL	Terminal Deoxynucleotidyl Transferase – Mediated Nick End Labeling
7 – AAD	7-Amino Actinomycin D
VMRCVM	Virginia-Maryland Regional College of Veterinary Medicine

PART I. INTRODUCTION

A. Hypothesis

Methylmercury (MeHg) is a highly toxic substance that has been reported as having a devastating impact on human health (1). Methylmercury causes adverse effects on both the nervous and immune systems in man and in experimental animals (2), (3). Increases as well as decreases in apoptotic processes in the immune system and the brain have been found in MeHg poisonings (1), (2). Toxoplasmic encephalitis is a life-threatening condition observed in some immunosuppressed individuals (i.e., those infected with the AIDS virus). It is caused by the reactivation of latent tissue cysts of *Toxoplasma gondii*, a protozoan parasite. Once reactivated, the organism (tachyzoites) released from these tissue cysts destroy neuronal tissue and cause progressive meningoencephalitis (3). There is substantial evidence that immunosuppressed patients are unable to destroy the actively dividing tachyzoite stage of the infection (4), (5).

Independently, both MeHg and *T. gondii* have been found to show effects on the nervous system as well as the immune system. Therefore, it was hypothesized that the combination of MeHg and *T. gondii* could show even more adverse effects than either alone. For this dissertation research, it was hypothesized that MeHg could alter the course of a *T. gondii* infection in a chronically infected mouse model, and that MeHg would affect neurological, immunological, and behavioral endpoints. Until

our study, the effects of methylmercury on manifestations of a chronic parasitic infection such as *T. gondii* were unknown. The questions we examined were: Does the heightened sensitivity of the immune system to an environmental contaminant (MeHg) cause a relapse of a disease state in a subject chronically infected with *T. gondii*? Will the effects of a *T. gondii* infection on the nervous system be made worse with MeHg because this immunotoxicant is also a neurotoxicant? Toxicological, immunological, and histological approaches were used to test the hypothesis.

B. Justification

The fields of neurotoxicology and immunotoxicology have long been related but not fully linked scientifically. Little data exist regarding the interactions of environmental toxicants that have the potential to be both immunosuppressive and neurotoxic. Even less information is known about the effects of immunotoxic and neurotoxic chemicals on chronically infected individuals. The use of *T. gondii* for studies of neurotoxicity and immunotoxicity is valuable because its virulence is well confirmed and it is considered a reliable tool for investigating host resistance following a chemical exposure (6).

The damaging consequences of MeHg, which contaminates lakes, rivers, and wetlands, are frequent. It is well recognized that MeHg is a causative agent of neurological symptoms (7). Recent reports have

suggested MeHg toxicity concerning the pathological features of arteriosclerosis (8) and increased rates of autism from exposure to childhood vaccines (9). MeHg has also been found to affect the immune system, as reduction in T cell activity and a decrease in thymus weight have been noted over 12-week exposure periods (10). The U.S. Environmental Protection Agency's (EPA's) Office of Research and Development (ORD) has recently announced its Mercury Research Strategy which outlines research that shows auditory and visual deficits resulting from developmental exposure to MeHg (www.epa.gov/appcdwww/crb/epa-60-R-01-066.pdf). Neurotoxic and immunotoxic environmental chemicals, such as MeHg, could have the potential to alter the course and increase the severity of a *T. gondii* infection, which also has effects on the immune system and on the nervous system. The potential for increased damage to the central nervous system (CNS) after a *T. gondii* infection with exposure to a neuroimmunotoxicant such as MeHg has not yet been investigated (1), (11). The potential for an agent that affects the immune system and the nervous system to alter the expression of an infectious disease exists. It may be possible that exposure to MeHg could alter progression of a chronic infection with *T. gondii*. It may also be possible that the presence of a chronic infection with an infectious agent such as *T. gondii* could alter the previously documented expression of adverse effects from a neuroimmunotoxicant such as MeHg.

The following studies investigated endpoints associated with immunotoxicity and neurotoxicity in mice exposed both to MeHg and *T. gondii*. Apoptosis in the nervous system (brain) and immune organs (thymus and spleen) in toxicant-exposed mice was examined. MeHg burden of experimental subjects was assessed in the brain and kidney. It has been established that MeHg causes an increase in apoptosis of T lymphocytes by mitochondrial-related events (12); similarly, *T. gondii* has also been found to trigger apoptosis by mediators of immune and inflammatory functions (30). These observations suggest that although apoptosis is a normal feature in the developing nervous and immune systems, it could also be a mechanism contributing to the detrimental effects induced by MeHg on these systems (13). Therefore, we hypothesized that MeHg could cause an increase in the severity of *T. gondii* effects on both the immune system and the nervous system by a common mechanism, apoptosis.

The importance of such work lies within the reality that chronic parasitic infections are likely to be better controlled when there is not concurrent exposure to chemicals and/or drugs that cause further detrimental effects on an already immunosuppressed individual (14). The research was proposed because there was potential to create data that may be used for risk assessment for patients with immunosuppressive conditions that stem from a chronic infection and combined exposure to an environmental toxicant. Furthermore, this study provided the benefits of

interdisciplinary collaborations between the fields of Toxicology and Parasitology with agents that target both the nervous and immune systems.

C. Specific Objectives

Specific Objectives were as follows:

1. To test the hypothesis above, we investigated apoptosis as a mechanism associated with cell damage in neuronal tissues and immune organs. This included determination of apoptosis in the brain, thymus, and spleen. Tissues from mice given *T. gondii* , MeHg, and *T. gondii* plus MeHg were used. Apoptosis was examined in the brain, spleen, and thymus with an In Situ Cell Death Detection Kit, (AP by Roche), and, for lymphocytes, with the 7-aminoactinomycin D (7-AAD) assay along with Annexin-V/PI staining. The advantages to using at least two methods of detecting apoptosis include distinguishing early and late phase events and distinguishing cells that are not viable because of mechanical damage. The use of the 7-AAD assay correlates well with the TUNEL assay, a widely accepted flow cytometric technique for quantifying apoptosis (15). Quantification of apoptosis was done by flow cytometric analysis of cell numbers present in treated animals (identified as healthy, necrotic, or apoptotic) compared to control animals.

2. To compare immune system effects of MeHg and *T. gondii* alone and in combination.

a) Evaluation of these effects included cytometric analyses of cell subpopulations. This involved quantification of single positive and double positive CD4+ and CD8+ T-cells as well as non-T cell populations in mice exposed to MeHg and *T. gondii*. Effects of immunotoxic agents on the thymus and spleen have been well characterized and are documented using this method (16). Documentation of decreases and, in some instances, increases in subpopulations have been proven to show the effects of these subpopulations on the ability of an individual to protect him or herself against an infection or an invasion by a foreign substance (17). These data were presented as the percentage of positive cells that express the surface marker of $CD4^+$ and/ or $CD8^+$.

b) Determination of organ weights and organ-to-body weight ratios were used to compare treated animals to controls. Spleen and thymus weight changes are considered a primary observation when investigating immunotoxicity and although they are not specific, they do provide a basis for comparing actual differences in treated versus untreated animals (6).

c) Histological evaluation was done to observe changes in organ/tissue architecture. The use of histopathology of lymphoid organs and the changes in the distribution of cell populations (i.e., T cell subpopulations) provides a straightforward, cost-effective approach to

identifying toxic effects on such organs. This type of evaluation can be expressed quantitatively or qualitatively (6).

3. To compare effects on the nervous system of mice caused by *T. gondii* and MeHg alone and by MeHg in combination with *T. gondii*.

a) Neurobehavioral studies have been proven to be reliable sources of toxicity assessment in mice experimentally exposed to MeHg (18). Behavioral alterations were determined using a standard series of observations, as previously done (Functional Observation Battery for Mice, Laboratory for Neurotoxicity Studies, VMRCVM, SOP 11-49). Additional tests (i.e., tumble, rod, and tilting plane test) designed to reveal slight deficits that could occur before the appearance of gross signs, such as a waddling gait or crossing of the hind limbs, were also included (19).

b) Tissue cyst enumeration was done by using homogenized brain tissue and microscopic assessment on half of the brain.

c) Microscopic alterations of the nervous system were also evaluated.

i) Hematoxylin and eosin (H&E) and luxol fast blue stains were used to determine the damage in cerebellar granule cells, the primary target sites of pathological damage in MeHg toxicity (7). Neuropathological examination has been done for decades and has been a standard means for substantiating neurological deficits (19). Slides of brains from control and treated groups were compared.

ii) Brain lesion scores were determined microscopically (using H&E slides) on the brain and scored based on a previously developed grading system without prior knowledge of the exposure status or the identity of the animal (20).

iii) Immunohistochemistry was used to evaluate levels of glial fibrillary acidic protein (GFAP) in brain sections. Evaluation included assessing antibodies against the GFAP that are released from injured astrocytes. This test is most sensitive when determining immunosuppression or immunostimulation. GFAP has been used to characterize the phenotype of cells and to highlight the degree of gliosis and demyelination in the brain (21).

PART II. LITERATURE REVIEW

Chapter 1. Literature Review

A. Concurrent Exposure to Infectious Agents and Toxicants

Infectious agents and toxicants can cause serious health impacts on both humans and animals worldwide. These problems are worse in developing countries. This is especially true with environmental toxicants because of several factors, including the failure of governments to enforce regulations controlling chemical pollution. This in turn increases the risk of exposure to toxic agents. The exposed populations from industrial and agricultural activities are found more often in lower social and economic classes (22). Published reports of concurrent exposure are rare since they often occur outside of the U.S., these problems have worldwide consequences in a global economy (22), (31). In spite of the fact that both parasitic infections and exposure to toxicants can be deleterious to human health and the environment, very little scientific data has been published on co-exposure to parasitic infections and environmental toxicants, even though they both often cause detrimental effects on similar systems. The nervous, immune and respiratory systems are frequent targets (23) (24).

One example of published data on concurrent exposure to a parasitic infection and to a toxicant is mice experimentally infected with *Toxocara canis* and lead. *T. canis* is the common roundworm of dogs and has caused changes in motor activity, sensory reactivity, and learning (25). *T. canis* completes its

lifecycle in dogs (26), as most parasitic infections use a mammalian definitive host (27). The larvae migrate throughout various organs including the brain, where they remain viable and mobile for extended lengths of time. It has been noted that the combination of lead exposure along with an infection by *T. canis* influences a pattern of behavioral changes in mice (27). When mice were exposed to both agents, lead altered the impact of the parasitic infection by attenuating the behavioral effects observed in mice given *T. canis* alone. These studies have also shown that the magnitude of both behavior and immune reactions may be suppressed if the host is exposed to multiple, versus acute doses of lead (26). Lead, as well as other environmental toxicants may alter the reactivity of the host in the presence of the parasite. This is especially notable when the serological indices suggested that 7% of the world population have antibodies to *T. canis* (26). Epidemiological studies noted that if *T. canis* and lead can both cause cognitive deficits and also have pica as a common risk factor (for children). Therefore, the results of some lead studies may have been confounded due to unrecognized *T. canis* infection (28).

Another example of an interaction between a parasite and environmental toxicant occurred with *Ascaris suum* and heavy metals. For this study, the effects of copper and mercury were examined on phagocytic cell activity and antibody levels in guinea pigs with experimental ascariasis (*Ascaris suum*) (29). *Ascaris suum* are large roundworms and the most common nematode of swine. The eggs contaminate crops grown in soil or fertilized with sewage that has not yet received chemical treatment. Clinical signs are frequently seen in young

feeder pigs that are between two and five months of age. The usual effect is decreased weight gains, which can be quite dramatic. Larval migration through the liver and lung make them the primary sites for lesions but the extent of these lesions depends on the magnitude of the infection. Pre-exposure to the infection as well as genetic background of both the host and the parasite can also make a difference in the display of lesions (30). Human infection often occurs through the consumption of raw produce or from infected food handlers who potentially could contaminate a wide array of food products. The occurrence of disease in humans (long-lasting allergic symptoms) is mainly found in urban areas of North America with a relatively high infection rate. Consumers of uncooked vegetables and fruits grown in or near soil fertilized with sewage are the highest target populations (31). An experimental study demonstrated that dual exposure of mercury and copper caused significant inhibition of peritoneal macrophage phagocytic function in mice infected with Ascaris. When exposed concurrently, both metals changed the phagocytic ability of leukocytes in the blood. This dual exposure also caused an increase in the Ascaris larvae in the lungs of infected animals when compared to infected non-exposed treatment groups (29).

Other examples of an interaction of a parasitic infection with an environmental pollutant are those that occur with methylmercury (MeHg). MeHg has been noted to cause an increased susceptibility of a host to a nononcogenic (encephalomyocarditis virus, EMCV), but not to an oncogenic (Rauscher leukemia virus, RLV) virus when given to 28-day old Swiss Webster mice (32). Results from these experiments demonstrated that MeHg increased the mortality

rate of mice infected with EMCV, but failed to affect those inoculated with RLV. These data suggest that MeHg is synergistic with nononcogenic, but not with oncogenic viruses. This suggests that MeHg not only poses a threat to public health as a toxic agent, but, at subclinical concentrations, it may augment infectious agents to produce cancer.

In addition to interactions with a virus, mercury has been found to enhance murine susceptibility to leishmaniasis (*Leishmania major*). Human leishmaniasis is an endemic health problem in Brazil, the Middle East, and Asia. These regions often geographically overlap with areas of high environmental mercury levels (33). *Leishmania* parasites are intracellular organisms that replicate within host tissue much like *Toxoplasma gondii*. Both can be reactivated by infection with HIV. The severity of disease is dependent not only on the strain of *Leishmania* but also the immune status of the host (34). Manifestations of the disease include cutaneous lesions (hypopigmented macules, erythema, and nodules) and fatal visceral infections of the liver, spleen, and bone marrow. Pretreatment of *L. major* resistant mice to subtoxic doses of mercuric chloride indicated that mercury upregulated production of cytokines and immunoglobulins. IL-4 production (*in vitro*) and serum IgE and IgG1 were increased (34). These data suggest that mercury exposure rapidly induces IL-4 secretion in susceptible mouse strains, and susceptibility of *Leishmania* depends on the presence of IL-4 within the first few days of the infection. Mercury induction of IL-4 – mediated Th2 responses (those that are mediated by $CD4^+$ T helper cells) could impair *Leishmania* control. This study also indicated that mercury may indirectly affect

macrophage function at low doses by altering antigen processing and presenting cryptic epitopes. These self-epitopes do not trigger deletion of autoreactive T cells, therefore they join the peripheral mature T-cell population (34). Despite the fact that animals and humans have differing susceptibilities to mercury toxicosis, the no effect level of MeHg intake in animals has been suggested to be 10 times that for humans (35). Evidence from this study demonstrated that mercury levels of humans living in *Leishmania* – endemic and mercury polluted areas are sufficient to result in similar depressions of cell – mediated immunity (34).

These studies illustrate that exposure (acute or chronic) to subclinical concentrations of the environmental contaminant pose a threat to human health as a toxic entity (causing suppression or stimulation of the immune system). Those same subclinical concentrations of MeHg could also alter the outcome of infectious agents (pre- or post – exposure) to produce more severe disease.

B. Parasitic Infection: *Toxoplasma gondii* (*T. gondii*)

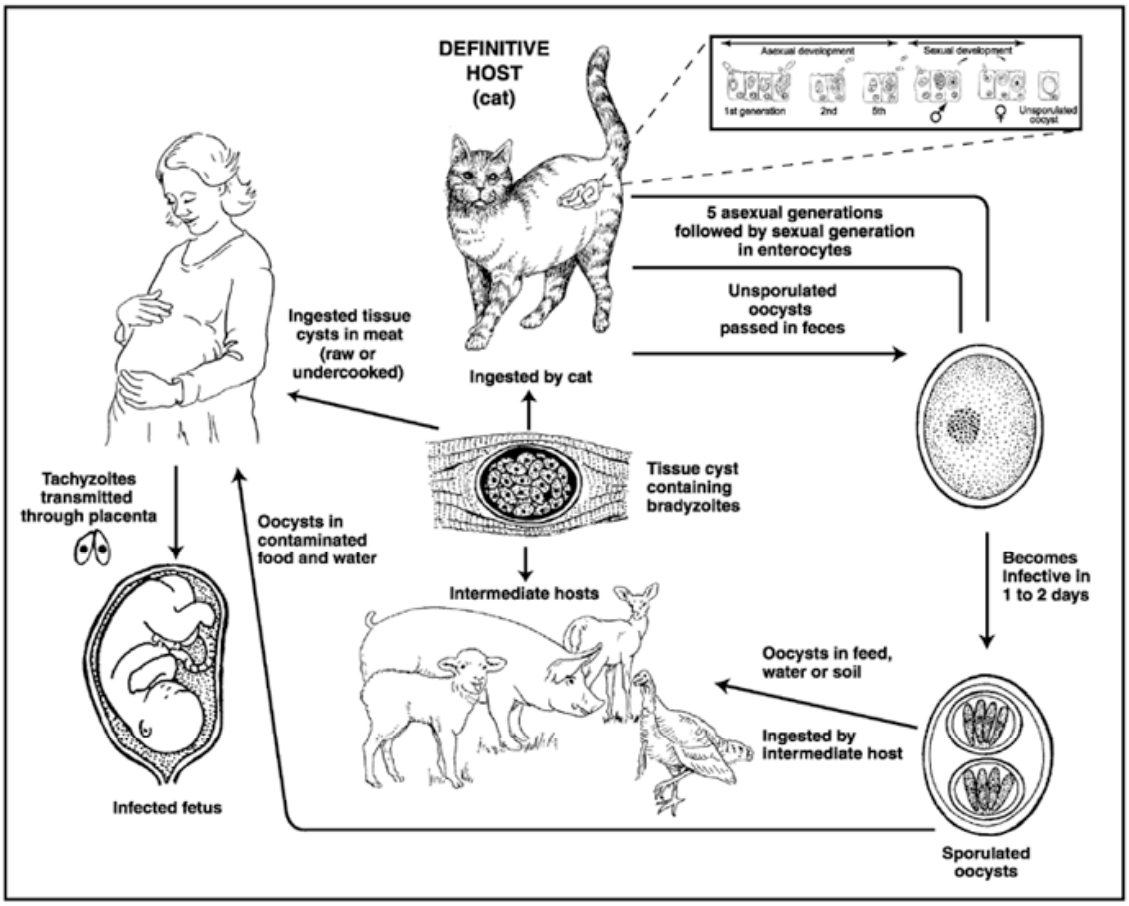
a. Pathogenesis and Course of Infection

Toxoplasma gondii is a protozoan parasite that causes infections that are prevalent worldwide in both humans and animals. Toxo means arc shaped, and plasma means cell. *T. gondii* was discovered in 1908 in Africa (in a rodent host) and in Brazil (in a rabbit host). The first case of human toxoplasmosis was reported in 1923 in an 11-month-old congenitally infected infant. It took several

decades to describe its life cycle, with definitive confirmation in 1970 (36). The life cycle of the parasite is complex and is dependent primarily on the developing stage that is ingested by the cat (Figure 1) (4).

Figure 1.

Toxoplasma Life Cycle



After ingestion of tissue cysts by the cat, oocysts are produced. Ingestion of oocysts is not very effective at producing more oocysts. Sporulated oocysts can stay viable for over a year or more. Sporulation occurs within 1-5 days after they have been shed. The ingestion of a sporulated oocyst initiates a *Toxoplasma gondii* infection (37) (4). Humans, sheep, and pigs most commonly are exposed to oocysts. These are the intermediate hosts for the parasite and inside them is where asexual replication occurs. Tissue cysts are 20 to 80 μm in size and can contain >100 unicellular bradyzoites. The bradyzoites (brady=slow) are the organisms that initiate infections by penetrating enterocytes or the intestinal wall. Enteroepithelial stages of *T. gondii* are common asexual and sexual coccidial stages found only in the small intestines of cats and develop only from bradyzoites (5). Bradyzoites transform into tachyzoites by endodygeny (a form of cell replication) in an intracellular vacuole within a few days. Bradyzoites replicate by endodyogeny and are the encysted stage of parasite that remains in the tissue for the life of the host. The tachyzoites (tachy=fast) are the rapidly multiplying stages of *T. gondii* infection that cause tissue damage and disseminate the infection to other organs. Tachyzoites eventually become bradyzoites in tissue cysts, but can covert back to their highly proliferative stage if reactivation occurs (36). The tissue cyst (bradyzoite) stage of the parasite's life cycle is infectious only if ingested or if inoculated. Tissue cyst stages are not excreted in the feces or body fluids. They are readily killed by water since they cannot osmoregulate (4).

There is an acute and a chronic stage of toxoplasmosis (the disease state from a *T. gondii* infection) in which both tachyzoites and bradyzoites are found. Cells become filled with tachyzoites, rupture, and are then released into the extracellular space where they are able to infect nucleated cells and continue to replicate. The tissue cysts from these tachyzoites develop in the eye, brain, and skeletal muscle. Infectious stages of tissue cysts are present as early as 8 hours after ingestion. Oocyst excretion from cat feces can occur anywhere from seven to >20 days, although most excretion occurs between days 5 and 8 after ingestion. The research done for this dissertation used the tissue cyst stage of *T. gondii* in mice. Murine models have been found susceptible to *T. gondii;* therefore, these models are used to duplicate the disease state found in humans (38).

Humans can be infected with *T. gondii* by ingestion of raw or undercooked meat (lamb and pork) infected with parasites. *T. gondii* can also be transmitted from vegetables, drinking water and from an infected mother to her fetus (congenital toxoplasmosis) (37) (39). After oral ingestion, the bradyzoites which are resistant to stomach pH, enter cells of the gut. Bradyzoites produce tachyzoites, which divide quickly, infect other cells immediately, and further disseminate the infection into the lymphatic system. After about 14 days the tachyzoites disappear and the clinical symptoms resolve. Because tissue cysts can stay dormant and viable in muscle tissue for many years, *T. gondii* infected hosts show little signs of severe disease and most normal appearing though infected adults (80-90%) are asymptomatic (37). Benign lymphadenopathy is the

most common clinical symptom seen in immune competent hosts. These hosts seem to recover from infection with the help of humoral and cell mediated immune (CMI) responses. Bradyzoites during this time have usually developed in some of the host tissues (brain, cardiac, and striated muscle, and in the retina). The population shift from tachyzoites to bradyzoites is often the effect of an immune response that develops against the parasite (40).

Parasites persist for the life of the host since dormant tissue cysts usually cause no inflammatory reaction. Suppression of the immune system can cause tissue cysts to rupture since the body is less likely to be able to ward off an infection. The release of bradyzoites and stage conversion to tachyzoites in vital organs such as the brain or the central nervous system (CNS) is often life threatening. Toxoplasmic encephalitis (TE) has been diagnosed in many patients with AIDS. Reactivation of a chronic infection from the rupturing of latent tissue cysts, due to a decrease in immune function is the cause of TE (40). Ocular toxoplasmosis is often identified in cases of uveitis, because *T. gondii* has been found the most common pathogen to infect the retinas of healthy individuals. Newly apparent ocular infections may actually stem from organisms that disseminated from non-ocular sites, with reactivation leaving only mild scars at first (41) (42).

The tachyzoite stage has both DNA and RNA, as well as organelles of an apical complex cell. After entering mammalian cells by active invasion or phagocytosis, the parasites multiply within a parasitophorous vacuole until the host cell ruptures and the freed organisms invade new cells. Strains of *T. gondii*

have similar morphology but are different in their degree of virulence. Virulent strains replicate rapidly and avirulent strains divide more slowly. The ability of *T. gondii* to readjust a cell's environment for its own metabolic purposes has not yet been completely explained. Genetic differences between *T. gondii* strains do exist (43) (44).

b. Prevention and Public Health Importance

The preventable costs caused by congenital toxoplasmosis contribute significantly to the public health burden (45). Acute infections of the nervous system with *T. gondii* are common features of patients with HIV infections (5). The combined use of sulfadiazine and pyrimethamine synergistically blocks folic acid metabolism in tachyzoites. These drugs, however, have been found ineffective against the cyst form of the parasite. Adverse reactions to these drugs include dermatitis, anemia, leukopenia, neutropenia, and thrombocytopenia with incidence ranging from 20% to 50%. The relapse rate of a treated infection is 40% to 80% (46). *T. gondii* is prevalent in the general population, with 10% to 15% of infected adults exhibiting symptoms and 20% to 70% of the US population demonstrating a serological positive reaction (47). Genetic factors of the host and the parasite may influence pathogenicity, while the host immune system is the most important factor in determining expression of clinical features of the infection (48).

Production of immunoglobulins IgM and IgG antibodies directed against *T. gondii* lead to killing of extracellular parasites by activating the complement pathway. In addition, the use of interferon gamma (IFN-γ), interleukins (IL-10), and TNF-α (see humoral and cell-mediated immunity section below) is being examined for their efficacy against *T. gondii*. Humoral responses are primarily responsible for defense in acute phases of infection, while T cells and macrophages most likely mediate the chronic phases (48). Exposure to *T. gondii* is dependent on a multitude of factors, which include but are not limited to geographical area, food preparation, hygienic practices, and contact with cat feces (49). This infection is commonly acquired by humans from food or water contamination, and not necessarily from exposure to cats (50). Although felines are the definitive hosts and can be intermediate hosts as well for this parasite, it is from their feces that the oocysts are shed (4). The association of cats and humans to *T. gondii* is not easily assessed, since soil rather than cats is the main entity by which the oocysts are spread (50).

Prevalent symptoms of *T. gondii* infection are myocarditis, pneumonitis, hepatitis, disseminated disease, and brain abscess formation. Congenitally infected babies have a delayed onset of the disease state and manifestations of the disease usually involve the CNS. The proximity of pregnant women and cats along with the possibility of a *T. gondii* infection has been a topic of discussion for many years. Some believe that proper precautions while around a cat may reduce the risk of acquiring the disease while others believe that no contact between a pregnant female and a feline should be risked (51). In the year 2000,

the Centers for Disease Control published findings that reported cases of *T. gondii*. There were 750 deaths, 5000 hospitalizations, and 225,000 illnesses with 112,500 of those being foodborne (52)

In AIDS patients, a *T. gondii* infection presents a range of clinical manifestations. Dementia related to cerebral lesions is the most prevalent. *T. gondii* has also been linked to liver dysfunction, cardiac enlargement, and pericarditis. In the past decade, the prevalence of *T. gondii* has decreased in adult humans and swine but congenital toxoplasmosis in children has not. Congenitally infected children (immune competent) suffer from mental retardation and vision impairment, while immunosuppressed patients often die (4). Estimated costs for special services for infected children born with toxoplasmosis each year is $221.9 million, while the cost of prevention averages $369 million annually in the U. S. (53). In the U.S. as well as in other countries there is a cost-benefit associated with screening for toxoplasmosis during pregnancy. The total annual costs of congenital toxoplasmosis without screening amount to $128/pregnancy/year, and with systematic serological screening, $95/pregnancy (54).

In addition to the effect on human health, a severe effect of a *T. gondii* infection is abortions in sheep and goats. This can have a devastating impact on the agriculture industry. The economic/public health burden of this loss has been calculated to be over $400 million annually in the United States alone (55).

c. Humoral and Cell-mediated Immunity

Humoral immunity develops when immunoglobulins (Igs) are produced in response to an encounter of a foreign entity and then presentation by antigens to the immune system. Bone marrow derived cells (B cells) initiate the production of the different classes of Igs (IgM, IgG, IgA, and IgE). In certain parasitic infections, such as congenital toxoplasmosis, there is often an increase in IgM and IgE (56). Early after a *T. gondii* infection, high titers of IgM are detected, whereas IgG is detected in its later stages and may be present throughout the life of the patient. When surveying patients with ocular toxoplasmosis, it has been recognized that IgA, not IgM, may be a useful indicator of this infection. IgA has been recently considered a diagnostic tool for specifying an acute phase of the disease, along with IgM. Antibodies present in sera also aid in the intracellular destruction of tachyzoites by way of macrophages (57) (58).

In congenital toxoplasmosis, IgM antibodies appear at about 3-5 weeks after onset of infection and elevated levels may continue for years. It has been recorded that some congenital cases don't show detectable IgM titers and the argument ensues that maternal IgG inhibits antibody formation to *Toxoplasma* in the fetus (41). IgG may block antibody recognition sites on the parasite itself, thereby delaying an immune response. Immunosuppressed patients with acute *T. gondii* infection could show high Ab titers, but patients with AIDS often show no IgM response and a range of differences occur within IgG titers. Protective immunity from passive transfer experiments currently demonstrates conflicting

results. Some research has shown no protection at all, while others claim to have found moderate protection against milder, less virulent strains (59). Variable amounts of Igs have been demonstrated in AIDS patients and, in HIV infected individuals, B cells are incapable of responding to signals that would normally trigger the release of antibodies in these individuals.

Cell-mediated immune (CMI) responses involve T lymphocytes that mature in the thymus. When T cells encounter an antigen (Ag) from an antigen-presenting cell, they generate interleukins that in turn initiate a cascade of immune reactions. T cells are also capable of assisting B cells in the humoral immune response. Delayed-type hypersensitivity (DTH), a CMI response, is used as the monitoring mechanism for *Toxoplasma* skin testing in experimental animals and humans (60). In DTH reactions antigens appear within a week of onset and last for several years. A T cell response may be heightened or suppressed, mainly because of phagocytosis. Acute and chronic phases of toxoplasmosis result in changes of natural killer (NK) cell activity. NK cells are found normally in the body; they can exert a cytotoxic effect on tumor or virus-infected cells. In the presence of a normal functioning immune system, *T. gondii* elicits an efficient immune response. Therefore, the infection is usually accompanied by minimal symptoms of fever, fatigue, and lymphadenopathy. T cells, also called T helper or $CD4^+$ cells, play an important role in infections caused both by virulent and by avirulent parasite strains. The lack of functional $CD4^+$ cells in mice causes extreme susceptibility to *T. gondii* resulting in reactivation of dormant tissue cysts in the CNS. Suppressor T cells, the

population of T cells that reduce the immune responses of other T and B cells, have been found to be involved in a protective type of immunity along with inhibition of *T. gondii* cyst formation (57).

Together $CD4^+$ and $CD8^+$ T cells serve a dual role in inducing one another's activity as well as modulating the production of cytokines and interleukins. It has been postulated that $CD4^+$ cells help their host develop a type of resistance during acute toxoplasmosis and that $CD8^+$ cells help the host maintain a protective immunity while inhibiting tissue cyst formation (58). Protection of naïve mice challenged with virulent *T. gondii* strains was demonstrated following transfer of $CD8^+$ cells. Cytokines and interleukins are reported to be important for controlling tachyzoite replication (61). Interferon γ and TNF-α both activate macrophage function during chronic and acute phases of infection. Interleukins-10 and 12 are effective in adjusting interferon gamma (INF-γ) *in vivo* and inhibiting it so that extensive tissue damage and inflammation do not occur. IL-10 regulates both IL-12 and INF-γ in an acute infection. NK cells are also a major source of INF-γ while IL-12 is a central mediator of NK cell synthesis. The *in vivo* treatments with IL-12, INF-γ, or TNF-α have a protective effect against *T. gondii* infection (57). TNF-α mediates immune and inflammatory functions, growth of cell types, and can trigger apoptosis. Recent studies have demonstrated that T cells can be activated without IL-12 (58). Various components of the immune system are responsible for recognition and control of infections. T cells, INF-γ, NK cells, macrophages, and several interleukins are responsible for strong T cell immunity development.

d. Neurotoxic Complications

The appearance of pathological abnormalities associated with *T. gondii* infection in the CNS of patients with AIDS is greater than 80%. Nearly half of these individuals demonstrate clinical symptoms of neurological dysfunction and blindness indicating the infection targets the brain and eyes. People afflicted with *T. gondii* without AIDS are presented as "normal", since they express no clinical symptoms. However, presently, the interaction of an infectious agent, such as *T. gondii* with an immunotoxicant and/or a neurotoxicant that may manifest relatively similar symptoms is unknown (37).

Glial filaments, detected by immunostaining of the glial fibrillary acidic protein (GFAP), were found to accumulate around the perimeter of *T. gondii* cysts as they developed in mouse astrocytes. The glial filaments wrapping cysts may play a role in bradyzoite differentiation and/or cyst stabilization in the host cell cytoplasm (62). When cell-mediated immunity is decreased, this leads to various symptoms including inflammatory abscesses of the nervous system. Descriptions of neuronal damage reveal multiple iso- or hypodense lesions located in the white matter or in the basal ganglia (63). In the brain stem and cerebellum there are extensive alterations, including necrosis of the brain parenchyma (64).

The disease state induced by *T. gondii* includes both acute and chronic phases. Acute phases can range from 2-8 days to as long as 14-21 days (38), while chronic stages appear over 30 days following a primary infection.

Treatment with sulfadiazine has been proven successful in preventing acute disease. However, *T. gondii* infections require continued treatment throughout the life of the host to prevent a relapse of both the active disease state and the neurological manifestations of the infection. Life long continued treatment does not guarantee absolute protection from a re-occurring acute infection (46).

C. Environmental Contaminant: Methylmercury (MeHg)

a. Organic and Inorganic Mercury

Millions of people are exposed to neurotoxicants each year and outbreaks of neurological disease in numerous populations usually occur after accidental exposures (65). Certain metals have been widely recognized as neurotoxicants. Common examples of metal neurotoxicants are lead, manganese, and mercury (especially methylmercury).

Mercury exposure in people, animals, and the environment often occurs from chemical contaminants while anthropogenic mercury emission is also causative (66). Mercury is also believed to travel over long distances by atmospheric currents which reach distant locations and is then, through localization in water supplies, i.e., lakes and oceans, converted by bacteria to methylmercury (MeHg) which bioaccumulates in fish and sea mammals. Biotransformation of mercury causes it to be changed from inorganic to organic mercury, which potentially has greater toxicity. One of the most toxic forms of mercury is MeHg (10). MeHg and inorganic mercury are both known to cause cerebellar syndrome (tremors and ataxia), chronic encephalopathy, visual

dysfunction, and peripheral neuropathy. A primary difference between the action of MeHg and inorganic mercury relates to their toxicokinetics. Inorganic mercury vapors are inhaled and deposited into the lung. If ingested, inorganic mercury is not well absorbed from the gastrointestinal (GI) tract, so its major route of excretion is through the feces. Although inorganic mercury does not cross the blood brain barrier, it does accumulate in the pituitary gland, and areas where the barrier is usually absent (67). In contrast, MeHg, a potent neurotoxicant, is efficiently absorbed by the GI tract and bound to hemoglobin and glutathione (GSH) in blood and tissues. MeHg has an affinity for sulfur and sulfhydryl groups, including those found in the tissues (kidney, liver, and brain) of humans and animals (10). This may be a transport mechanism of MeHg. It is this binding that causes most of the biochemical effects of mercury compounds.

The major uses for both inorganic and organic mercury include, but are not limited to, electric lighting, batteries, paint manufacturing, and dental amalgams (fillings). Occupational laboratory exposure also occurs. People are exposed to organic mercury primarily through the diet and exposure to dental amalgam. Inorganic mercury can undergo methylation by microorganisms in water and soil, resulting in transformation to MeHg. MeHg remains in the environment for long periods of time, which contributes to bioaccumulation. MeHg in particular was first detected in occupational settings but most of its notoriety developed in relation to the environmental exposures resulting from agricultural applications (i.e., as fungicides and pesticides) (10), (68).

b. Etiology and Exposure to MeHg

MeHg causes organic mercury intoxication, or Minamata disease. Clinical signs of Minamata disease are often neuronal, and include severe mental and motor dysfunction and cognitive deficits. In laboratory animals, changes are seen in reflexive development, locomotor activity, and cognitive function (18). Release into the atmosphere from soil along with anthropogenic sources can result in increased MeHg concentrations in fish by way of bioaccumulation. Exposure of the public to MeHg most often occurs when the compound is found in drinking water and in food. Approximately 30% of the daily water intake for an adult contain traces of MeHg, while almost 90% of the total mercury intake is attributed to seafood harvested by recreational fisherman is MeHg.

MeHg is considered the most toxic to humans of any of the mercury compounds since it causes severe biochemical abnormalities, such as increasing intracellular calcium uptake at synapses. MeHg disrupts mitochondrial calcium uptake and inhibits mitochondrial respiration, as well as causing neurotransmitter metabolism to be disrupted by changing calcium release (7). Another important chemical feature of methylmercury is its tendency to form covalent rather than ionic bonds; covalent bonds increase stability.

In Japan in 1950, a chemical plant in Minamata Bay discharged inorganic mercury into the bay as sludge. The inorganic mercury was methylated to MeHg resulting in contamination of fish and shellfish, which were a main source of food for inhabitants of that area (69). Another epidemic occurred in Iraq in 1971,

when the local government switched its wheat order from the Mexican government to a more resilient variety. A single letter typographical error was made when labeling the fungicide to be used in the treatment of the wheat; consequently, the wheat was treated with poisonous MeHg. On arrival in Iraq, persons receiving the shipment were obviously unaware of the significance of skull and cross bones and colored dye additives on the wheat to warn them of danger. They proceeded to use the wheat for baking bread. In just a few weeks the MeHg intoxication was widespread and had a devastating impact with about 450 reported deaths (70).

Exposure to MeHg has detrimental affects on various body systems and tissues. Animals treated with MeHg were found to exhibit stress intolerance, suggesting adrenal gland dysfunction (71). Mean serum levels of corticosterone in MeHg treated mice were only half the levels found in control mice. Impaired swimming abilities were partially restored upon glucocorticoid therapy. Destruction of the adrenal gland was demonstrated in rats exposed to MeHg via drinking water (20mg/L) or by intraperitoneal injections of 100μg or 200μg daily from 7 – 180 days. Mercury accumulation was demonstrated by light and electron microscopy with a highly sensitive histochemical technique (72). The amount and location of the mercury recovered were dependent on method of administration, the amount administered, and the length of exposure. Rats treated for greater than 180 days showed necrosis of cells within the cortical zone as well as of the cytoplasmic vacuoles of the zona fasciculata. Mercury has been noted as the most likely heavy metal to exert an adverse effect on the

viability of isolated rat adrenal capsular and decapsular cells. It also damages Leydig cells of the testis. A decrease in cell viability paralleled by a reduction in corticotropin – stimulated corticosterone production indicated a direct toxic action of mercury on steroid – producing cells in the adrenal gland and the testis (73). Additional studies claim that there may be a connection between the symptoms of acute mercury intoxication and its adrenal accumulation (74). On the other hand, it has been reported that the acute effects of a single low dose (12 mg/kg, subcutaneously) of MeHg in rats were completely reversible between a period of 1 hour through 10 days (75) . Exposure to MeHg has been found to cause a diminished capacity for metabolism of the corticosterone *in vitro* due to a loss of liver mass. Adrenal function and plasma cell levels of corticosterone were found to be unaffected by MeHg treatment in this study (76).

c. Neurotoxicity of MeHg

One of the many cell types that play a significant role in MeHg neurotoxicity is the astrocyte. Although this is not the only cell type affected, MeHg has been shown to preferentially accumulate in the astrocyte, thereby causing inhibition of glutamate uptake as well as neuronal swelling (70). Inorganic mercury crosses the blood brain barrier poorly, but biotransformation of MeHg into mercury occurs after MeHg crosses the blood brain barrier, and this can cause persistent and continued brain damage. Blood brain barrier dysfunction often occurs during HIV associated dementia with macrophage brain infiltration and modulation of monocytes by microglia, which could accelerate the

uptake of mercury into the CNS (77). Histopathological evidence in mice indicates that the cerebellum is often the site most affected by MeHg intoxication. Atrophy, nerve cell degeneration, gliosis, and macrophage aggregation also occurs (67). Neuronal proliferation and migration are the principal effects of MeHg in the neonate. Since it is highly lipophilic and has a high affinity for sulfhydryl groups, MeHg is thought to interfere with membrane components in the cell. This interference could possibly cause disassembly of microtubules at the mitotic spindle. It is also believed to inhibit the assembly process of tubulin monomers, causing depolymerization (67). MeHg neurotoxicity is characterized by abnormal mitosis, ribosome dissolution, and loss of microtubules in neuronal cells (67). Following acute MeHg exposure, most of the mercury in the brain is in the organic form; however, with chronic exposures there is a greater proportion of inorganic mercury. This would suggest that demethylation increases with long-term exposure (77). Although MeHg is extremely toxic to the nervous system, the kidney often contains the highest concentration (78).

d. Immune System Effects of MeHg

There is limited information on effects on immune function with MeHg, but research has indicated that T cell depletion causes increases in susceptibility to MeHg induced apoptosis (1). MeHg also inhibits lymphocyte functions, such as the expression of cell activation markers on cell surfaces and the production of cytokines (79). These studies suggest the possibility of interaction between

MeHg and *T. gondii* because other immunotoxicants have been demonstrated to alter response to infectious agents. Examples of this include combinations of cycloheximide and acetaldehyde or dibutyltin dichloride (80).

Once in the body, both inorganic and organic mercury causes enhanced mortality of laboratory animals challenged with an infectious virus (pathogen). MeHg decreases antibody synthesis as well as circulating antibodies in both mice and rabbits (81). Decreases in NK T – cell activity and reduced thymic weight have been observed in female mice after 14 weeks of continuous exposure (82). At six weeks, exposure to MeHg chloride produced increases in body weights, splenic weights, and thymic weights in rats. On the other hand MeHg sulfide produced only an increase in thymic weight and had no significant effect on NK T – cell or splenocyte activity in the rat (83). These data reflect the uncertainty of distribution of the sulfide form of MeHg or its affinity for different targets in the immune system. To date these differences are unknown and the conclusion is that low doses of MeHg chloride appear to have an effect on splenocytes and NK T – cell activity.

2,3,7,8-Tetradichloro-dibenzo-p-dioxin (TCDD)

a. Etiology and Exposure

Dioxins, polychlorinated biphenyls (PCBs) and halogenated aromatic hydrocarbons (HAHs) have no commercial usefulness. They are formed during combustion processes such as the manufacturing of herbicides and paper bleaching with chlorine. Waste incineration, forest fires, and backyard trash

burning also produce these compounds. These unwanted bi-products have been found in coffee filters, milk cartons, and diapers (84). Dioxin was a contaminant of Agent Orange used as a defoliant by the United States in the war against Vietnam. As recently as three years ago imports from Belgium included dioxin-contaminated chicken, meat, eggs, and those products containing more than 2% egg product were considered unsafe (85). This class of environmental chemical has been proven to be both carcinogenic and immunotoxic (86).

2,3,7,8-Tetrachloro-dibenzo-p-dioxin or dioxin (TCDD) is the most toxic of HAHs. Dioxin's profound stability has influenced its persistent presence in the environment. Its presence there has led to its bioaccumulation in fatty tissues of organisms (birds, reptiles, fish and mammals) and biomagnification within the food chain. Dioxin has poor biodegradability and is extremely lipophilic. Dioxin produces its effects by entering a cell and binding to a protein present in cells known as the Ah receptor. This receptor, when bound to dioxin, can then bind to DNA and alter the expression of some genes. This then leads to changes in the level of specific proteins and enzymes in the cell. These properties have resulted in severe wasting syndrome in acutely exposed animals (87),(88). Because dioxin causes a loss of body mass, it is thought to adversely affect the regulatory systems for both body weights and /or feed intake. This further leads to the belief that there is CNS toxicity involvement. Studies have suggested that dioxin exposure at high levels to chemical workers leads to an increase in cancer (89). Additional studies have indicated that reproductive and developmental problems increased heart disease and increased diabetes. Teratogenicity has not yet

been established in humans but laboratory mice have been used to demonstrate that dioxin and similar chemicals can produce congenital defects (90). TCDD-induced toxicity has been reported as causing hepatotoxicity, gastric lesions, reproductive toxicity, teratogenicity, and embryo toxicity. A hallmark symptom of TCDD in humans is chloracne, which often follows dermal or systemic exposure to (91).

b. Immune System Effects

Some of the most distinctive features of TCDD – induced toxicity involve the immune system. Effects on immune function have been confirmed to be among the earliest and most responsive indicators of 2,3,7,8-TCDD-induced toxicity. Recent evidence has indicated that exposure to 2,3,7,8-TCDD caused changes in innate immunity in addition to the changes in acquired immunity (this includes effects on both cell-mediated and humoral immunity). Effects of 2,3,7,8-TCDD on specific indicators of immune function have been linked with changes in host resistance capabilities, which are often thought to be more holistic indicators of immunocompetence (92). Immunosuppression has been noted as a primary toxic effect of dioxin in laboratory animals (90). Atrophy of the lymphoid organs (thymus, spleen, and testis), concurrent suppression of cell mediated immunity (CMI); humoral immunity and B cell differentiation (16) have all been described after low-level exposure to TCDD. These doses may be so low that they may not necessarily induce organ toxicity. More recently, it has been suggested that one

mechanism of TCDD toxicity may be apoptosis. It has been found that apoptosis of thymocytes *in vivo* after TCDD exposure occurs, although rapid clearing of apoptotic cell bodies caused difficulties in proving this hypothesis (93).

PART III. MATERIALS AND METHODS

Chapter 2. Materials and Methods

A. Experimental Design

General Overview

The experimental animals were divided into 6 groups of 10 animals per group, with the following groups being observed:

	Vehicle	DEX	MeHg
T. gondii (-)	Group 1	Group 2	Group 3
T. gondii (+)	Group 4	Group 5	Group 6

1) Negative control – orally administered dimethylsulfoxide (DMSO)/phosphate buffered saline (PBS) or Hanks' Balance Salt Solution (HBSS)
2) Positive control, immunosuppression– dexamethasone (DEX, topically, 100 mg/kg body weight)
3) Neurotoxicant treated – MeHg by oral gavage (up to 20mg/kg body weight)
4) *T. gondii* infected – 25 tissue cysts/mouse by oral gavage
5) Immunosuppressive combination – dexamethasone by topical application and *T. gondii* by oral gavage
6) Combination – MeHg and *T. gondii* dosed by oral gavage

Acute studies were performed with endpoints determined on day 14 after *T. gondii* infection. In acute experiments, MeHg dosing occurred once, 2 days prior to parasitic infection.

Chronic infections were the primary focus of this dissertation research. In initial chronic experiments, a single treatment with MeHg occurred on day 42 after an infection has been established (*T. gondii* infection is considered "chronic" at 6-7 weeks) (80). Initially, chronic studies were carried out over a 49-day period, allowing for a more apparent *T. gondii* infection that targets the CNS to emerge. The mice were observed over the 2-week period that follows the initial infection. At the time of sacrifice, mice were anesthetized with halothane. Tissues collected included: the brain, spleen, thymus, and the kidney. Since problems within the nervous system occur after long-term exposure to high levels of inorganic mercury (10), MeHg exposure could interfere with the chronic infection of the parasite. In this instance, difficulty arose in determining whether the effect was due to the infection or the treatment. In order to investigate this further, the experimental design was adjusted accordingly. It became necessary to perform multiple dosing studies with MeHg every 2-3 days with control groups with and without the infection, continuing this treatment for up to 2 weeks. This attempted to delineate neurological and immunological signs as well as to better identify which entity, the toxicant or the parasite, was the causative agent.

1. Murine Model

Female six-week old CBA/J mice obtained from Jackson Laboratories (Wilmington, MA) were used for experimental infection and methylmercury (MeHg) exposure. Mice were maintained in wire topped polystyrene cages under controlled conditions of temperature (22°C), humidity (40-60%), lighting (12 hour light/dark cycle) and provided with standard mouse chow and water ad libitum throughout the course of the experiments.

CBA/J mice have been proven resistant to acute *T. gondii* infection but susceptible to the chronic stage. Their propensity to develop both encephalitis and high numbers of brain cysts make this mouse model desirable for experimental purposes (38). This susceptibility also plays an important role in identifying the critical responses necessary to initiate protection and ultimately survival from a *T. gondii* infection (94). Studies have demonstrated that strains of mice differ in their immune responses to *T. gondii* (95). Several other murine strains were considered and were found not as useful when attempting to observe both immunotoxic and neurotoxic effects. For example, C57BL/6J mice are extremely susceptible to an acute *T. gondii* infection and mice often died within two weeks after infection, unless treated with sodium sulfadiazine (King, M. D., and Lindsay, D. S., unpublished data). There was also concern about response of this strain of mice to MeHg. For example, research has been published (18) to show that neurobehavioral changes of the male offspring from C57BL/6J female mice treated with MeHg (18) had no significant differences in

locomotor activities between control and treated mice of this strain. Therefore, we chose the CBA/J model, as it was more likely to provide an opportunity to gain tangible data to visualize the alterations that may occur with the combination of a toxicant and a chronic infection (96). At the time of sacrifice on day 14 or 49-post parasitic infection, tissues and organs were harvested. The tissues of the mice used included those of the nervous system (brain) and the immune system (thymus and spleen). The kidney and half of the brain were also removed to determine the MeHg content.

2. Parasitic Infection

a. *T. gondii* Infection

Chronic *T. gondii* infection was established with tissue cysts of the ME-49 strain of *T gondii*. The infection is considered chronic within a six -week period, as previously demonstrated (97). Tissue cysts were collected from the brains of International Cancer Research (ICR) strain mice infected 8 to 26 weeks previously. The entire brain from 2 to 3 ICR mice were then removed and placed in 2-4 milliliters of Hanks balanced salt solution (HBSS) and homogenized in a Stomacher 80, Laboratory Blender by Seward or a Black & Decker ®, 7.2 Volt 300/600 rpm drill. The numbers of tissue cysts were determined by counting the numbers present in a 50 μl aliquot of 2 ml total volume of brain homogenate.

CBA/J mice were infected with *T. gondii* tissue cysts in Hanks Balanced Salt Solution (HBSS). The tissue cysts were given by using a mouse feeding needle, 22X1” gauge (Popper & Sons, New Hyde Park, NY). Tissue cyst volume

is dependent on total cyst burden counted, from that; each mouse received 25 tissue cysts. Preparation and oral infection of *T. gondii* parasite tissue cysts were performed under the supervision of Dr. David Lindsay (parasitologist), of the dissertation committee. The laboratory area was disinfected with a 70% alcohol solution after cyst preparation to kill of any remaining *T. gondii*. Various protective measures to avoid accidental parasitic infection were followed and included: wearing gloves while inoculating mice and during necropsy procedures and then autoclaving the gloves after use. The mouse carcasses were either frozen or immediately incinerated to kill tissue cysts, and the serological status of individuals working with *T. gondii* in the Parasitology Laboratory was also monitored using the modified direct agglutination test.

3. Toxicant Dosing

a. MeHg Dosing

MeHg was given as a single oral dose by gavage needle, unless otherwise stated (see Chapter 6). A single dose of 20 mg/kg was chosen based on literature that reported this dose of MeHg was sufficient to cause neuronal dysfunction (98). The toxicant was dissolved in 84 µL DMSO and then in 8.3 ml of PBS to prepare a final concentration of 20 mg/ml. Methylmercury (II) chloride powder (CH_3HgCl, purchased from the Alfa Aesar Company, Ward Hill, MA) was used as the experimental neurotoxicant. As indicated by the Standard Operating Procedure (SOP) for the Toxicology Laboratory at the College of Veterinary Medicine at Virginia Tech, the preparation and storage of MeHg was done under

the supervision of a board-certified toxicologist. Dosing amounts were prepared prior to each experiment, so as not to have excessive amounts of a MeHg go unused. Protective clothing such as lab coats, gloves, and dust/mist respirators were worn. Items used in MeHg solution preparation went through a decontamination process with a cysteine solution before disposal, since cysteine binds and inactivates the MeHg. The Hazardous Waste System of Virginia Tech routinely picks up waste from the Laboratory of Toxicology, which includes MeHg. Treated animal carcasses were properly labeled as such and disposed of by incineration, following University Safety Protocols.

MeHg quantification was done by cold vapor generation accessory method of atomic absorption by trained technical staff of the Toxicology Laboratory of the VMRCVM. This assay determines the concentration of mercury by using the Varian SpectraAA – 220FS. Sample preparation included: weighing of tissue, tissue digestion with nitric acid and antifoam emulsion.

b. TCDD dosing

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was donated by Dr. Stephen Safe, Texas A&M University, College Station, TX, and stored at –20ºC. TCDD was pre-dissolved in acetone and diluted in corn oil (50 μg TCDD in 50 μl of chloroform). The solution was gently heated with stirring to evaporate the acetone. Mice were administered 50 μg/kg body weight of TCDD by gavage six weeks after exposure to *T. gondii*. Controls received corn oil by oral gavage (50

μg/kg body weight). Mice were euthanized by carbon dioxide gas 14 days after TCDD or vehicle treatment.

All housing and experimental procedures involving animals were approved prior to initiation of experiments by the Virginia Tech Animal Care and Use Committee, in accordance with Virginia Polytechnic Institute and State University guidelines.

B. Experimental Protocols

1. Evaluating Apoptosis

Quantitative and qualitative assessment of immunotoxicity and neurotoxicity was performed by examining apoptosis in the immune system organs (spleen, and thymus) and in the brain using a staining protocol and flow cytometry.

a. Immunohistochemical Staining Protocol

The staining protocol was used to detect apoptotic bodies within tissue sections of brain, spleen, and thymus. A commercial In Situ Cell Death Detection Kit, AP (Alkaline phosphatase) or POD (Peroxidase) (Roche Molecular Biochemicals, Mannheim, Germany) was used to assess the single and double stranded DNA breaks that occur in the early stages of apoptosis. The hallmark of apoptosis is DNA degradation, which is characterized by DNA cleavage of double and single stranded DNA breaks (nicks). These types of breaks can be

detected by attachment of fluorescein-dUTP to free 3'OH ends of the DNA. This method is referred to as the **T**dT – mediated d**U**TP-X **n**ick **e**nd **l**abeling or TUNEL assay. After incubation with the TUNEL reaction mixture, the slides are washed to remove any unbound label. An anti-fluorescein antibody conjugated with the AP or POD then marks the label that is incorporated at the damaged sites of the DNA. After washing to remove unbound enzyme conjugate, the AP or POD remaining in the immune complex is then viewed by a substrate reaction mixture. Toluidine blue was used as a counterstain (to facilitate the visualization process) and cells were then rinsed and mounted for histology. Evidence of apoptosis (darkened area of various spots in tissue) was obtained with the use of a Nikon model C-LP camera/microscope (Tokyo, Japan). A minimum of n=5 per treatment group was used for organ fixation and slide preparation to be stained with an *in situ* cell detection kit for quantification of apoptosis. In addition to observing apoptotic bodies, areas of cerebellar white matter of *reactive* astrocytes were counted as they were viewed under the microscope. These data were reported as qualitative and descriptive endpoints.

b. Flow Cytometric Analysis

The second method for evaluation of apoptosis used 7-aminoactinomycin D (7-AAD). This technique discriminates between live, early apoptotic, and late apoptotic/necrotic cells (46). It also distinguishes cells that are not viable because of mechanical damage. The 7-AAD assay was used for evaluation of

apoptosis in cells of the thymus and spleen. Both splenocytes and thymocytes were incubated for five minutes at room temperature in Red Blood Cell Lysing buffer (Sigma, St. Louis, MO). Cells were counted on an electronic CASY-1 cell sorter and a series of rinsing steps completed. The cells were then suspended in 7-AAD dissolved in methanol (concentration of 10 µg/ml of 7-AAD) from Molecular Probes, Eugene, OR (99). Cells were then incubated overnight at 4°C in 3% paraformaldehyde and run on a flow cytometer the next day. Apoptotic cells were identified and quantified by a method described by Schmid (17). This assay was modified and a combination of three dyes, i.e. FITC- anti CD4, PE-anti CD8, and 7-AAD, were used to stain apoptotic target cells. This modified assay makes it possible to concurrently quantitate the percentage of cell lysis and the phenotype of the target cell. A Coulter Epics XL flow cytometer (Hilaeah, FL) was used to perform the analysis. Five thousand events (cells) were analyzed per sample. These data we presented as percent mean ± SE of cells that express surface markers of $CD4^+CD8^-$, $CD4^-CD8^-$ (DN – double negative), $CD4^+CD8^+$ (DP – double positive), and $CD4^-CD8^+$.

2. Evaluating Immune Parameters

Immunotoxicity assessment employed the technique of flow cytometry to measure cellular effects of *T. gondii* infection with concurrent MeHg exposure. In addition the organs of the immune system were weighed and examined histologically.

a. Flow Cytometric Determination of Lymphocyte Phenotypes

Flow cytometry was used to examine lymphocyte population ratios. Alterations can indicate depression of the immune system caused by MeHg and its correlation to the manifestations seen during a chronic *T. gondii* infection. The thymus and spleen were made into single cell suspensions containing two million thymocytes or splenocytes/ml, respectively, from vehicle control, *T. gondii* infected, MeHg and non-MeHg treated mice. Cells were also obtained from dexamethasone treated mice with and without a *T. gondii* infection following this protocol. Splenocyte and thymocyte single cells were released using the screen method and then counted using the CASY-1 Cell Counter (Scharfe System, GMbH, Germany) (99). Lymphocytes from the thymus and spleen were washed in phosphate buffered saline and $2x10^6$ cells were aliquoted into a total volume of 2 ml. The gates for flow cytometric analyses were set based on autofluorescence of unstained cells from each organ. The cells were then incubated for 60 min on ice with a concentration of 0.5 µg/µl fluorescein isothiocyanate (FITC) conjugated anti-CD4 monoclonal antibody (mAb) and 0.2 µg/µl phycoerythrin (PE) conjugated anti-CD8 mAb (BD PharMingen, San Diego, CA), and 7-aminoactinomycin D (7-AAD) (Molecular Probes Eugene, OR). The 7-AAD was dissolved in methanol for a final concentration of 10 µg/ml. Cells were then incubated overnight at 4°C in 300 µl of 3% paraformaldehyde. Following this, cells were washed and the fluorescence (470-600 nm) was measured on a

Coulter Epics XL flow cytometer (Hilaeah, FL). Five thousand events (cells) were analyzed per sample. Flow cytometry was performed to detect alterations in subpopulations of T cells ($CD4^+/CD8^+$/DN/DP) as an endpoint for immunotoxicity as described previously (100). These data we presented as percent mean ± SE of cells that express surface markers of $CD4^+CD8^-$, $CD4^-CD8^-$, $CD4^+CD8^+$, and $CD4^-CD8^+$. Immunotoxicity is often characterized by looking at the ratio between the $CD4^+CD8^-$ and $CD4^-CD8^+$ subtypes (101). The general linear model (GLM) procedure of the SAS package (SAS Institute Inc., Cary, NC) was used to assess the changes in the percentage of cells that expressed different phenotypic markers in the various treatment groups. When there was a significant interaction, the SLICE option was used to test the effect of *T. gondii* within each level of MeHg. Results were considered significant at $p<0.05$.

b. Organ-to-body Weight Ratios and Histology

Assessment of weight changes is typically done when attempting to determine immunosuppression or stimulation. The reduction or enlargement of immune organs (thymus and spleen) often indicates abnormalities and possibly dysfunction of those organs (6). A decrease in thymus weight, especially when expressed as thymus-to-body weight ratio denotes a decrease in total number of thymocytes. The same is true for splenocytes from the spleen (6). These data were reported as mean ± SE, and comparisons made between treated and control animals. Histological examination was used to make comparisons between structures of organs of the immune system (spleen and thymus) from

control and treated animals. These descriptions were used to note any membrane degradation, swelling, and/or shrinkage of organelles. Descriptions of any lesions and or abnormal structural alterations were included.

3. Evaluating Neurotoxicity

a. Behavioral Assessment

Behavioral analysis was used to score the severity of the clinical manifestations of toxicant-induced neurotoxicity in living mice (Acute Oral Toxicity Study in Mice with Neurobehavioral Examinations, unpublished protocol of the Laboratory for Neurotoxicity Studies, 1993). Examinations were done during the dark cycle of the standard 12-hour light/ 12-hour dark cycle with a red light lamp for visualization. Briefly, observations of mice occurred at varied time points dependent on MeHg dosing. With single dosing of MeHg, observations were done each day from the time of dosing until 7 days later, the time of sacrifice. Times included the following days in chronic *T. gondii* infection with multiple doses of MeHg: 0, 7,14, 21, 28, 35, and 49. Observations included, but were not limited to, a decrease or increase in body weight, cage viability (alive or dead), cage activity (normal or abnormal), ataxia (coordination or lack of), posture (stiff, hunched, or normal), respiration (labored, slow, or normal), and cyanosis (bluish color of extremities or mouth area). Additional endpoints evaluated are listed in Table I.

Observations were done with the examiner being unaware of the mouse treatment group (19). The observational assessments and manipulative tests were reported as descriptive data or quantitative data, where possible.

Table I

Acute Oral Toxicity Neurobehavioral Examination Responses

Response Variable	**Assessment Score**
Home cage viability	Y / N – dead or alive
Home cage activity	Nor / Abn – moving or not moving
Righting reaction	Nor / Abn – able to return to normal stance or not able to
Click response	Y / N – responded to sound or not respond
Menace response	Y / N – responded to head on approach or not
Wooden rod	Y / N – able to stay on rod for 10 seconds or not
Posture	Nor / Abn – normal unless stiff, hunched, or head tilt
Coat condition	Nor / Abn – normal unless ungroomed or ruffled
Tail condition	Nor / Abn – normal unless swollen, discolored, or tip lost
Respiration	Nor / Abn – normal unless labored, gasping, rapid or slow
Vocalization	Y / N – present or not present
Diarrhea	Y / N – present or not present
Soft stool	Y / N – present or not present
Fecal stains	Y / N – present or not present
Urine stains	Y / N – present or not present
Salivation	Y / N – present or not present
Nasal discharge	Y / N – present or not present
Oral discharge	Y / N – present or not present
Lacrimation	Y / N – present or not present
Ocular discharge	Y / N – present or not present
Pilorection	Y / N – present or not present
Dehydration	Y / N – present or not present
Cool to touch	Y / N – notable or not notable
Cyanosis	Y / N – present or not present
Corneal bulging	Y / N – present or not present
Weight	Actual numerical data recorded

Y=Yes, N=No, Nor=Normal, Abn=Abnormal

Fishers Exact test was used to compare each treatment group to all other treatment groups by running a cross tabulation table which gave the percentages of each group that had significant differences on each of the six days of observation. Due to a small sample size (n=5-9) statistics of pair wise comparisons were not able to be performed, however; an overall treatment effect was analyzed ($P < 0.05$).

b. Tissue Cyst Enumeration

The entire brain was removed intact and split evenly with a mid-saggital cut with a razor. The numbers of tissue cysts in the left half of the brain were enumerated to determine if treatment with MeHg influences tissue cyst numbers (which thereby indirectly indicates reactivation of the previously dormant infection). H&E sections do not contain an adequate amount of tissue for meaningful evaluation of tissue cyst numbers. Therefore, the left side of the brain was removed and placed in 4 ml of HBSS and homogenized using a Stomacher 80 or a Black & Decker ®, 7.2 Volt 300/600 rpm drill. The number of tissue cysts present in 50 μl of homogenate was counted using a bright-field microscope. Tissue cysts have a characteristic dark appearance and are spherical structures. The numbers of tissue cysts present in brain from control and treated mice were evaluated using a Kruskal-Wallis non-parametric test and a distribution-free multiple comparison method. The remaining portion of the brain homogenate and the entire kidney were used for analysis of mercury content using atomic absorption as described above (VMRCVM Laboratory of Toxicology).

c. Brain Histology and Lesion Scoring

The right half of the brain was stained by H&E to determine damage to astrocytes and cerebellar granule cells. Astrocytes are often the initial cells that

respond to injury of the CNS. These cells respond by swelling and causing gliosis (hypertrophy of astrocytes) (47). Gliosis has been reported in chronic *T. gondii* infections (45). In order to characterize the extent of neuronal damage in the cerebellum and other brain regions, mid-sagittal cuts of the brain were assessed. Glial scarring and hypertrophic cells with enlarged cytoplasmic processes were also used as a pathological endpoint when describing neuronal alterations. This endpoint is reported as descriptive data.

The brains from mice were also examined microscopically after routine processing for histology to apply a semi-quantitative assessment to determine the severity of lesions induced by MeHg alone, by *T. gondii* alone and by a combination of MeHg and *T. gondii*. The brains from control mice (mice not given MeHg or *T. gondii*) were used for comparison. Hematoxylin and eosin (H&E) stained tissue sections of brains were coded and brain lesions determined in a blinded fashion without knowledge of mouse treatment. The brain lesions were scored based on the following severity of lesions: none = grade 1, slight = grade 2, mild = grade 3, moderate = grade 4, and marked = grade 5. This grading system has previously been developed and used for the evaluation of the effects *T. gondii* in the brain (20). The numbers of mice in each treatment with lesions were compared using Fisher's exact test. Mean lesion scores were evaluated using a Kruskal-Wallis non-parametric test and a distribution-free multiple comparison test. Significance was established at a cutoff of $P < 0.05$ prior to conducting analysis of these data.

d. Glial Fibrillary Acidic Protein

Glial fibrillary acidic protein (GFAP) is a cytoskeletal intermediate protein found only in astrocytes (102). This protein helps the astrocyte to maintain its structure. After insult or injury (exposure to parasite or chemical) astrocytes often swell. Detectable levels of GFAP have been found to appear as early as 3-7 days after a neuronal injury (103). A staining protocol was used as an immunohistochemical marker for injury of astrocytes with antibodies to GFAP. Identification of the immunoreactivity of GFAP was performed using a previously approved protocol (104). This procedure used nervous tissue slides incubated with anti-GFAP mouse monoclonal antibodies. Tissue sections were microwaved and incubated with normal goat serum at room temperature for 30 minutes. A primary antibody was added and the tissues were allowed to incubate overnight at 4°C. Tissues were then run through a series of incubations with mouse Clono PAP and then rinsed before a substrate was added (105). Finally, slides were stained with Gill's hematoxylin a background stain (to facilitate the visualization process), and cells were then rinsed and coverslips were mounted on each slide for histology. This endpoint was graded as normal or increased using a plus/minus scale.

C. Data Analysis

Three different types of data were assessed in this dissertation. The first type was continuous, or data that could take on many values. These variables

were flow cytometric data, tissue cyst counts, organ weights, apoptosis staining, and GFAP staining. The second type was categorical or ordered data; these data could fit into the subcategories of binomial, ordered, or nominal. Binomial data take on yes/no or +/- values. These data included some endpoints from the behavioral observations. Ordered data were rated on a scale, such as lesion scoring, which takes into account severity. Nominal data were named and not ordered or described by severity, such as some response variables from within the behavioral battery of tests (i.e., posture- hunched, normal, or stiff). The third type of data was descriptive or written, which give details about alterations of tissues. Endpoints in this category were brain, spleen, and thymus pathology, and apoptosis. Statistical analyses for this study was by restriction and random allocation to control factors that are normally difficult to control (i.e., genetic history of animal model). In order to ensure that any significant association between a factor and a disease state existed we used relative risk in our testing protocol. We incorporated standardized testing procedures in our study. Quantitative data were analyzed by a generalized linear model (GLM), which tests for the main effects of MeHg, *T. gondii* and the interaction between the two. When a significant interaction exists, SAS software and $P<0.05$ were used to test the effect of *T. gondii* within each level of MeHg. Pair wise comparisons such as vehicle control mice versus MeHg only and MeHg only versus *T. gondii* only mice were also determined with the use of Tukeys post-hoc test. This type of model is flexible and accommodates for analysis of variance (ANOVA), regression, and

continuous data. All other methods are described in the proceeding methodology section.

PART IV. RESULTS

Chapter 3. Effect of 2,3,7,8-Tetrachloro-di-benzo-p-dioxin on Chronic *Toxoplasma gondii* Infection in Mice

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Chapter 3. Effect of 2,3,7,8-Tetrachloro-di-benzo-p-dioxin on Chronic *Toxoplasma gondii* Infection in Mice

Abstract

In the current study, the effect of exposure to the environmental pollutant, 2,3,7,8-Tetrachloro-dibenzo-p-dioxin (TCDD) on mice having chronic infection with *Toxoplasma gondii* was investigated. For this purpose, groups of mice were used – mice treated with vehicle, mice treated with TCDD alone, mice infected with *T. gondii* alone, and mice receiving a combination of TCDD treatment and *T. gondii* infection. Histological examination and tissue cyst enumeration were performed to indicate the level of infection of the brain. The immune status was studied by enumerating the cellularity as well as the percentages and absolute numbers of the lymphocyte subsets based on the expression of CD4 and CD8 markers in the thymus and spleen. Our studies demonstrated that there was a significant decrease in the total number of thymocytes in TCDD-treated mice that were either uninfected or infected with *T. gondii*, when compared to vehicle controls. However, there was no significant difference observed in the thymic cellularity in mice that were infected with *T. gondii* alone when compared to the uninfected vehicle controls. In addition, the ratio and the total numbers of CD4+, CD8+, CD4-CD8- (double negative, DN) and $CD4^{+}CD8^{+}$ (double-positive, DP) T cell subsets in the thymus from various groups were determined. There was no change in the percentages of T cell subsets in TCDD-treated mice or *T. gondii* infected mice when compared to the

vehicle controls. However, there was a decrease in the percentage of DP T cells and an increase in the DN and $CD4^{-}CD8^{+}$ T cells in mice that received a combination of TCDD-treatment and *T. gondii* infection when compared to mice receiving the vehicle or TCDD-treatment alone or infection with *T. gondii* alone. There was also a decrease in the absolute numbers of the DP and $CD4^{+}CD8^{-}$ T cells and an increase in the $CD4^{-}CD8^{+}$ T cells in the thymus of mice receiving the combination of TCDD-treatment and *T. gondii* infection when compared to vehicle controls. The splenic cellularity as well as the percentage and absolute numbers of the CD4+ and CD8+ T cell subsets and the non-T cells were not altered in all the groups tested. The natural history of *T. gondii* infection was not altered following TCDD-treatment as demonstrated by no significant differences in brain lesion scores and the number of tissue cysts in the brains of the groups of mice. This work was supported in part by grants NIH R01 ES09098 and K02 AI01392 to MN and by Virginia-Maryland Regional College of Veterinary Medicine.

Introduction

Environmental pollutants such as dioxin have been shown to have significant immunosuppressive effects (Holsapple et al., 1991; Kerkvliet,1995). 2,3,7,8-Tetrachloro-dibenzo-p-dioxin (TCDD) is a prototype halogenated aromatic hydrocarbon, which is highly immunotoxic even at very low concentrations (Nagarkatti et al., 1984; Holladay et al., 1991). The thymus is a primary

lymphoid organ that is highly sensitive to TCDD-induced immunotoxicity. T cells develop in the thymus following migration of the prothymocytes from the bone marrow or fetal liver. They differentiate in the thymus and undergo positive and negative selection and the mature cells then emigrate to the peripheral lymphoid organs. Mature T cells are the single positive, CD4+ or CD8+ T cells that develop from the $CD4^{+}CD8^{+}$, double positive (DP) thymocytes that in turn differentiate from the $CD4^{-}CD8^{-}$, double negative (DN) cells in the thymus. Earlier studies from our lab have demonstrated that TCDD induced apoptosis in the thymocytes (Kamath et al., 1997; 1998; 1999). Other studies have also demonstrated that apoptosis is induced in the thymocytes following TCDD treatment *in vitro* (McConkey et al., 1988). However, several other mechanisms have also been suggested to explain dioxin-induced thymic atrophy, including the inability of the prothymocytes to seed to the thymus (Fine et al., 1990a;1990b). Other studies have demonstrated that TCDD may affect thymocyte differentiation (Holladay et al., 1991; Kremer et al., 1994).) The nature of the phenotypes of thymocytes affected by TCDD has been controversial. It has also been shown that TCDD may target the epithelial cells that may indirectly affect the thymocytes (Greenlee et al., 1985).

There has also been a great deal of controversy as to which thymocyte subsets are affected by TCDD. Some studies have indicated that there is a decrease in the DP T cells and an increase in the DN and CD8+ T cells (Lai et al., 1998; Holladay et al., 1991), while others have demonstrated that TCDD affects all the subsets of thymocytes (Silverstone et al., 1994a; Lundberg et al.,

1990a; 1990b; Rhile et al, 1996, Kamath et al., 1998). The reason for this variation in the findings is not clear.

The main purpose of our current study was to evaluate the interaction of chemical-induced immunosuppression on the outcome of a chronic *Toxoplasma gondii* infection. In addition, we wished to determine whether the sensitivity of thymus to TCDD would alter during a chronic infection. Protection against the development of toxoplasmosis has been attributed to the CD8+ T cells (Khan and Casciotti, 1999; Ely et al., 1999) as well as to the CD4+ T cells (Gazzinelli et al., 1992). Several investigators have demonstrated that immunosuppression induced by a variety of agents could lead to the development of a lethal infection with *T. gondii* (Venturini, 1996; Goetz et al., 1996; Nicoll et al., 1997; Schluter et al., 1998; Chappell et al., 1992). Thus any xenobiotic that affects T cell development resulting in immunosuppression should have profound effects on the outcome of a chronic infection with *T. gondii*.

In the current study, we tested whether exposure to the immunosuppressive environmental contaminant, TCDD would cause reactivation of infection of mice that were chronically infected with *T. gondii.* Such a study was undertaken to provide insight into why patients with severe immunosuppression, as seen in AIDS, are found to have reactivated toxoplasmosis manifested as toxoplasmic encephalitis (TE). In fact, the identification of TE is often an initial finding that leads to the diagnosis of AIDS (Garly et al., 1997; Reiter-Owona et al., 1998). Thus it is important to determine whether environmental contaminants may further adversely affect these patients.

Toxoplasmosis in the central nervous system is a common cause of brain lesions in AIDS patients. In a number of patients suffering from TE, the infection may be lethal (Belanger et al., 1999; Daubener and Hading, 1997). This condition has been shown to be caused by reactivation of latent tissue cysts, which upon activation leads to tachyzoite-induced destruction of neuronal tissue (Frenkel and Escajadillo, 1987). In addition, other sites may also be affected, leading to ocular, pulmonary and liver toxoplasmosis. In the current study, we examined the extent of brain lesions in *T. gondii* infected mice treated with TCDD, but our experiments demonstrated that TCDD-exposure did not exacerbate the outcome of the disease.

Materials and Methods

Four to six week old female C57BL/6 mice were purchased from Charles River Laboratories, Raleigh, NC. They were housed in groups of five in polyethylene cages containing wood shavings and provided with rodent chow and tap water ad libitum.

The procedure for infecting mice with *T. gondii* was as follows: Tissue cysts of the ME-49 strain of *T. gondii* were collected from the brains of mice infected 8 to 26 weeks previously. This strain was chosen because the majority of laboratories working on immunity to *T. gondii* use this strain. The brains of 2 mice were pooled and ground in Hank's balanced salt solution (HBSS) with a teflon-coated tissue grinder. The number of tissue cysts present was determined

by counting those present in 50μl aliquots. Mice were gavaged with 25 cysts of *T. gondii* in HBSS. Controls received HBSS. Mice were given sodium sulfadiazine (1mg/ml) in the drinking water beginning 7 days after *T. gondii* or HBSS exposure. The medicated water was provided for 14 days. This allowed most mice to survive the acute stage of infection.

TCDD was generously donated by Dr. Stephen Safe, Texas A&M University, College Station, TX, and stored at –20ºC. TCDD was dissolved in acetone and diluted in corn oil. The solution was gently heated with stirring to evaporate the acetone. Mice were administered 50μg/kg body weight of TCDD by gavage six weeks after exposure to *T. gondii*. Controls received corn oil. Mice were sacrificed 14 days after TCDD or vehicle treatment.

Mice from control and experimental groups were euthanized by CO_2 and the thymus and spleens were surgically removed. Organs were placed in RPMI-1640 medium (GIBCO Lab, Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 10mM HEPES, 1mM glutamine, 40mg/ml of gentamicin sulfate and 50μM 2-mercaptoethanol. Single cell suspensions were made with a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati, OH) and kept on ice. Cells were pelleted by centrifugation and resuspended in Tris-ammonium chloride solution to lyse erythrocytes. Cells were further washed twice in medium. Twenty-five microliters of the single cell suspension was added to trypan blue dye and viable cells were counted by dye exclusion using an inverted phase contrast microscope.

The cells were washed in phosphate buffered saline and $2x10^6$ cells were incubated with fluorescein isothiocyanate (FITC) conjugated anti-CD4 monoclonal antibody (mAb) and phycoerythrin (PE) conjugated anti-CD8 mAb (Pharmigen, San Diego, CA) for 30 min at 4°C. The cells were washed and the fluorescence was measured on an Epics V flow cytometer as described (Kamath et al., 1998). Ten thousand cells were analyzed per sample. The gates for flow cytometric analyses were set based on autofluorescence of the negative control consisting of autofluorescence and positive controls consisting of normal thymocytes stained with FITC- anti-CD4 and PE-anti-CD8 mAbs.

The brains were harvested 2 weeks after TCDD or sham treatment and split in half and the right side fixed in 10% neutral buffered formalin for histopathological examination. The tissue sections were stained with hematoxylin and eosin and the lesions scored in a blinded fashion by one author (DSL). The brain lesions were scored based on the following criteria: scores of 1 if no lesions were present, 2 if minimal inflammation and tissue cysts were present, 3 if moderate inflammation and tissue cysts were present and 4 if severe inflammation and tissue cysts were present. For tissue cyst enumeration, the other half of the brains were homogenized in 2mls of HBSS and the number of cysts in 50μl were counted under a bright field microscope.

In the current study, groups of 7 to 8 mice were used. The mean ± standard errors were calculated for the lymphocyte data and groups were compared using an ANOVA. Brain lesion scores were compared using Fisher's Exact test. Values were considered to be statistically significant if $p<0.05$.

Results

We first examined whether TCDD-treatment of mice chronically infected with *T. gondii*, caused a decrease in the thymic cellularity and increased susceptibility to the parasite. For this purpose, groups of C57BL/6 mice were fed tissue cysts of *T. gondii*, then treated with sodium sulfadiazine to prevent lethal infection, and gavaged 6 weeks later with 50 μg/kg body weight of TCDD or corn oil. The thymi from these mice were harvested 14 days later and the cellularity determined. Control mice used in the study were treated with HBSS as well as corn oil and are referred to as vehicle-treated mice. As shown in Table 1, the thymic cellularity in mice receiving TCDD alone was decreased when compared to the vehicle controls. There was no significant change in the cellularity of the thymus in mice receiving *T. gondii* alone when compared to the controls. Following combination of TCDD-treatment and *T. gondii* infection, significant thymic atrophy was observed. However, these decreases in cell numbers were similar in magnitude when compared to mice that received TCDD alone. These data suggested that *T. gondii* infection did not affect the ability of TCDD to induce thymic atrophy.

In order to determine which of the subsets of thymocytes are affected following TCDD exposure of *T. gondii* infected mice, the thymi from control and experimental groups of mice were stained with FITC- conjugated anti-CD4 and PE-conjugated anti-CD8 monoclonal antibodies. The flow cytometric data from representative examples from each group are depicted in Fig. 1. Following either

TCDD-treatment alone or after *T. gondii* infection alone, there was no significant change in the percentages of CD4+, CD8+, double negative (DN, CD4-CD8-) and double-positive (DP, $CD4^+CD8^+$) cells in the thymus when compared to the controls (Table II). There was no change in the percentage of CD4+ T cells in mice that received the combination of TCDD-treatment and *T. gondii*-infection. However, there was a significant decrease in the percentage of DP ($p<0.05$) cells as well as an increase in the percentages of DN ($p<0.05$) and CD8+ ($p<0.05$) T cells in mice receiving a combination of TCDD and *T. gondii*, when compared to mice that received vehicle alone, TCDD alone or *T. gondii* alone. These studies, therefore, demonstrated that TCDD-treatment acts synergistically with *T. gondii* infection to cause significant alterations in the thymic cell subpopulations.

A change in the absolute numbers of T cell subsets may also occur particularly when there is a change in the total cellularity of the thymus as well as the ratios of the various subpopulations. In order to examine this, the absolute number of each T cell subset found in the thymus was determined by multiplying the percentage of the T cell subpopulation with the total number of cells in the thymus. TCDD-treatment alone led to a significant decrease in the absolute numbers of CD4+ and DP T cell subsets whereas there was no change in the absolute numbers of all the T cell subpopulations in mice infected with *T. gondii* alone, when compared to the uninfected controls (Table I). However, following the combination of TCDD-treatment and *T. gondii* infection, there was a dramatic decrease in the DP T cells when compared to the vehicle controls or mice infected with *T. gondii* alone. These data demonstrated that TCDD-treatment

decreased the absolute numbers of CD4+ and DP T cells whereas a combination of TCDD and *T. gondii* did not further influence the absolute numbers of T cell subsets.

In order to examine the effect of TCDD-treatment on peripheral lymphoid tissues, spleen cells from various groups of mice were studied for cellularity and T cell subsets. The cellularity of the spleen (Table III), as well as the percentages (Table IV) and absolute numbers (Table III) of the CD4+ and CD8+ T cell subsets and the non T cells were not altered in any of the groups tested when compared to controls.

The brains from various groups of mice were examined histologically for lesions. The brains of *T. gondii* infected mice were also examined for the presence of tissue cysts. As shown in Table V, there were no significant differences in the lesion scores in all groups of mice tested. Furthermore, the number of tissue cysts observed in brains of *T. gondii* infected mice in the presence or absence of TCDD-treatment was not significantly altered. These data therefore demonstrated that TCDD-treatment may not adversely affect the natural history of a *T. gondii* infection.

TABLE I

Total thymic cellularity and absolute numbers of T cell subsets in the thymus of mice treated with TCDD and infected with *T. gondii* *

Treatment	Total cellularity	Cellularity of T cell subpopulation			
		$CD4^+CD8^-$	$CD4^-CD8^+$	$CD4^-CD8^-$	$CD4^+CD8^+$
Control	127.3 ± 10.6	17.0 ± 3.34	6.20 ± 1.01	6.62 ± 2.56	97.4 ± 8.39
TCDD	56.3 ± 11.9[a]	7.01 ± 0.979[a]	3.40 ± 0.613	2.49 ± 0.157	43.5 ± 11.0[a]
T. gondii	133.9 ± 17.5	20.1 ± 1.76	9.79 ± 1.47	9.43 ± 5.29	94.7 ± 17.5
T. gondii/TCDD	53.7 ± 9.5[a,b]	8.73 ± 1.50[b]	8.55 ± 1.47[c||]	12.6 ± 3.26	23.9 ± 7.40[a,b]

* All data are expressed as mean x 10^6 ± standard error per mouse thymus.

a Statistically significant ($P < 0.05$) when compared to control

b Statistically significant ($P < 0.05$) when compared to *T*. gondii-infected mice

c Statistically significant ($P < 0.05$) when compared to TCDD-treated mice

TABLE II

Percentages of T cell subsets in thymus of mice treated with TCDD and infected with *T. gondii*.

Treatment	**Percentages of T cell subsets***			
	$CD4^+CD8^-$	**$CD4^-CD8^+$**	**$CD4^-CD8^-$**	**$CD4^+CD8^+$**
Control	13.66 ± 2.89	4.79 ± 0.60	4.85 ± 1.70	76.7 ± 3.30
TCDD	14.23 ± 2.15	6.86 ± 0.92	5.64 ± 1.00	73.24 ± 3.68
T. gondii	17.00 ± 3.37	7.87 ± 1.35	7.71 ± 3.80	67.41 ± 5.64
T. gondii/TCDD	17.50 ± 1.43	17.71 ± 2.18[a,b,c]	24.9 ± 5.85[a,b,c]	39.86 ±6.97[a,b,c]

* All data are expressed as mean percentages ± standard error for T cell subpopulations following staining with mAbs against CD4 and CD8 markers.

a Statistically significant difference ($P < 0.05$) when compared to controls

b Statistically significant difference ($P < 0.05$) when compared to *T*. gondii-infected mice

c Statistically significant difference ($P < 0.05$) when compared to TCDD-treated mice

TABLE III

Total splenic cellularity and absolute numbers of T cells subsets and non-T cells in spleens of mice treated with TCDD and injected with *T. gondii**.

Treatment	**Total cellularity**	**Cellularity of T cell and non-T cell subpopulations**		
		CD4	**CD8**	**non-T cells**
Control	153.4 ± 22.6	28.9 ± 3.87	27.4 ± 5.21	94.0 ± 13.60
TCDD	116.7 ± 12.4	23.0 ± 3.75	18.9 ± 3.11	72.8 ± 8.21
T. gondii	188.9 ± 25.3	30.9 ± 2.77	26.7 ± 3.21	125 ± 20.70
T. gondii/TCDD	176.2 ± 19.2	31.9 ± 2.82	24.4 ± 3.37	114 ± 16.80

* All data are expressed as mean x 10^6 ± standard error per mouse spleen.

TABLE IV

Percentages of T cell subsets and non-T cells in spleens of mice treated with TCDD and injected with *T. gondii*.

	Percentage of T cell subsets and non-T cells*		
Treatment	**CD4**	**CD8**	**non-T cells**
Control	19.15 ± 1.07	17.36 ± 0.92	61.33 ± 1.52
TCDD	19.14 ± 1.84	16.09 ± 2.05	62.74 ± 3.79
T. gondii	17.03 ± 1.01	15.07 ± 1.81	64.69 ± 2.36
T. gondii/TCDD	18.59 ± 1.07	14.31 ± 1.70	63.54 ± 2.62

* All data are expressed as mean percent ± standard error for T cell subpopulations and non-T cells per mouse spleen.

TABLE V

Mean lesion scores and tissue cyst counts for mice given *T. gondii* and/or *T. gondii* and 50µg/kg TCDD.*

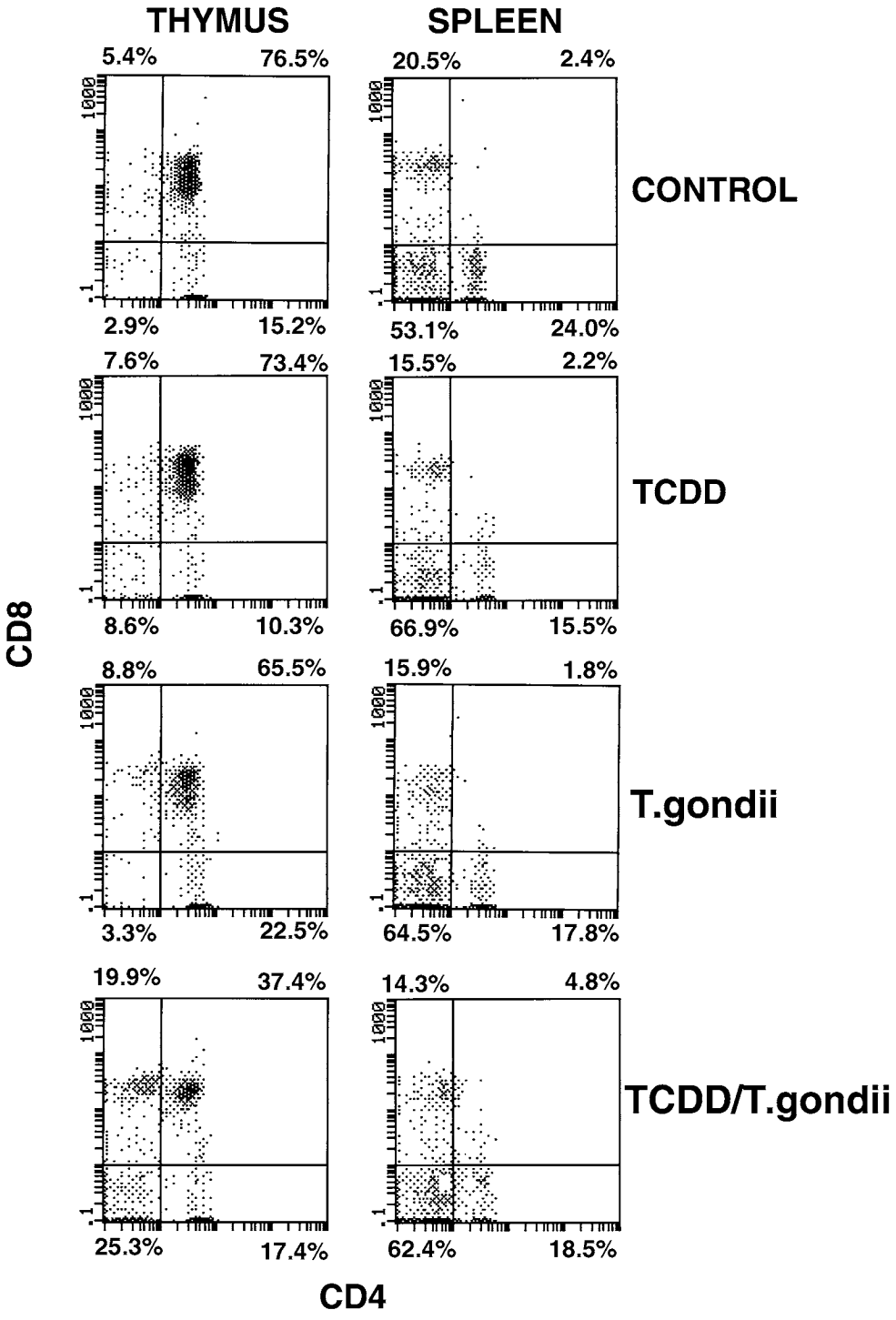
Treatment	**Mean lesion score**	**Mean tissue cyst**
Control	1.0 ± 0	NA[a]
T. gondii	2.0 ± 0.2	1.9 ± 0.6
TCDD	1.0 ± 0	NA[a]
TCDD/*T. gondii*	1.6 ± 0.2	3.3 ± 0.8

* No significant (P>0.05) differences due to TCDD treatment were present.

a NA = Not applicable.

FIGURE 1

Flow cytometric analysis of T cell subsets in the thymus and spleen. Mice were treated with vehicle, TCDD, *T. gondii* and a combination of TCDD and *T. gondii* as described in Materials and Methods. After treatment, the thymocytes and splenocytes were harvested and stained with FITC-anti CD4 and PE-anti CD8 mAbs. Histogram parameters were based on negative controls consisting of cells stained with FITC- or PE-conjugated normal antibody isotypes. The upper left quadrant denotes $CD8^-$ T cells, the lower left, $CD4^-$ $CD8^-$ T cells; upper right, $CD4^+CD8^+$ T cells, and lower right, $CD4^+$ T cells.


THYMUS
SPLEEN
5.4%
76.5%
20.5%
2.4%
CONTROL
2.9%
15.2%
53.1%
24.0%
7.6%
73.4%
15.5%
2.2%
TCDD
8.6%
10.3%
66.9%
15.5%
8.8%
65.5%
15.9%
1.8%
T.gondii
3.3%
22.5%
64.5%
17.8%
19.9%
37.4%
14.3%
4.8%
TCDD/T.gondii
25.3%
17.4%
62.4%
18.5%
CD8
CD4
1000
1
-1

Discussion

In the current study, we demonstrated that TCDD-treatment of mice that were chronically infected with *T. gondii* did not lead to reactivation of the parasite and development of TE despite the observed thymic atrophy and changes in the thymocyte subpopulations. This may be attributed to the fact that while alterations were seen in the thymus, similar changes were not observed in the peripheral lymphoid organs such as the spleen.

As reported by Silverstone et al. (1994b), it is possible that extrathymic mechanisms of T cell development may operate to compensate for the decreased export of the mature T cells from the thymus following TCDD treatment. Alternatively, in the peripheral lymphoid organs of *T. gondii*-infected mice following TCDD-treatment, cell replenishment may be due to direct activation of peripheral mature T cells (Lang et al, 1994; Neumann et al, 1993; Sharma and Gehring, 1979) or indirect stimulation mediated through cytokines (Prell et al., 1995; Karras et al., 1995; Lang et al, 1998; Khan and Casciotti. 1999; Ely et al, 1999; Daubener and Hadding, 1997)

In the current study, we have demonstrated that TCDD-treatment of C57BL/6 mice led to thymic hypoplasia, although the percentage of T cell subsets was not altered. These results confirmed earlier studies from our lab and that carried out elsewhere (Kamath et al., 1997; Rhile et al., 1996; Fine et al., 1989). TCDD has been shown to cause thymic involution, although the exact mechanism by which it acts is not clear. Several mechanisms have been proposed: First, it has been hypothesized that TCDD may inhibit the seeding of

the bone marrow precursors (Silverstone et al., 1994a; Fine et al., 1990a). Other investigators have demonstrated that TCDD-treatment may lead to an alteration in the intrathymic development. These processes include induction of apoptosis (Kamath et al, 1997; McConkey et al., 1988; Kurl et al., 1993) and inhibition of T cell proliferation or differentiation (Lundberg et al., 1990a, Lai et al., 1994, Kremer et al., 1995, Holladay et al., 1991). Furthermore it may be possible that the nonlymphoid cells in the thymus may be affected by TCDD (Nagarkatti, et al., 1984; Greenlee et al., 1985; Kremer et al., 1994, 1995; De Heer et al., 1994).

There has been a great deal of controversy as to which thymocyte phenotypes are affected by TCDD. Several investigators have demonstrated that the percentages of thymocyte subtypes are not altered following dioxin exposure (Rhile et al., 1996; Silverstone et al., 1994a). In contrast, others have found that TCDD decreases the percentage of DP T cells and increases DN and CD8+ T cells (Kremer et al., 1995; Kerkvliet and Brauner, 1990; Lundberg et al., 1990a, De Heer et al., 1994; De Waal et al., 1993; Lai, et al., 1998). Furthermore, TCDD has been shown to affect a subset of CD8+ T cells (Blaylock et al., 1992, Holladay et al., 1991, Fine et al, 1990a). It should be noted that in these reports, the experimental conditions varied.

Our data demonstrated that TCDD-treatment of *T. gondii*-infected mice also led to thymic atrophy similar to that seen in mice treated with TCDD alone. Interestingly, however, in mice treated with TCDD and infected with *T. gondii* but not TCDD alone showed a decrease in the percentage of DP T cells and an increase in the DN and CD8+ T cells in the thymus. These studies may help to

resolve the earlier controversy on the effect of TCDD on the percentage of T cell subsets. Our studies demonstrate that TCDD can alter the percentage of T cell subsets if there is an extrinsic factor such as infection. The DN T cells present in the thymus acquire the CD8 marker and then differentiate into the DP T cells. These DP, which in turn lose either the CD4 or CD8 antigens, become the single positive CD8+ or CD4+ T cells respectively. These single positive cells are the mature T cells, which then immigrate to the periphery. The block in the differentiation of the DN T cells to the DP T cell stage or increased seeding of the bone marrow precursors may have resulted in the accumulation of the DN T cell subpopulation. Furthermore, a decrease in the DP T cells could indicate an arrest in the differentiation of the DN T cells to the DP stage or it may be due to enhanced differentiation from the DP T cell stage to the mature T cells. However, there is also the possibility that the differentiation of the DP T cells may be skewed towards CD8+ T cell subpopulation. The increase in the CD8+ T cells observed may be due to either a block in the export of this subset to the periphery or due to the arrest of the differentiation of DN T cells at the CD4-CD8+ immature T cell stage (Holladay et al., 1991).

Gazzinelli et al. (1992) determined that reactivation of toxoplasmosis in mice occurred within 2 weeks of simultaneous depletion of CD4+ and CD8+ T cells in C57BL/6 mice and their findings were the bases of our experimental infections in the present study. TCDD treatment did not lead to the level of depletion of these T cell subpopulations, which was induced by the use of antibodies directed against CD4 and CD8 markers in their study.

Our studies demonstrated that following *T. gondii* infection in mice did not result in changes in the thymic cellularity and thymocyte subpopulations. This may be because treatment with sodium sulfadiazine led to development of a chronic infection with *T. gondii*. Acute infection with *T. gondii* has been shown to be immunosuppressive (Khan et al., 1996; Channon and Kasper, 1996).

The lack of changes in the spleen cellularity and splenic T cells subsets as well as the non T cells seen following treatment with TCDD or infection with *T. gondii* or a combination of the two, may not be indicative of the functional status of the peripheral lymphocyte populations. In the current study, there were no changes in the percentages and numbers of the non-T cells, which may include the B cells and natural killer cells. It should be noted that macrophages and neutrophils were gated out of the lymphoid cell population based on the size and granularity of the cells during flow cytometric analysis.

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Chapter 4. Effects of Recent Methylmercury Exposure on Acute Toxoplasmosis in CBA/J Mice

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CHAPTER 4. Effects of Recent Methylmercury Exposure on Acute Toxoplasmosis in CBA/J Mice

Abstract

Toxoplasma gondii is a protozoan parasite that can cause life-threatening disease in congenitally infected infants and immunocompromised patients, especially those with AIDS. Toxoplasmic encephalitis is a common presenting condition in an AIDS infection. People become infected with *T. gondii* by ingesting tissue cysts in undercooked meat or by ingesting oocysts excreted by cats. Methyl mercury is a well-documented neurotoxicant that accumulates in the brain and causes severe mental and visual dysfunction, including chronic encephalopathy. Consumption of fish, grains, and seeds are common sources of human exposure to methyl mercury, as is dental amalgam. The present study was conducted to determine how and if exposure to a single 20-mg/kg-body weight dose of methyl mercury would influence the course of an acute *T. gondii* infection. Four groups of six week old, female CBA/J mice were treated orally with methyl mercury on day 0 and then fed 25 *T. gondii* tissue cysts on day 2. CBA/J mice are resistant to acute infection with *T. gondii* but are prone to develop encephalitis during chronic infection. The mice were sacrificed on day 14, post *T. gondii* infection. None of the mice in any group developed signs of acute toxoplasmosis as would be expected if the methyl mercury made them more susceptible to acute infection. The results suggest that exposure to methyl mercury does not increase the susceptibility of CBA/J to acute toxoplasmosis.

Flow cytometric examination of lymphocyte populations (CD4+ and CD8+ lymphocytes) in the spleen and thymus demonstrated differences in the *T. gondii* infected mice when compared to controls and mice given only methyl mercury. Studies are underway to determine if exposure to methyl mercury influences the course of a chronic infection in these encephalitis-prone mice.

Introduction

Toxoplasma gondii is a protozoan parasite that can cause life-threatening disease in congenitally infected infants and immunocompromised patients. Toxoplasmic encephalitis is a common presenting condition in an AIDS infection. People become infected with *T. gondii* by ingesting tissue cysts in undercooked meat or by ingesting oocysts excreted by cats (106). Methyl mercury (MeHg) is a well- documented neurotoxicant that accumulates in the brain and causes severe mental and visual dysfunction, including chronic encephalopathy (12). Consumption of fish, grains, and seeds are common sources of human exposure to MeHg, as is dental amalgam. The present study was conducted to determine whether MeHg exposure would alter the course of an acute *T. gondii* infection in CBA/J mice. CBA/J mice are resistant to acute infection with *T. gondii* but are prone to develop encephalitis during chronic infection.

Materials and Methods

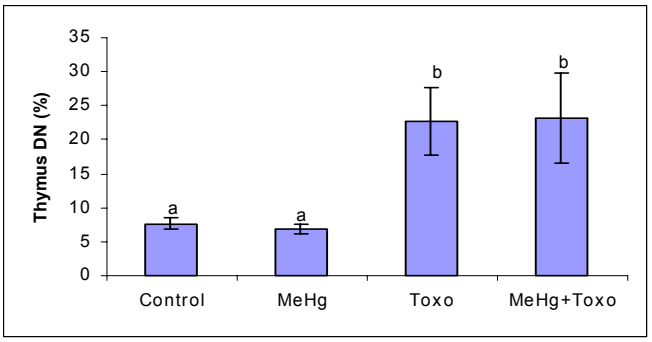
We established a chronic *T. gondii* infection with tissue cysts collected from the brains of International Cancer Research (ICR) strain mice, infected 8 to

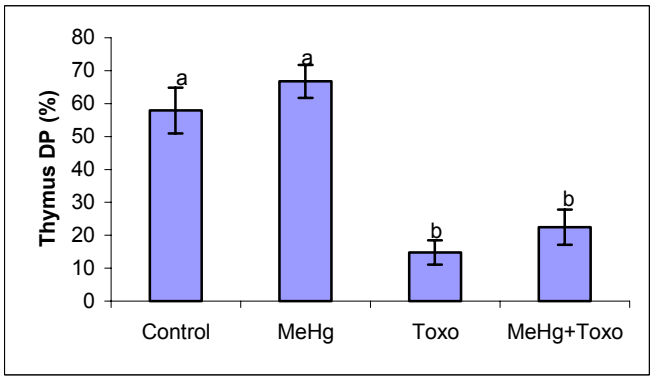
26 weeks previously. A single 20-mg/kg-body weight dose of MeHg was given (78) to cause immunosuppression in the CBA/J mice. MeHg was dissolved in a PBS and DMSO solution. Four groups, including a control and a combination group of six week old, female CBA/J mice were treated orally with MeHg or vehicle control on day 0 and then fed 25 *T. gondii* tissue cysts on day 2. The mice were sacrificed on day 14, post *T. gondii* infection. The brain and kidney were removed and sent to a commercial laboratory for MeHg quantification. The thymus and spleen were removed and made into single cell suspensions of two million thymocytes and splenocytes, respectively, from MeHg and non-MeHg treated mice. Lymphocytes were incubated with fluorescein isothiocyanate (FITC) conjugated anti-CD4+ monoclonal antibody and phycoerythrin (PE) conjugated anti-CD8+ monoclonal antibody. We used an Epics V, model 752, flow cytometer for sample analyses. Statistical analysis was performed with the use of the general linear model (GLM) from the SAS package (SAS Institute Inc., Cary, NC 27513). Significant differences of results with $P<0.05$ were reported.

Results and Discussion

Flow cytometric examination of lymphocyte populations (CD4+ and CD8+ lymphocytes) in the spleen and thymus demonstrated differences in the *T. gondii* infected mice when compared to controls and mice given only MeHg (2). *T. gondii* results show significant differences between thymic T cell subpopulations with the exception of thymus CD8+ cells (Figure 1). *T. gondii* caused a decrease in the percentage of double positive (DP) cells, while there was an increase in

the double negative (DN) and CD4+ cells of the thymus (9). Spleen T cell subpopulations results (not shown) were different in the DP, CD4+ and CD8+ cells of *T. gondii* infected mice, but there was no difference in the non-T cell subpopulation. MeHg was found in both brain and kidneys of all mice given single 20-mg/kg dose. Co-exposure of MeHg and *T. gondii* demonstrated that there were significant differences between the main effects of *T. gondii* and interactions between groups. Future studies will include acute infection vs. chronic infection status when analyzing immune function, nervous function and apoptosis.





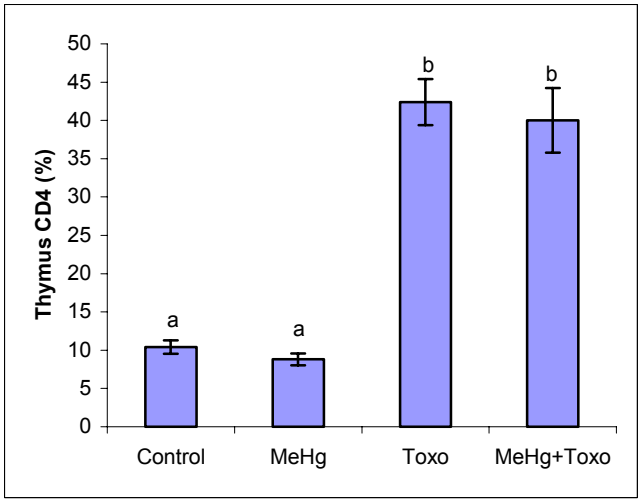


Figure 1. Thymic T-cell Subpopulations (mean ± standard error) 14-day *T. gondii* exposure. Thymus T cell subpopulations, with the exception of CD8+ cells, show those groups without *T. gondii* differ from groups with *T. gondii* at $P<0.05$. Bars with different letters are different from one another; bars with the same letter are not.

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Chapter 5. Neurotoxicity and Immunotoxicity Assessment in CBA/J Mice with Chronic *Toxoplasma gondii* Infection and Single Dose Exposure to Methylmercury

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CHAPTER 5. Neurotoxicity and Immunotoxicity Assessment in CBA/J Mice with Chronic *Toxoplasma gondii* Infection and Single Dose Exposure to Methylmercury

Abstract

Toxoplasma gondii is a protozoan parasite that localizes in the brain where it can cause life-threatening disease. Methylmercury (MeHg) is a well-documented neurotoxicant that accumulates in the brain. We investigated endpoints associated with immunotoxicity and neurotoxicity in mice exposed to MeHg during a chronic *T. gondii* infection. Two groups of six-week-old, female CBA/J mice were either fed 25*T. gondii* tissue cysts of the ME-49 strain or given vehicle. Six weeks later, half of the mice in each group (*T. gondii* and vehicle control) were orally gavaged with a single dose of 20 mg/kg body weight of MeHg, creating four groups of mice (vehicle control, *T. gondii*, MeHg, and *T. gondii*/MeHg) . Mice were sacrificed seven days post MeHg exposure. MeHg increased splenic cellularity and spleen-to-body weight ratios. Thymic $CD4^+CD8^+$ T-cell subpopulations were decreased (P <0.05) by MeHg with or without a concurrent *T. gondii* infection. MeHg had no significant effect on the percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, or non-T-cell subpopulations in the spleen; however, it did cause a significant decrease in mouse body weight. MeHg dosed mice demonstrated an absolute increase in numbers of splenic $CD4^+$, $CD8^+$, or non-T-cells when compared to mice in control and *T. gondii* infected groups. There was a significant (P <0.05) increase in brain tissue cyst counts within the

group exposed to both MeHg and *T. gondii* (16 ± 4, mean ± SE, n=7) versus *T. gondii* alone (4 ± 1, n=8). Histopathological examination demonstrated encephalitis, gliosis, and meningitis in brains from mice infected with *T. gondii*. These data indicate that exposure to both MeHg and *T. gondii* has synergistic effects on the immune system.

Introduction

Toxoplasma gondii is a protozoan parasite that can cause life-threatening disease in congenitally infected infants and immunocompromised patients. People become infected with *T. gondii* by ingesting tissue cysts in undercooked meat or by ingesting oocysts excreted by cats. Toxoplasmosis in the central nervous system is a common cause of brain lesions in AIDS patients. In a number of patients suffering from toxoplasmic encephalitis, the infection may be lethal (107), (48). Toxoplasmic encephalitis is caused by the reactivation of latent tissue cysts of *Toxoplasma gondii*, a protozoan parasite. Once reactivated, the organisms (tachyzoites) released from these tissue cysts destroy nervous tissue and cause progressive meningoencephalitis (3).

Neurotoxicants also damage nervous tissue, but studies examining interactions of *T. gondii* and neurotoxicants have not appeared in the literature. For example, methylmercury (MeHg) is a well-documented neurotoxicant that accumulates in the brain and causes severe mental and visual dysfunction, including chronic encephalopathy (67), (10). Consumption of contaminated fish, grains, and seeds are common sources of human exposure to MeHg. MeHg is

sufficiently neurotoxic that some authors have suggested limiting the consumption of fish and seafood, although these foods have health benefits (6). Independently, both MeHg and *T. gondii* have been found to show effects on the nervous system as well as the immune system. Because both *T. gondii* and MeHg accumulate in the central nervous system and cause nervous system dysfunction, there is the possibility that concurrent exposure could exacerbate a chronic *T. gondii* infection. The present study was conducted to determine whether an acute MeHg exposure would alter the course of a chronic *T. gondii* infection in CBA/J mice. CBA/J mice were used for this study because they are resistant to acute infection but are prone to develop encephalitis during a chronic infection (38).

Materials and Methods

Animals, Parasitic Infection, Chemical Exposure

Female six-week old CBA/J mice (approximately 24-gram body weight) obtained from Jackson Laboratories (Wilmington, MA) were used for experimental infection and methylmercury (MeHg) exposure. Mice were maintained in wire topped polystyrene cages under controlled conditions of temperature (22°C), humidity (40-60%), lighting (12 hour light/dark cycle) and provided with standard mouse chow and water ad libitum throughout the course of the experiments. International Cancer Research (ICR) mice that had been infected 8 to 26 weeks previously were used for maintenance of the ME-49 strain of *T. gondii* (provided by J.P. Dubey, United States Department of Agriculture,

Beltsville, MD). A chronic *T. gondii* infection was initiated in CBA/J mice with 25 tissue cysts from the ICR mice. The cysts were homogenized in Hanks balanced salt solution (HBSS) and given with a 20-gauge feeding needle (Popper & Son, New Hyde Park, NY) to the CBA/J mice. Methylmercury II Chloride (Alfa Aesar, Ward Hill, MA) was dissolved at room temperature in DMSO and diluted with phosphate buffered saline (PBS, 1:100). A single oral dose of 20 mg/kg was provided in a volume of 4 ml/kg (approximately, 100 μl/mouse). Vehicle controls received 4 ml/kg of the DMSO:PBS. Six groups of 10 mice were dosed, including vehicle control, positive control for immunosuppression (dexamethasone, 100 mg/kg topically) (108), *T. gondii*, MeHg, *T. gondii*/MeHg, and a combination of *T.gondii*/dexamethasone. A single 20-mg/kg-body weight dose of MeHg was given orally as this has been reported to cause immunosuppression (109) (19). All housing and experimental procedures involving animals were approved prior to initiation of experiments by the Virginia Tech Animal Care and Use Committee, in accordance with Virginia Polytechnic Institute and State University guidelines.

Organ Preparation

Mice from control and experimental groups were euthanized by CO_2 inhalation 7 days after MeHg exposure and the spleen, thymus, brain, and left kidney were removed. The left half of the brain and the left kidney were used for analysis of MeHg concentration using a cold vapor accessory method of atomic absorption. Briefly, tissues were weighed in a beaker, 5 ml of concentrated nitric acid was added and then gently heating until approximately 1 ml of liquid

remained. The beaker was then removed and allowed to cool, diluted with distilled water to 10-20 ml. Antifoam emulsion (100µL) was added to end the digestion process. Trained technical staff of the Toxicology Laboratory performed the atomic absorption (AA) spectrophotometry analysis. After removal, the thymus and spleen were immediately weighed and placed into a sterile Petri dish for lymphocyte collection. For histopathology, three spleen and thymus tissue samples out of each group were prepared for staining with hematoxylin & eosin. These tissues for lymphocyte collection were dissociated using a stainless steel sieve screen using curved forceps in 10 ml of RPMI-1640 culture medium (GIBCO Lab, Grand Island, NY). Erythrocytes were removed from samples by placing the tissues in Red Blood Cell Lysis Buffer (Sigma Chemical, St. Louis, MO) for 5 minutes at room temperature. Cells were then washed two times in RPMI-1640 medium followed by centrifugation (1300 rpm) at 4°C. Cells were counted electronically using a CASY-1 cell counter (Scharfe System GmbH, Germany).

Detection of Surface Cell Markers and Apoptotic Cells in the Immune System

Lymphocyte cell suspensions from the thymus and spleen were washed in phosphate buffered saline and then $2x10^6$ cells were aliquoted into a total volume of 2 ml. The gates for flow cytometric analyses were set based on autofluorescence of unstained cells from each organ. The cells were incubated for 60 min on ice with a concentration of 0.5 µg/µl fluorescein isothiocyanate (FITC) conjugated anti-$CD4^+$ monoclonal antibody (mAb) and 0.2 µg/µl

phycoerythrin (PE) conjugated anti-$CD8^+$ mAb (PharMingen, San Diego, CA), and 7-aminoactinomycin D (7-AAD) (Molecular Probes Eugene, OR). 7-AAD was dissolved in methanol for a final concentration of 10 µg/ml. Cells were then incubated overnight at 4°C in 300 µl of 3% paraformaldehyde. Following this cells were washed and the fluorescence (470-600 nm) was measured on a Coulter Epics XL flow cytometer (Hialeah, FL) (93). Five thousand events (cells) were analyzed per sample. These data are presented as mean ± SE from the percentage of cells that express the surface marker of $CD4^+CD8^-$, $CD4^-CD8^-$ (DN = double negative), $CD4^+CD8^+$ (DP = double positive), and $CD4^-CD8^+$, along with the uptake of 7-AAD, which shows apoptosis for individual phenotypes. Statistical analysis was performed with the use of the general linear model (GLM) analysis of variance (ANOVA) from the SAS package (SAS Institute Inc., Cary, NC). Significant differences among each experimental group with $P<0.05$ were reported.

Dexamethasone Exposure

Mice were given a single topical dose of 100 mg/kg dose of dexamethasone (DEX) (108), DEX-21 phosphate disodium salt (Sigma Chemical, St. Louis, MO) and examined 48 hours later. Dexamethasone was dissolved in distilled water and placed directly on mice that had been shaved on their interscalpular region of the upper back (M.R. Prater, personal communication).

Detection of *T. gondii* and MeHg Effects in Brain

Mice brains were split into right and left halves after removal. The left half was used for tissue cyst enumeration and MeHg quantification. Tissue cyst enumeration was performed by placing half of the brain in 4 ml of Hanks balanced salt solution and homogenizing using a Stomacher 80 or a Black & Decker ®, 7.2 Volt 300/600 rpm drill. The number of tissue cysts present in 50 μl of homogenate was counted using a bright-field microscope. Paraffin blocks were prepared from the right half of the brains, and from them slides were stained with hematoxylin & eosin (H&E), labeled with TdT – mediated dUTP-X nick end marker (TUNEL, Roche Molecular Biochemicals, Mannheim, Germany) and antibody to glial fibrillary acidic protein, GFAP (21). The primary antibodies used for GFAP visualization were specific at a dilution of 1:100 (110). This procedure used paraffin embedded nervous tissue slides incubated with anti-GFAP mouse monoclonal antibodies. Tissue sections were microwaved and incubated with normal goat serum at room temperature for 30 minutes. A primary antibody was added and the tissues were allowed to incubate overnight at 4°C. Tissues were then ran through a series of incubations with mouse Clono PAP® at a dilution of 1:200 (Sternberger Monoclonals, Lutherville, MD) before being washed one time. Slides were then incubated with diaminobenzidine tetrahydrochloride (DAB) with hydrogen peroxide, added at a dilution of 0.5 ml of stock DAB plus 2.7 μl of 30% hydrogen peroxide. Finally, slides were stained with Gill's hematoxylin as a background stain (to facilitate the visualization process) and cells were dehydrated and covered with Permount and mounted for histology using the

protocol from Sternberger Mononclonals Inc. This endpoint was graded as normal or increased using a plus/minus scale.

Behavioral Assessment

Mice were examined for clinical deficits indicative of neurotoxicity following MeHg exposure. Neurobehavioral assessment was performed daily for six days from the day of MeHg dosing and on the seventh day, the mice were sacrificed. Examinations were done during the dark cycle of the standard 12-hour light/ 12-hour dark cycle with a red light lamp for visualization. Behavioral analysis was used to score the severity of the clinical manifestations of toxicant-induced neurotoxicity in living mice. Behavioral response tests were obtained and modified using an established protocol from the Laboratory for Neurotoxicity Studies (31).

Observed responses included: cage viability (alive or dead); cage activity (normal or abnormal); click response and menace response (reaction or lack of); tail condition (normal or abnormal – damaged, limp, or erect); posture (normal or abnormal – hunched, stiff); respiration (normal or abnormal – labored or slow), and a wooden rod test (ability or inability to hang on to wooden rod for 30 seconds).

All tests were administered with the examiner being unaware of the mouse treatment group (30). The observational assessments and manipulative tests were reported as descriptive data or quantitative data, where possible (19). All experimental procedures and methods were reviewed and approved by the

Virginia Tech Animal Care and Use Committee, in accordance with Virginia Polytechnic Institute and State University guidelines.

Results

Body weight, lymphoid organ weight, cellularity, and pathology

Chronic *T. gondii* infection, *T. gondii* infection plus MeHg and MeHg alone resulted in a loss of 2, 1, and 4 mice per group, respectively. These data indicate that CBA/J mice may be more sensitive to MeHg than other mouse strains. Body weights of the mice in both control and *T. gondii* infected groups exposed to MeHg were significantly less than the vehicle control group. Body weights in mice given MeHg were also lower than *T. gondii* alone ($P<0.05$) (Table I). There was no difference in body weight between vehicle control mice and mice given *T. gondii* alone. Thymus weights were largely unaffected by the exposure (Table I), while spleen organ weights showed a marked increase in those groups exposed to MeHg, which correlates to an increase in cellularity (Figure 1). Spleen-to-body-weight ratios were significantly increased in all treated mice. All treatments resulted in a loss of cell viability in the spleen and MeHg decreased cell viability in the thymus (Figure 2). Hematoxylin & eosin stained sections of thymus and spleen, from mice dosed with MeHg, *T. gondii*, or combinations of those agents were qualitatively similar to the vehicle controls (data not shown).

Table I

Body, Spleen, and Thymus Weights of CBA/J Mice Infected with *T. gondii* and/or *T. gondii* and MeHg*

Treatment	Body weight (g)	Spleen weight (mg)	Thymus weight (mg)
Control	24.1 ± 0.7	48.0 ± 6.5	20.0 ± 10.2
MeHg	20.0 ± 1.8[a]	195.0 ± 32.8[a]	27.5 ± 7.0
T. gondii	23.0 ± 0.5	126.3 ± 19.0[a]	37.6 ± 9.9
T. gondii/MeHg	17.8 ± 0.9[a]	233.0 ± 24.7[a,b]	27.0 ± 5.7

[*] Female CBA/J mice 7 days after oral exposure to 20 mg/kg MeHg (n=5-9)

All data are expressed as mean weight ± standard error

[a] Significantly different from control group ($P < 0.05$), using ANOVA

[b] Significantly different from *T. gondii*-infected mice ($P < 0.05$), using ANOVA

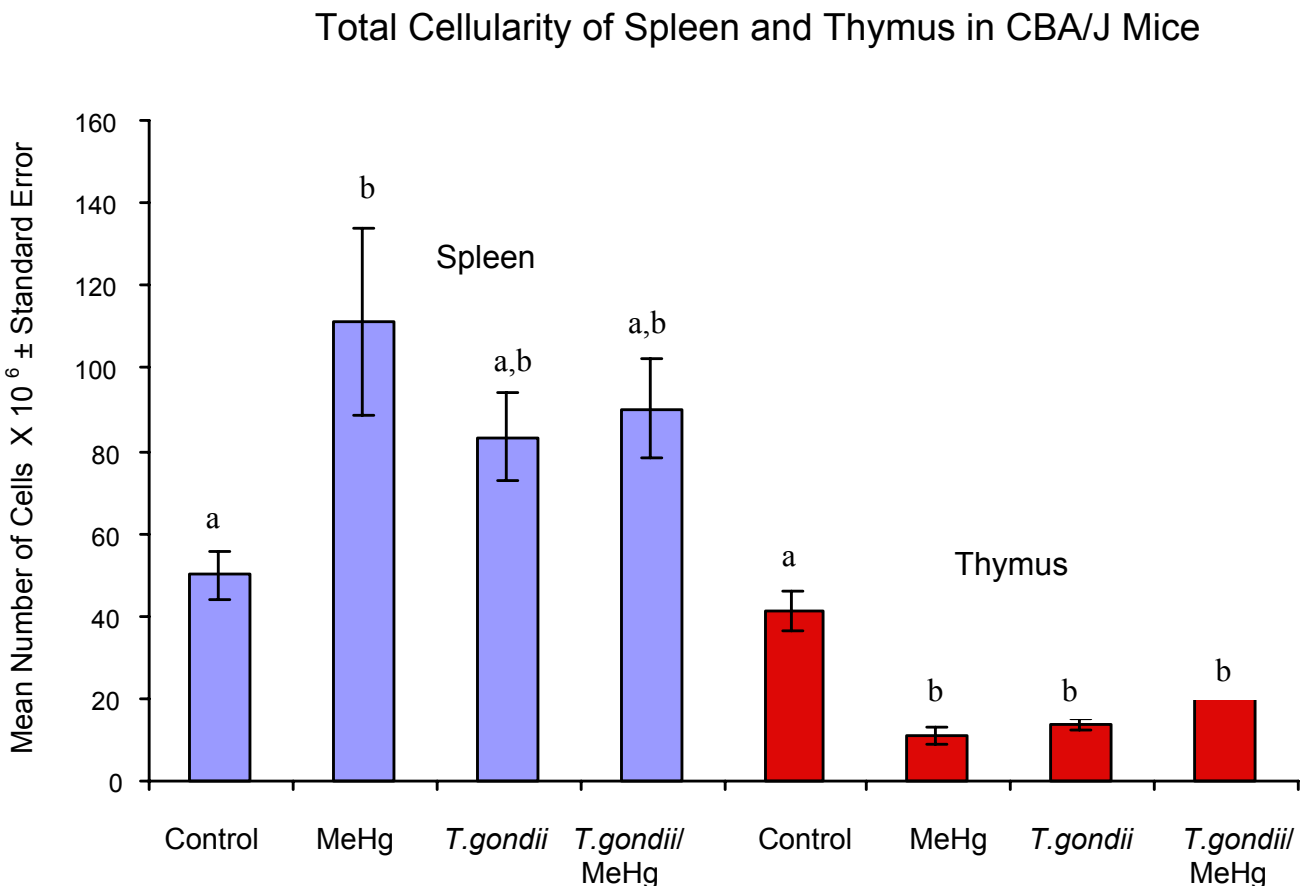

Figure 1. MeHg exposure increased total cellularity of the spleen with or without a concurrent *T. gondii* infection as measured by a CASY-1 electronic cell sorter. Mice were sacrificed 7 days post exposure to a single oral 20 mg/kg dose of MeHg. Thymic cellularity for all treatment groups decreased significantly ($P< 0.05$) compared to control. Each experimental group included 5-9 mice. Comparisons among groups were performed using a Tukey's non – parametric test. For each organ no shared letters above bars implies groups are significantly different (a< 0.05).

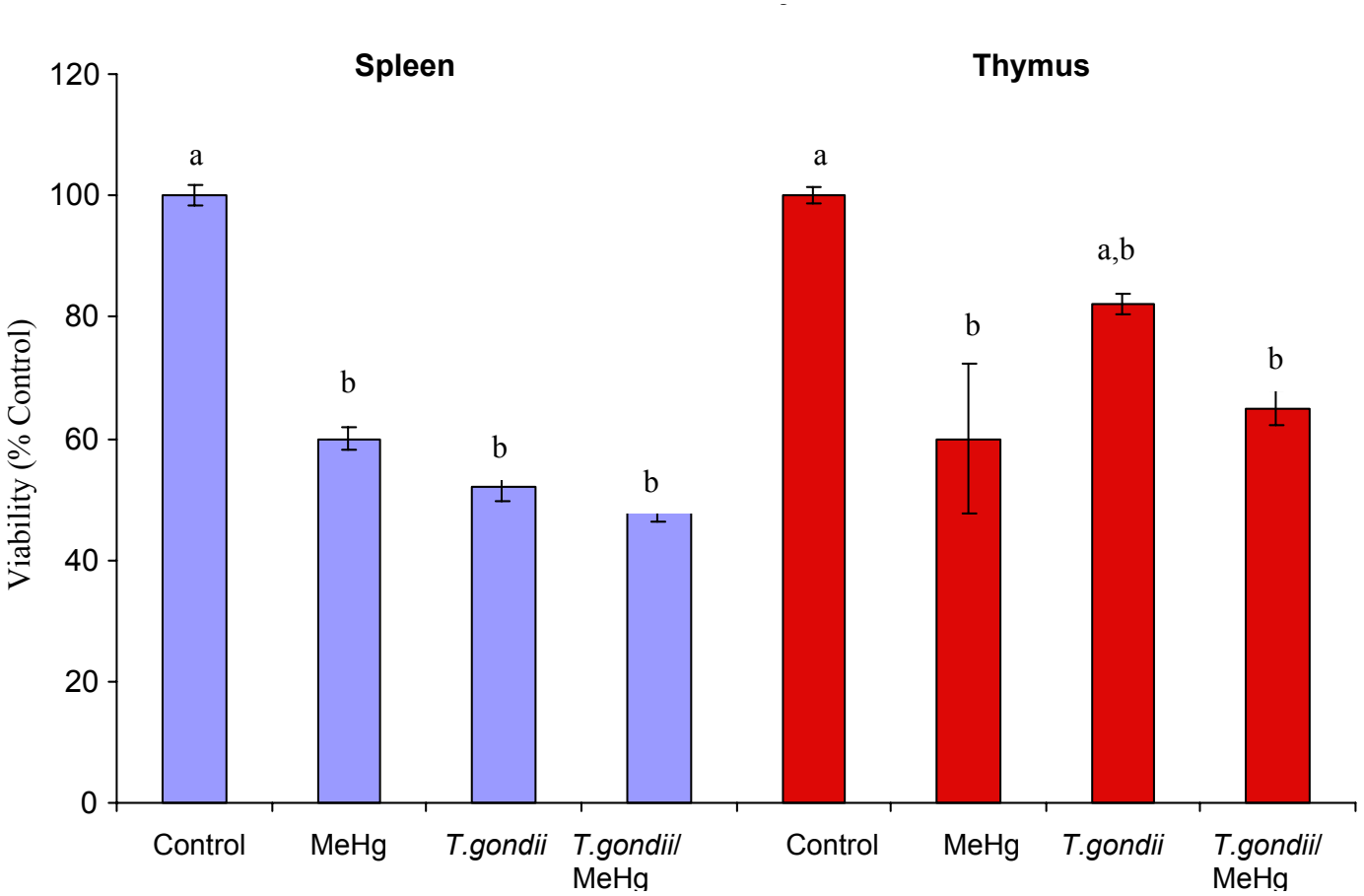

Figure 2. Mice dosed with 20 mg/kg MeHg orally after 6-week T. gondii chronic infection were sacrificed day 7 post MeHg exposure. Viability of cells, expressed as percent of total cells present within the spleen and thymus, was determined by flow cytometric analysis. In both the spleen and thymus, MeHg decreased viability to 60% of control. Similar decreases were seen in the spleen for *T. gondii* or MeHg + *T. gondii* exposures ($P < 0.05$). Between 5-9 mice were included in each experimental group. Comparisons among groups were

performed using a Tukey's non – parametric test. For each organ no shared letters above bars implies groups are significantly different ($a < 0.05$).

Lymphocyte Subpopulations in the Thymus and Spleen

Data from mice co-exposed to *T. gondii* and MeHg showed an increase in thymic $CD4^+$ and $CD4^-CD8^-$ T-cells, $CD4^+CD8^+$ T-cells were decreased. Splenocytes from mice co-exposed to *T. gondii* and MeHg had a significant increase ($P < 0.05$) in the absolute numbers when compared to vehicle control (Table II).

Flow cytometric analysis of thymocyte subpopulations of both groups exposed to MeHg (those with or without a concurrent *T. gondii* infection) showed significant increases ($P < 0.05$) in the percentage of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ T-cell subpopulations; however, $CD4^+CD8^+$ T-cells were markedly decreased (Table III). Splenic T-cell percentages showed no significant changes among any of the experimental groups.

TABLE II

Absolute Numbers of T-cell Subpopulations in Thymus and Spleen from Mice Infected with *T. gondii* and/or *T. gondii* and MeHg*

Treatment	**Cellularity of Thymic T-cell Subpopulation**			
	$CD4^+CD8-$	**$CD4-CD8^+$**	**$CD4^-CD8^-$**	**$CD4^+CD8^+$**
Control	9.9 ± 1.5	0.3 ± 0.1	0.6 ± 0.1	30.2 ± 4.0
MeHg	4.9 ± 4.2	0.1 ± 0.1	0.3 ± 0.2[a]	3.7 ± 2.9[a]
T. gondii	2.9 ± 0.3[a]	0.3 ± 0.4	0.2 ± 0.5[a]	10.7 ± 1.2[a]
T. gondii/MeHg	12.0 ± 0.2[a]	1.0 ± 0.1	1.0 ± 0.2[a]	6.5 ± 0.1[a,b]

Treatment	**Cellularity of Splenic T & non-T-cell subpopulations**		
	$CD4^+CD8-$	**$CD4-CD8^+$**	**non-T-cells**
Control	12.5 ± 2.1	12.6 ± 1.4	24.7 ± 2.9
MeHg	31.8 ± 9.1[a,b]	21.8 ± 4.9[a,b]	57.4 ± 20.9[b]
T. gondii	18.1 ± 1.3	24.4 ± 1.6[a]	39.7 ± 9.0
T. gondii/MeHg	26.0 ± 6.5[a,b]	22.0 ± 3.6[a,b]	41.0 ± 9.5[b]

[*] Female CBA/J mice 7 days after oral exposure to 20 mg/kg MeHg (n=5-9)

All data are expressed as mean x 10^6 cells ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

[c] Statistically significant difference ($P < 0.05$) when compared to MeHg-treated mice

TABLE III

T-cell Subpopulations as a Percent of Total Cells in the Thymus and Spleen of Mice Infected with *T. gondii* and/or *T. gondii* and MeHg*

Treatment	**Percentages of Thymic T-cell subpopulations**			
	$CD4^+CD8-$	**$CD4-CD8^+$**	**$CD4^-CD8^-$**	**$CD4^+CD8^+$**
Control	24.6 ± 3.0	1.0 ± 0.4	1.7 ± 0.3	72.8 ± 3.1
MeHg	46.8 ± 11.6[a,b]	3.5 ± 1.5[a,b]	4.5 ± 1.5[a,b]	45.2 ± 13.7[a,b]
T. gondii	22.2 ± 2.5	1.1 ± 0.3	1.3 ± 0.3	75.5 ± 2.7
T. gondii/MeHg	58.1 ± 2.7[a,b]	4.7 ± 0.4[a,b]	4.6 ± 0.6[a,b]	32.7 ± 3.3[a,b]
	Percentages of Splenic T-cell subpopulations			
	$CD4^+CD8-$	**$CD4-CD8^+$**	**Non-T-cells**	
Control	24.5 ± 1.6	25.5 ± 1.3	49.6 ± 1.7	
MeHg	30.3 ± 6.2	22.1 ± 5.1	47.5 ± 10.9	
T. gondii	23.4 ± 2.1	31.9 ± 3.2	43.2 ± 5.1	
T. gondii/MeHg	31.5 ± 7.2	25.4 ± 2.9	42.5 ± 9.5	

* Female CBA/J mice 7 days after oral exposure to 20 mg/kg MeHg (n=5-9)

All data are expressed as percent mean ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

[c] Statistically significant difference ($P < 0.05$) when compared to MeHg-treated mice

Dexamethasone Positive Control

Dexamethasone (DEX) at 100 mg/kg administered topically for 48 hours provided similar results to MeHg exposure when analyzing spleen and thymus lymphocyte populations. Spleen-to-body weights in mice given DEX, and *T. gondii* /DEX were 0.029 ± 0.004 and 0.057 ± 0.005, respectively, with both showing statistically significant ($P < 0.05$) decreases when compared to vehicle control (0.073 ± 0.003) and *T. gondii* only infected mice (0.079 ± 0.005). Thymus-to-body weights were also decreased significantly in DEX and *T. gondii*/DEX treated mice (0.028 ± 0.003 and 0.021 ± 0.001, respectively) when compared to vehicle control or *T. gondii* alone (0.043 ± 0.004 and 0.036 ± 0.005, respectively). Thymic cellularity (mean x 10^6 cells ± standard error) measured by an electronic cell counter showed a decrease in DEX treated groups with (1.7 ± 0.4) or without a T. gondii infection (1.8 ± 0.5) versus control and T. gondii only infected groups (7.0 ± 1.3 and 8.3 ± 0.5, respectively). Splenic cellularity had a significant increase of 12.4 ± 2.9 in the *T. gondii*/DEX treated group ($P < 0.05$) when compared to control and *T. gondii* only infected mice (5.2 ± 0.6 and 6.9 ± 0.6, respectively). The DEX only group showed a numerical increase in splenic cellularity to 8.1 ± 1.3, that was not statistically significant when compared to other experimental groups.

Flow cytometric analysis of actual percentages of spleen and thymus T-cell subpopulations treated with DEX showed similar data trends as MeHg exposed groups. Thymic $CD4^+$ T-cells in DEX treated groups with or without a concurrent *T. gondii* infection had significant increases ($P < 0.05$) as did $CD4^-$

$CD8^-$ T-cell subpopulations. The $CD4^+CD8^+$ T-cells had a marked decrease in all groups exposed to DEX with or without a concurrent *T. gondii* infection (Table IV). Splenic $CD4^+$ and non- T-cell subpopulations had differences in *T. gondii*, DEX, and *T. gondii*/DEX treated groups only when compared to the vehicle control. There were no differences in the $CD8^+$ T-cell population of the spleen (Table IV).

TABLE IV

T-cell Subpopulations as a Percent of Total Cells in the Thymus and Spleen of Mice Infected with *T. gondii* and/or *T. gondii* and Dexamethasone (DEX) *

Treatment	**Percentages of Thymic T-cell subpopulations**			
	$CD4^+CD8-$	**$CD4-CD8^+$**	**$CD4^-CD8^-$**	**$CD4^+CD8^+$**
Control	19.9 ± 4.9	5.3 ± 0.7	2.9 ± 0.5	72.0 ± 4.8
DEX	37.6 ± 2.7[a,b]	12.3 ± 3.6	43.2 ± 6.1[a,b]	7.7 ± 1.1[a,b]
T. gondii	15.1 ± 3.6	5.4 ± 0.7	2.2 ± 0.3	77.3 ± 3.4
T. gondii/DEX	47.7 ± 3.1[a,b]	18.0 ± 3.4[a,b]	24.4 ± 4.6[a,b,c]	10.1 ± 2.1[a,b]
Treatment	**Percentages of Splenic T-cell subpopulations**			
	$CD4^+CD8-$	**$CD4-CD8^+$**	**Non-T cells**	
Control	44.5 ± 1.9	28.8 ± 1.0	26.2 ± 2.8	
DEX	27.9 ± 2.9[a]	22.7 ± 3.8	42.7 ± 5.0[a]	
T. gondii	31.2 ± 2.3[a]	23.5 ± 2.3	44.0 ± 4.5[a]	
T. gondii/DEX	31.3 ± 4.5[a]	26.2 ± 2.6	38.5 ± 6.2	

[*] Female CBA/J mice exposed topically to 100 mg/kg DEX for 48 hours (n=10)

All data are expressed as percent mean ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

[c] Statistically significant difference ($P < 0.05$) when compared to MeHg-treated mice

Apoptosis in Thymocytes and Splenocytes

Flow cytometric analysis with a triple color stain allowed for determination of apoptosis in individual T-cell subpopulations of the thymus and spleen (Table V and VI). In the thymus, MeHg exposure resulted in significant increases in early apoptotic and late apoptotic/necrotic $CD4^+CD8^+$ T-cells, with no difference in cells from mice infected with *T. gondii*. *T. gondii* infection and *T. gondii*/MeHg co-exposure increased the percentage of late apoptotic/necrotic $CD4^+$ T-cells, but only *T. gondii* infection alone had this effect on $CD8^+$ T-cells (Table V). In the spleen, $CD8^+$, $CD4^+$ and non-T-cell subpopulations in mice treated with *T. gondii* alone underwent apoptosis more readily when those subpopulations were compared to control group or MeHg only treated mice. Splenocytes showed a greater susceptibility to apoptosis in all T-cell subpopulations in *T. gondii*/MeHg co-exposed mice when compared to MeHg alone (Table VI). Apoptosis staining (TUNEL) of the spleen and thymus revealed no differences among treated versus non-treated groups of mice (data not shown).

TABLE V

Apoptosis of Thymocytes in mice infected *T. gondii* and/or *T. gondii* and MeHg *

Treatment	**Percentages of $CD4^+CD8^+$ T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	6.5 ± 1.0	75.2 ± 1.1	18.4 ± 1.3
MeHg	3.1 ± 1.4[a]	63.1 ± 6.5[a]	33.8 ± 5.8[a]
T. gondii	1.8 ± 0.3[a]	72.3 ± 1.6	25.9 ± 1.7
T. gondii/MeHg	2.0 ± 0.2[a]	61.4 ± 2.1[a,b]	36.7 ± 2.1[a,b]
	Percentages of $CD4^+CD8^-$ T-cells		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	70.3± 2.1	14.2 ± 1.1	15.5 ± 1.2
MeHg	67.0 ± 4.3[a]	11.1 ± 2.5	22.0 ± 2.5
T. gondii	52.7 ± 2.5[a,c]	17.3 ± 1.5	30.0 ± 1.6[a,c]
T. gondii/MeHg	70.1 ± 2.7[b]	6.4 ± 1.2[a,b]	23.5 ± 1.7[a,b]
Treatment	**Percentages of $CD4^-CD8^+$ T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	16.4 ± 3.4	77.5 ± 3.6	6.1 ± 1.1
MeHg	12.6 ± 6.6	69.0 ± 2.1	18.4 ± 7.6
T. gondii	8.5 ± 1.0	72.4 ± 2.6	19.1 ± 3.0[a]
T. gondii/MeHg	8.2 ± 1.0	73.8 ± 2.0	18.0 ± 2.5

[*] Female CBA/J mice 7 days after oral exposure to 20 mg/kg MeHg (n=5-9)

All data are expressed as percent mean ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

[c] Statistically significant difference ($P < 0.05$) when compared to MeHg-treated mice

TABLE VI

Apoptosis of Splenocytes in mice infected *T. gondii* and/or *T. gondii* and MeHg *

Treatment	**Percentages of $CD4^+CD8^-$ T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	50.2 ± 5.3	19.5 ± 0.6	30.3 ± 4.9
MeHg	75.8 ± 7.3[a,b]	18.0 ± 6.7	6.3 ± 1.8[a,b]
T. gondii	25.9 ± 3.4[a]	23.2 ± 3.3	50.9 ± 5.5[a]
T. gondii/MeHg	54.5 ± 8.0[b]	24.4 ± 3.4	20.9 ± 5.11[b]
Treatment	**Percentages of $CD4^-CD8^+$ T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	4.8 ± 0.4	57.4 ± 5.5	37.8 ± 5.7
MeHg	19.2 ± 6.0	58.0 ± 1.9	22.8 ± 5.1
T. gondii	4.3 ± 0.6[c]	34.9 ± 4.6[a,c]	60.8 ± 5.0[a,c]
T. gondii/MeHg	12.7 ± 3.8	47.1 ± 3.9	40.3 ± 5.3[b]
Treatment	**Percentages of Non - T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	62.2 ± 3.4	16.4 ± 1.1	21.4 ± 3.7
MeHg	87.2 ± 2.1[a,b]	7.5 ± 1.1[a]	5.3 ± 5.9[b]
T. gondii	39.4 ± 5.4[a]	13.6 ± 2.2	46.9 ± 1.5[a]
T. gondii/MeHg	72.5 ± 3.6[b]	14.5 ± 1.5	13.0 ± 2.8[b]

[*] Female CBA/J mice 7 days after oral exposure to 20 mg/kg MeHg (n=5-9)

All data are expressed as percent mean ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

[c] Statistically significant difference ($P < 0.05$) when compared to MeHg-treated mice

Behavioral Assessment

Statistical analysis using cross tabulation allowed for testing of repeated behavioral responses giving a percentage of mice exhibiting a certain response on each day. Two-day pre-treatment baseline values were used to compare behavioral responses to each day post MeHg exposure based upon effects that deviated from normal responses. Multiple day analysis indicated that 10 out of 27 responses showed a change when comparing *T. gondii*/MeHg treated mice (Table VII) to control mice, which showed no change in responses. Responses from mice with co-exposure exhibited differences that occurred primarily on day 1 (first day after MeHg exposure) and on day 5 post MeHg exposure. These results are presented in Table VII. The most significant response that differed between control and *T. gondii*/MeHg treated mice was home cage activity on day one, with 100% of the mice not active. Other responses seen at that time included: 55% with audible vocalization, and 33% with difficulty balancing on the wooden rod, and 22% with visibly poor tail conditions. None of the control mice displayed these responses. On day five 37% of mice were not active in their cages. Lack of response to click and to menace occurred in 25% and 37%, respectively, of *T. gondii*/MeHg mice, but none of the controls.

In addition to the items noted in Table VII, *T. gondii* infected mice not given MeHg had poor coat conditions (22%) and difficulty balancing on a wooden rod (11%) on Day 4. Mice receiving only MeHg had a 40% death rate by day 5 while those concurrently exposed to *T. gondii*/MeHg had a 20% death rate. Only those variables with significant differences have been listed with percent

response in Table VII. Those variables on either day that were not significant when *T. gondii*/MeHg mice were compared to controls are denoted as n.s.

TABLE VII

Mice Exposed to *T. gondii/*MeHg Showing Response that Differed from Baseline

Responses *T. gondii*/MeHg	Day 1 - % of mice showing response	Day 5 - % of mice showing response
Home cage inactivity	100	37.5
Click response	n.s.	25
Menace response	n.s.	37.5
Wooden rod	33.3	n.s.
Posture	n.s.	25
Tail condition	22.2	n.s.
Respiration	n.s.	12.5
Vocalization	55.6	n.s.

Tissue cysts, lesion scoring, and MeHg quantification

Tissue cyst counts indicated significant differences between the mice that had concurrent exposure to *T. gondii*/MeHg versus those that had only a *T. gondii* infection (Table VIII). Mean lesion scoring was similar across all treatment groups including vehicle control (Table VIII). Cysts were visible upon histopathological examination (Figure 3). All mice dosed with MeHg had quantifiable amounts of MeHg in both their brain and kidney (Table IX).

Table VIII

Mean tissue cyst counts and lesion scores for mice given *T. gondii* and/or *T. gondii* and 20 mg/kg MeHg

Treatment	**Mean tissue cysts**	**Mean lesion score**
Control	NA	1.0 ± 0
T. gondii	4.0 ± 1.0	2.0 ± 0.2
MeHg	NA	1.0 ± 0
T. gondii/MeHg	16.0 ± 4.0*	1.6 ± 0.2

* Significant (P<0.05) differences when MeHg exposure was present.

NA = Not applicable.

TABLE IX

Mercury burden in the brain and kidney of CBA/J Mice treated with 20 mg/kg MeHg only or infected with *T. gondii* and MeHg*

Treatment	**brain mercury burden**	**kidney mercury burden**
MeHg	0.35 ± 0.30	8.25 ± 1.9
T. gondii/MeHg	0.04 ± 0.01[a]	5.39 ± 1.8

Levels of MeHg in mice not treated with 20 mg/kg MeHg were non-detectable

*All values are given in mean ± standard error of parts per million (n = 5-9)

[a] Statistical difference of (P < 0.05) when compared to MeHg only

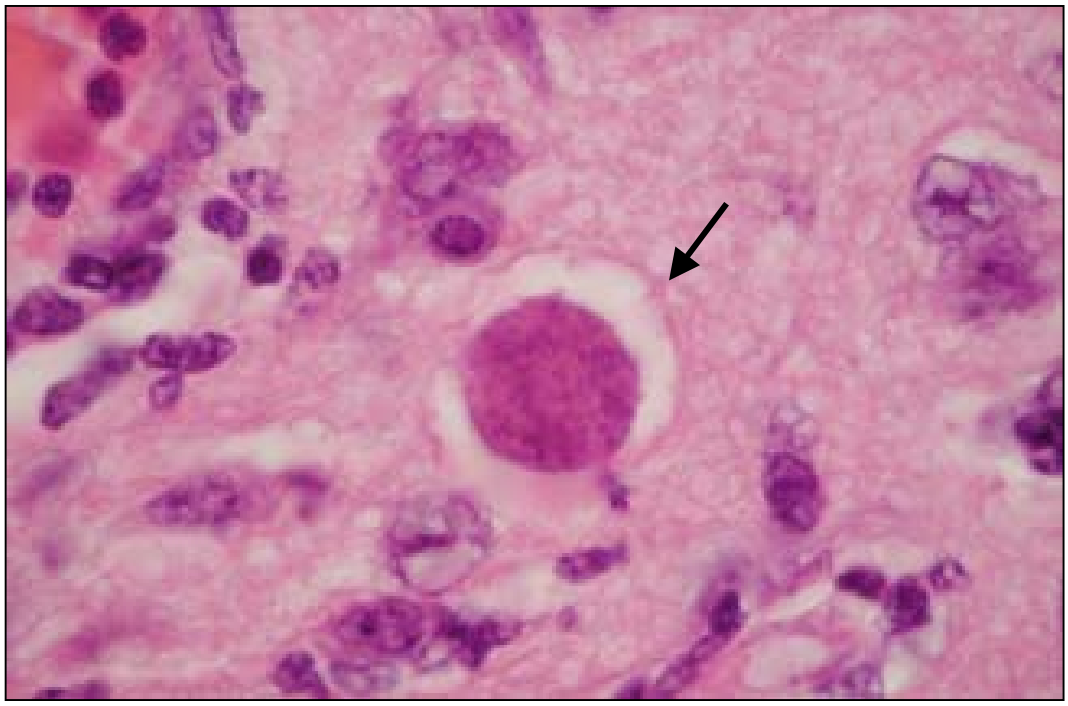

Figure 3. Tissue cyst (arrow) in an immunocompetent mouse chronically infected with *T. gondii*. Bradyzoites are present within cysts and are isolated from the host immune system by the cyst wall. Scattered adjacent inflammation provides strong evidence that an immune response has been mounted. Hematoxylin – eosin, X 400.

Histopathology of the Brain

A single 20 mg/kg dose of methylmercury administered 7 days prior to sacrifice did not elicit any histologic changes in the brain as determined by H & E (Figures 4 and 5) or TUNEL staining for DNA strand breaks (not shown) when compared to vehicle control mice. In mice only infected with *T. gondii* there was prominent meningitis with a predominance of lymphocytes and plasma cells in the exudate. In the brain, there were multiple regions of inflammation. Some of these were small focal infiltrates of lymphocytes, plasma cells, microglia, and macrophages. In other regions, the exudate was more extensive, with associated regions of necrosis. Foci of dystrophic calcification and perivascular lymphocytic and plasmacytic exudates were seen. *T. gondii* cysts are not uncommon (Figure 3). Some granulomas are seen in inflamed regions. Lesions were noted in all regions of the brain. The mice administered both *T. gondii* and MeHg had similar lesions. The degree of inflammation and dystrophic calcification were less prominent in mice only given *T. gondii*.

Immunohistochemical staining for GFAP showed variation in staining intensity within the MeHg dosed and control groups (Table X). GFAP – reactive astrocytes were assessed in the cerebellar subcortical white matter and in the external capsule. There was marked variation of staining intensity in the cerebellar region but this was related more to the degree of staining reaction. There were some qualitative differences in the appearances of the astrocytes. These often appeared as typical cells (Figure 5). One MeHg dosed animal had many rounded GFAP positive cells resembling macrophages and representing a degradative change in astrocytes.

The mice only infected with *T. gondii* had a much more prominent astrocytic reaction (gliosis) with many prominent hypertrophic cells (Table X). This was usually seen within or adjacent to parasite – induced regions of inflammation (Figure 6 and 7). This often led to diffuse GFAP – positive regions, possibly reflecting marked staining of glial processes. Some fragmentations of astrocytic processes, as noted above, were also seen. In addition, many mice in this group had diffuse gliosis of structures such as the hippocampus, superficial cerebral cortex (often beneath regions of meningitis) and cerebellar cortex (Figure 7). On occasion intact tissue cysts had a narrow collar of astrocytic processes.

The two regions, external capsule and subcortical white matter, that were examined in the control and MeHg dosed mice had much more extensive gliosis, even in the absence of local *T. gondii* – induced inflammation (Table X). There was marked variation in the intensity of GFAP staining among the mice dosed with both *T. gondii* and MeHg (Table X). The basis for this is not clear. As a group, these mice appear to have less intensive GFAP staining of astrocytes than those given *T. gondii* alone (Table X).

TABLE X

GFAP Staining Scores in Brains of mice infected with or without *T. gondii* and/or 20 mg/kg of MeHg after 7-day exposure*

Mouse #	Cerebellar White Matter	External Capsule	Overall Staining Intensity
L1	+1	0	+2
L2	+1	+1	+1
L3	+2	0	+2
L4	0	0	+1
L6	+2	0	+3
L8	0	0	+2
L9	+3	+1	+4
M2	+2	+1	+2
M3	0	0	0
M6	0	+1	+1
M8	+1	0	+1
M9	+3	+3	+3
T2	+4	+4	+4
T3	+2	+3	+3
T4	+4	+4	+4
T7	+3	+4	+4
T8	+4	+3	+3
C1	0	0	0
C2	+2	+1	+2
C3	+2	0	+1
C4	NP	0	+1
C5	+2	+1	+2
C6	+3	+1	+3
C9	+3	+3	+3
C10	+4	+4	+4

* L- vehicle control
M- MeHg
T- *T. gondii*
C- *T. gondii*/MeHg

NP – brain tissue region not present in section
0 – no staining
+1 – minimal staining
+2 – mild staining
+3 – moderate staining
+4 – marked staining

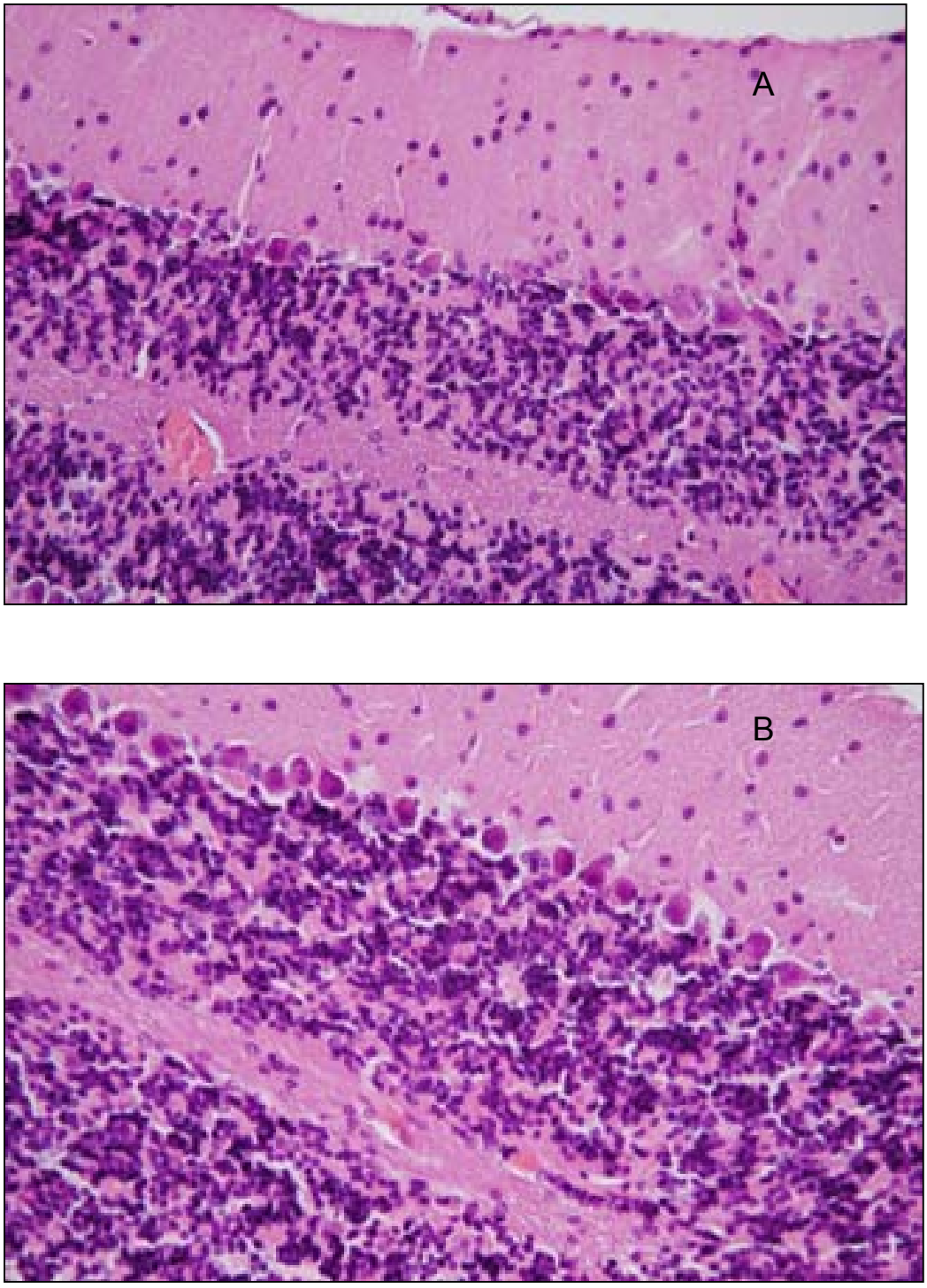

Figure 4. Cerebellar cortex from a vehicle control (L1) and a MeHg dosed (M3) mouse. Significant differences were not noted between these two specimens. Hematoxylin – eosin staining 200X.

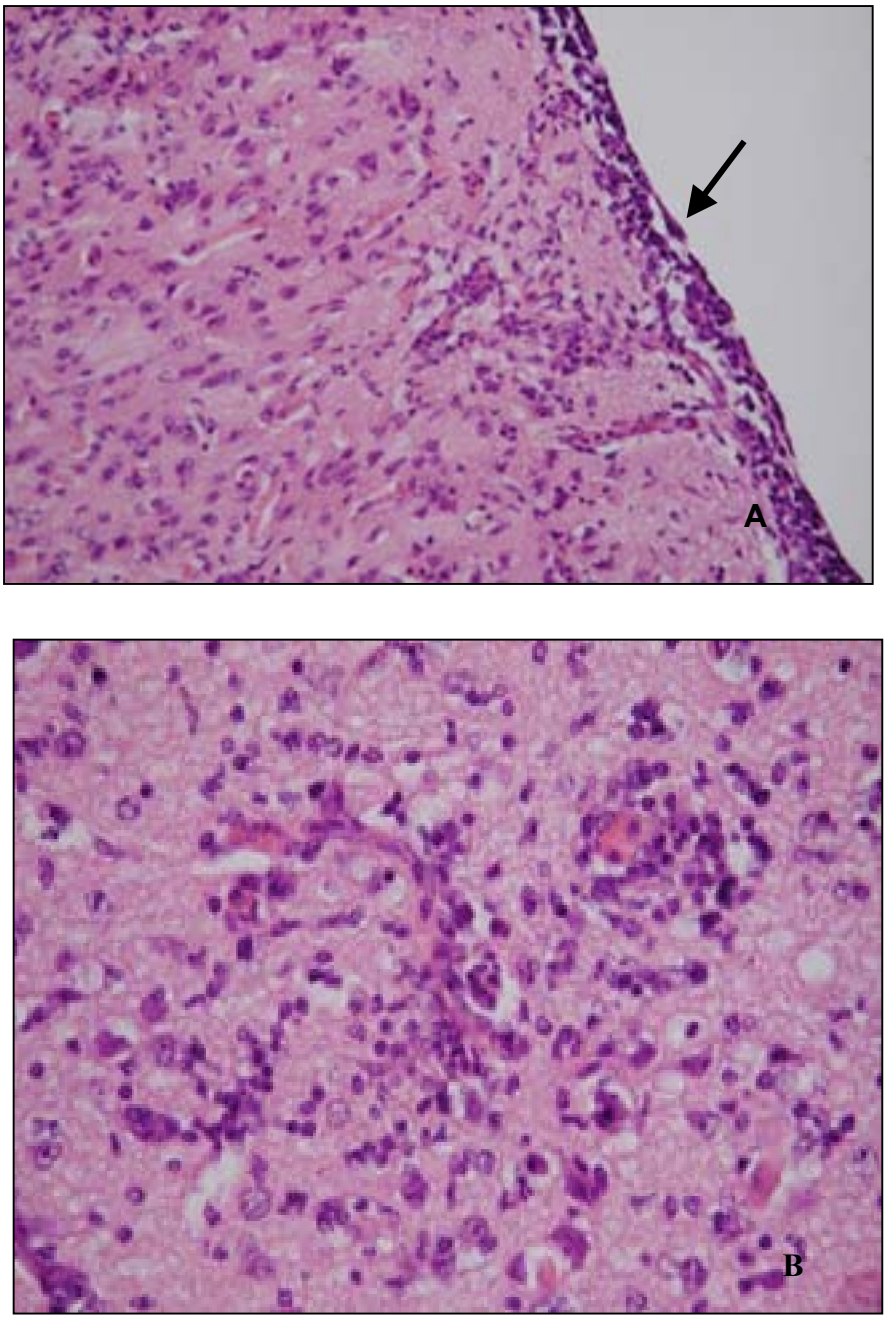

Figure 5. *Toxoplasma gondii* inflammation. Cerebral cortex of mouse given 25 tissue cysts 7 weeks earlier. There is prominent meningitis (A, arrow). The underlying brain tissue has prominent blood vessels and intense inflammation (B) is a higher magnification. The perivascular exudate is predominantly lymphocytic and plasmocytic. Hematoxylin – eosin.

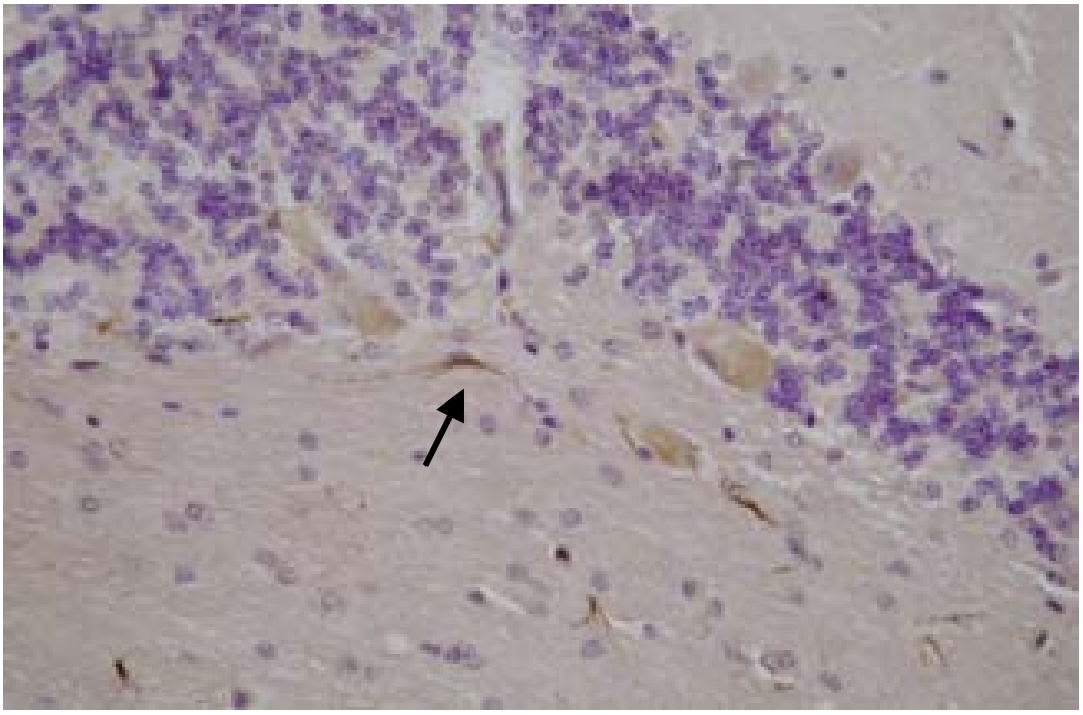

Figure 6. GFAP – positive astrocytes (arrow) in the cerebellar subcortical white matter of a MeHg dosed mouse. This was consistent with the vehicle control mice. GFAP stain.

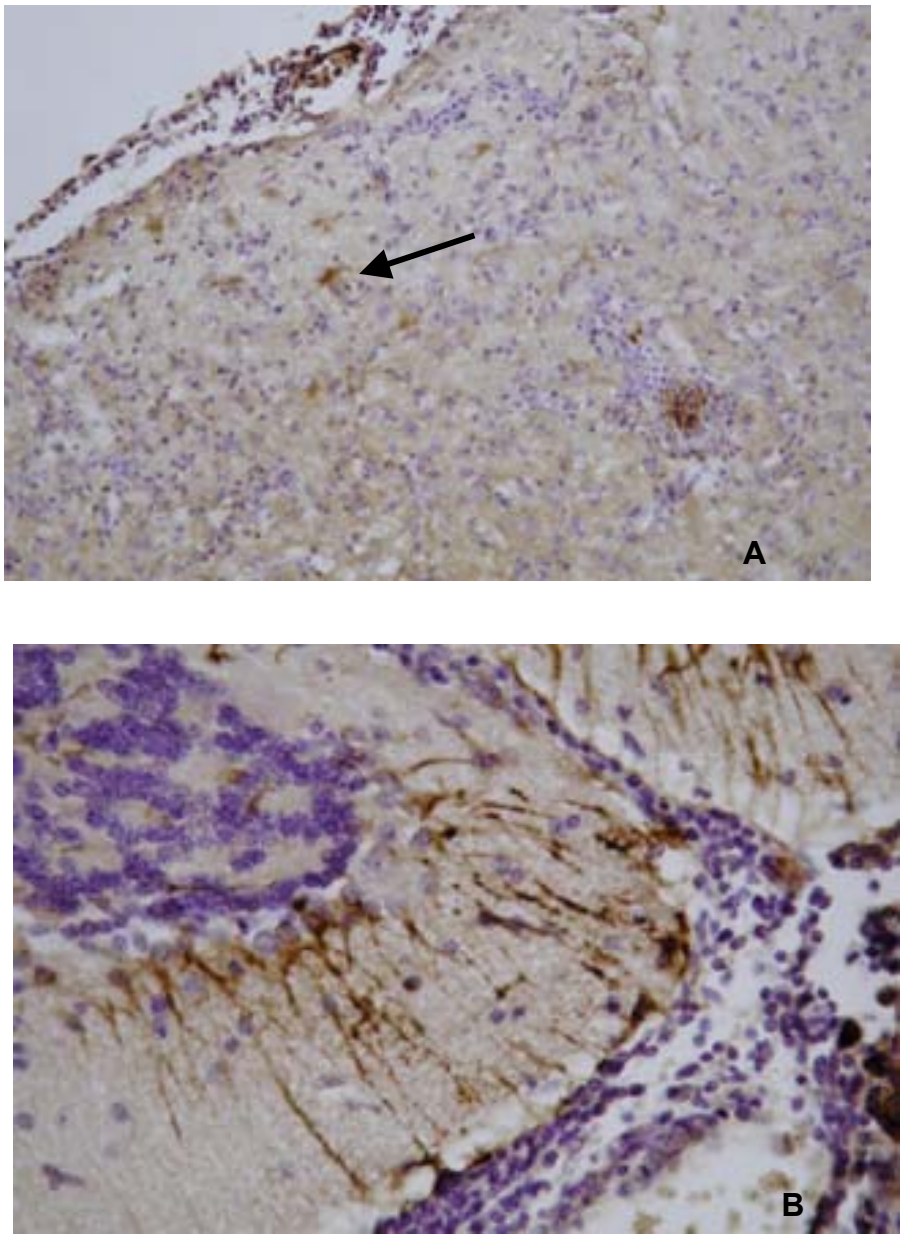

Figure 7. Hypertrophic astrocytes (arrows) are seen in an inflamed region of cerebral cortex (A). Similar cells are seen in the cerebellar molecular layer beneath a region of meninges (B) a higher magnification. GFAP stain.

Discussion

In this study, we tested whether a single exposure to an environmental contaminant, MeHg, could adversely affect a chronic *Toxoplasma gondii* infection. Reactivation of a chronic infection from the rupturing of latent tissue cysts, due to a decrease in immune function, is the cause of toxoplasmic encephalitis (TE) (40) and conceivably could be induced by MeHg exposure. Results indicated that the single dose of MeHg had a detrimental effect on the CBA/J mouse immune system. In this regard, MeHg has been reported to inhibit lymphocyte functions, such as the expression of cell activation markers on cell surfaces and the production of cytokines (79). We saw that MeHg could affect several lymphocyte subpopulations. Results of this study further demonstrate that in CBA/J mice a relapse of toxoplasmosis could be induced by a single dose of MeHg. This was demonstrated by a marked increase in the number of *T. gondii* cysts in the brain. This relapse of a chronic *T. gondii* infection also provides a new model to elucidate mechanisms *in vivo* that underlie the immunosuppressive effects of MeHg. Since both these agents cause detrimental effects on similar systems, nervous and immune (48) (77), it was reasonable to expect that the co – existence of a chronic infection and an environmental contaminant such as those examined here would worsen the initial disease state.

Factors that contribute to the observed interaction of *T. gondii* and MeHg probably relate to effects of MeHg on the immune system. MeHg increased the number of mature $CD4^{-}CD8^{+}$ lymphocytes. The double negative (DN, $CD4^{-}CD8^{-}$) thymocytes present in the thymus acquire the CD8 marker and then differentiate

into the double positive (DP, $CD4^+CD8^+$) T-cells. These DP cells, which in turn lose either the CD4 or CD8 antigens, become the single positive $CD4^-CD8^+$ or $CD4^+CD8^-$ T-cells. Single positive cells are functionally mature T-cells that then immigrate to the periphery. A block in the differentiation of the DP T-cell stage in the present mice may have resulted in the accumulation of the DN T-cell subpopulation since increased apoptosis of DP cells was not apparent (data not depicted). The increase in the percentage of thymic $CD4^-CD8^+$ T-cells observed in this study may be due to a block in the export of this subset to the periphery. However, in mice with *T. gondii* infection, apoptosis of $CD4^-CD8^+$ T cells was increased.

In our studies, dexamethasone (DEX) was used as a positive control for immunosuppression for cells of the immune system (16), (108). Because it is known to cause apoptosis as well, it provided a model for comparison of immunosuppression and apoptosis caused by MeHg exposure. Results of our study with MeHg denoted similar increases and decreases of percentages in T-cell subpopulations for all groups exposed to MeHg when comparing results to DEX treated mice with and without a *T. gondii* infection. These data further substantiate MeHg induced immunosuppression and suggest that this depression of the immune system contributed to alterations in the T-cell subpopulations. DEX can induce apoptosis, but phagocytic cells rapidly clear dying cells causing difficulty in detecting cell death in the thymus (16). This could also be why apoptotic cells were not detected using the TUNEL staining on slides from the spleen, thymus, and brain tissue in MeHg treated mice.

Other effects of *T. gondii* and MeHg seen in the present study are supported by previous studies. We found a decrease in body weight of groups of mice exposed to MeHg regardless of a *T. gondii* infection. This has been previously shown as an initial symptom of MeHg intoxication (111). The toxicity indicated by a decrease in overall body weight for MeHg exposed groups may have contributed to the increases in splenic weights and cellularity, and the decrease in splenic cell viability when compared to vehicle control (111).

In our studies, we did not note many behavioral alterations within the 7 days following a single oral dose of 20 mg/kg MeHg. Previous studies indicated that large doses of MeHg (100 mg/kg) over time (17 days) caused an increase in abnormal neurological symptoms such as the inability to perform a righting reflex, stay on a rod, or maintain their head posture. Death occurred shortly thereafter. In that study, lower doses given over longer periods of time (38 days) resulted in a delayed appearance of symptoms of neurotoxicity (112), (113). Studies reported here, with a single MeHg exposure did not show this same effect. It is possible that the single dose of MeHg is more “reversible” (114) or possibly, a toxic effect may be more delayed (115). In an unexpected occurrence, the 20 mg/kg dose was high enough to cause death of some mice in the MeHg treated group. In another study, we found that all mice survived multiple doses of 8 mg/kg MeHg every 2-3 days for 14 days and did not show neurobehavioral effects (see Chapter 6). Concentrations of MeHg greater than 10 mg/kg (10-week exposure) are usually accompanied by neurological symptoms. Concentrations in the brain increase when the kidney is no longer able to handle

the whole body burden of mercury or when there is a decrease in mercury excretion by the kidney (18). Delayed effects from longer-term exposure to MeHg have been observed following environmental MeHg exposure in humans (116). This includes Minamata disease in Japan (117) and effects noted in people following consumption of contaminated grain in Iraq (118).

Brains from mice exposed to MeHg showed less intensive staining to GFAP compared to brains from mice exposed to *T. gondii* only, verifying reports from another study (1). This in part supports the decrease seen in lesion scores and the concurrent increase in tissue cysts seen in brains of mice exposed to *T. gondii*/MeHg, which was likely to have been caused by a decrease in the inflammatory response of MeHg exposed mice. It is well known that the pathogenicity of a *T. gondii* infection is partially regulated by the genetics of the parasite (38). The ME-49 strain of *T. gondii* is known to produce more tissue cysts than the RH or BK strains (4). The present increase of ME-49 tissue cysts seen in the co-exposure group most likely reflects the ability of MeHg to cause immunosuppression. These data further support immunosuppression as a pathogenic mechanism related to toxoplasmic encephalitis (119). The strain of mouse may play a role in the prominence of tissue cysts, although the efficiency of the host immune system is still the main factor controlling the clinical features of toxoplasmosis (38).

Apoptosis was not seen in the brains of mice given MeHg or *T. gondii*. The TUNEL method showed that positive DNA strand breaks were not prominent in the cerebellar granule cell layer of the MeHg exposed mice (120). This type of

assay did not prove useful when attempting to elucidate our hypothesis, that MeHg would increase apoptosis in the brain of mice chronically infected with *T. gondii*. Furthermore, it is well understood that there is great difficulty in experimentally separating pathologic changes caused by the parasite through direct tissue destruction versus damage caused by toxicant – induced exposure (58).

In summary, the present experiments have confirmed that a single dose of MeHg causes a chronic *T. gondii* infection to relapse. This is indicated both by thymic T-cell subset increases in those cell phenotypes ($CD4^-CD8^+$ and $CD4^+CD8^-$) that have been implicated as having a primary response to the parasite and by increases in the numbers of *T. gondii* tissue cysts in the brain. The effects on the brain have potential to contribute to nervous system dysfunction.

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Chapter 6. Neurotoxicity and Immunotoxicity Assessment in CBA/J Mice with Chronic *Toxoplasma gondii* Infection and Multiple Exposures to Methylmercury

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CHAPTER 6. Neurotoxicity and Immunotoxicity Assessment in CBA/J Mice with Chronic *Toxoplasma gondii* Infection and Multiple Exposures to Methylmercury

Abstract

Toxoplasma gondii is a protozoan parasite that causes life-threatening disease in congenitally infected infants and immunocompromised patients, especially those with AIDS. Methylmercury (MeHg) is a well-documented neurotoxicant that accumulates in the brain and causes severe mental and visual dysfunction. The present study was conducted to determine the effect of multiple low doses of MeHg on the course of a chronic *T. gondii* infection. Four groups of six-week-old, female CBA/J mice were either fed 25*T. gondii* tissue cysts of the ME-49 strain or given vehicle. Six weeks later, half of each group (*T. gondii* and vehicle control) was orally gavaged with 8-mg/kg body weight doses of MeHg on days 0, 2,4,7,10,13. All mice were sacrificed on day 17 or 18 post MeHg exposure. There was no mortality in any of the experimental groups of mice (n=10). MeHg caused a significant increase in spleen weights while thymic weights remained unchanged. Flow cytometric analysis of lymphocyte subpopulations in the thymus demonstrated a significant increase in the percentage of $CD8^+$ T-cells in groups exposed to MeHg with a concurrent *T. gondii* infection. Groups of mice exposed to MeHg showed a decrease in total thymic cellularity and cellularity of each T-cell subpopulation when compared to control mice, but viability of these cells was unaffected. Splenic cell viability was decreased in mice exposed to MeHg, but alterations in T-cell subpopulations were not noted. Histopathological examination of the brain did not demonstrate

differences in lesion scores or differences in tissue cyst numbers in mice co-exposed to *T. gondii* and MeHg compared to mice exposed to *T. gondii* alone. These data indicate that multiple low doses of MeHg did not exacerbate chronic toxoplasmosis in CBA/J mice, although MeHg-induced effects on the immune system were evident.

Introduction

Toxoplasma gondii is a protozoan parasite that can cause life-threatening disease in congenitally infected infants and immunocompromised patients. People become infected with *T. gondii* by ingesting tissue cysts in undercooked meat or by ingesting oocysts excreted by cats. Toxoplasmosis in the central nervous system is a common cause of brain lesions in AIDS patients. In a number of patients suffering from toxoplasmic encephalitis, the infection may be lethal (107), (48). Toxoplasmic encephalitis is caused by the reactivation of latent tissue cysts of *Toxoplasma gondii*, a protozoan parasite. Once reactivated, the organisms (tachyzoites) released from these tissue cysts destroy nervous tissue and cause progressive meningoencephalitis (3).

Neurotoxicants also damage nervous tissue, but studies examining interactions of *T. gondii* and neurotoxicants have not appeared in the literature. For example, methylmercury (MeHg) is a well-documented neurotoxicant that accumulates in the brain and causes severe mental and visual dysfunction, including chronic encephalopathy (67), (10). Consumption of contaminated fish, grains, and seeds are common sources of human exposure to MeHg. MeHg is

sufficiently neurotoxic that some authors have suggested limiting the consumption of fish and seafood, even though these limitations reduce the consumption of foods with health benefits (6). Independently, both MeHg and *T. gondii* have been found to show effects on the nervous system as well as the immune system. Therefore, it was hypothesized that the combination of MeHg accumulation could worsen a chronic *T. gondii* infection. Because both *T. gondii* and MeHg accumulate in the central nervous system causing dysfunction, there is the possibility that concurrent exposure could exacerbate a chronic *T. gondii* infection. The present study was conducted to determine whether exposure to multiple doses of MeHg would alter the course of a chronic *T. gondii* infection in CBA/J mice.

Materials and Methods

Animals, Parasitic Infection, Chemical Exposure

Female six-week old CBA/J mice (approximately 24 gram body weight) obtained from Jackson Laboratories (Wilmington, MA) were used for experimental infection and MeHg exposure. Mice in groups of 10 each were maintained in wire topped polystyrene cages under controlled conditions of temperature (22°C), humidity (40-60%), lighting (12 hour light/dark cycle) and provided with standard mouse chow and water ad libitum throughout the course of the experiments. International Cancer Research (ICR) mice that had been infected 8 to 26 weeks previously were used for maintenance of the ME-49 strain of *T. gondii*. A chronic *T. gondii* infection was initiated in CBA/J mice with 25

tissue cysts obtained from the brains of ICR mice. The tissue cysts were in Hanks balanced salt solution (HBSS) and given with a 20-gauge feeding needle (Popper & Son, New Hyde Park, NY) to the CBA/J mice. CBA/J mice were used for this study because they are resistant to acute infection but are prone to develop encephalitis during a chronic infection (38). Methylmercury II Chloride (Alfa Aesar, Ward Hill, MA) was dissolved at room temperature in DMSO and diluted with phosphate buffered saline (PBS, 1:100). Multiple oral doses of 8 mg/kg body weight were given to each mouse on days 0, 2, 4, 7, 10, 13. Vehicle controls received a DMSO/PBS suspension. Four groups of mice were dosed, including vehicle control, *T. gondii*, MeHg, and *T. gondii*/MeHg. The 8 mg/kg dosage chosen for multiple oral exposure was based on a dose response study done previously in our laboratory (see appended data, M.D. King).

Organ Preparation

Mice from control and experimental groups were euthanized by CO_2 inhalation 17-18 days after initiating MeHg exposure and the spleen, thymus, brain, and left kidney were removed. The left half of the brain and the left kidney were used for analysis of MeHg concentration using a cold vapor accessory method of atomic absorption. After removal, the thymus and spleen were immediately weighed and placed into a sterile Petri dish for lymphocyte collection. These tissues were dissociated using a stainless steel sieve screen and curved forceps in 10 ml of RPMI-1640 culture medium (GIBCO Lab, Grand Island, NY). Erythrocytes were removed from samples by placing the tissues in Red Blood Cell Lysis Buffer (Sigma Chemical, St. Louis, MO) for 5 minutes at

room temperature. Cells were then washed two times in RPMI-1640 medium by centrifugation (1300 rpm) at 4°C. Cells were counted electronically using a CASY-1 cell counter (Scharfe System GmbH, Germany).

Detection of Cell Surface Markers and Apoptotic Cells in the Immune System

Lymphocyte cell suspensions from the thymus and spleen were washed in phosphate buffered saline and then $2x10^6$ cells were aliquoted into a total volume of 2 ml. The gates for flow cytometric analyses were set based on autofluorescence of unstained cells from each organ. The cells were incubated for 60 min on ice with a concentration of 0.5 µg/µl fluorescein isothiocyanate (FITC) conjugated anti-CD4 monoclonal antibody (mAb) and 0.2 µg/µl phycoerythrin (PE) conjugated anti-CD8 mAb (PharMingen, San Diego, CA), and 7-aminoactinomycin D (7-AAD) (Molecular Probes Eugene, OR). 7-AAD was dissolved in methanol for a final concentration of 10 µg/ml. Cells were then incubated overnight at 4°C in 300 µl of 3% paraformaldehyde. Following this cells were washed and the fluorescence (470-600 nm) was measured on a Coulter Epics XL flow cytometer (Hialeah, FL) (93). Five thousand events (cells) were analyzed per sample. These data are presented as mean ± SE from the percentage of cells that express the surface marker of $CD4^+CD8^-$, CD4-CD8- (DN = double negative), $CD4^+CD8^+$ (DP = double positive), and $CD4^-CD8^+$ along with the uptake of 7-AAD, which shows apoptosis for individual phenotypes. Statistical analysis was performed with the use of the general linear model (GLM)

analysis of variance (ANOVA) from the SAS package (SAS Institute Inc., Cary, NC). Significant differences among each experimental group with $P<0.05$ were reported.

Detection of *T. gondii* and MeHg Effects in Brain

Mice brains were split into right and left halves after removal. The left half was used for tissue cyst enumeration and MeHg quantification. Tissue cyst enumeration was performed by placing half of the brain in 4 ml of HBSS and homogenizing using a Stomacher 80 or a Black & Decker ®, 7.2 Volt 300/600 rpm drill (21). The number of tissue cysts present in 50 µl of homogenate was counted using a bright-field microscope. Paraffin blocks were prepared from the right half of the brains, and from them slides were stained with hematoxylin & eosin (H&E), labeled with TdT – mediated dUTP-X nick end marker (TUNEL, Roche Molecular Biochemicals, Mannheim, Germany) and antibody to glial fibrillary acidic protein, GFAP (21). The primary antibodies used for GFAP visualization were specific at a dilution of 1:100 (110). This procedure used paraffin embedded nervous tissue slides incubated with anti-GFAP mouse monoclonal antibodies. Tissue sections were microwaved and incubated with normal goat serum at room temperature for 30 minutes. A primary antibody was added and the tissues were allowed to incubate overnight at 4°C. Tissues were then run through a series of incubations with mouse Clono PAP® at a dilution of 1:200 (Sternberger Monoclonals, Lutherville, MD) before being washed one time.

Slides were then incubated with diaminobenzidine tetrahydrochloride (DAB) with hydrogen peroxide, added at a dilution of 0.5 ml of stock DAB plus 2.7 μl of 30% hydrogen peroxide. Finally, slides were stained with Gill's hematoxylin a background stain (to facilitate the visualization process) and cells were dehydrated and covered with Permount and mounted for histology using the protocol of Sternberger Mononclonals Inc. This endpoint was graded as normal or increased using a plus/minus scale.

Behavioral Assessment

Mice were examined for clinical deficits indicative of neurotoxicity following MeHg exposure. Neurobehavioral assessment was performed every 2 days prior to MeHg dosing (6 doses total), and on the seventeenth or eighteenth day the mice were sacrificed. Assessments included the following days in chronic *T. gondii* infection with multiple doses of MeHg: 0, 7,14, 21, 28, 35, and 49. Examinations were done during the dark cycle of the standard 12-hour light/ 12-hour dark cycle with a red light lamp for visualization. Behavioral analysis was used to score the severity of the clinical manifestations of toxicant-induced neurotoxicity in living mice. Behavioral response tests were obtained and modified using an established protocol from the Laboratory for Neurotoxicity Studies (22).

Observed responses included: cage viability (alive or dead); cage activity (normal or abnormal); click response and menace response (reaction or lack of); tail condition (normal or abnormal – damaged, limp, or erect); posture (normal or abnormal – hunched, stiff); respiration (normal or abnormal – labored or slow),

and a wooden rod test (ability or inability to hang on to wooden rod for 30 seconds).

All tests were administered with the examiner being unaware of the mouse treatment group (19). The observational assessments and manipulative tests were reported as descriptive data or quantitative data, where possible. All experimental procedures and methods were reviewed and approved by the Virginia Tech Animal Care and Use Committee, in accordance with Virginia Polytechnic Institute and State University guidelines.

Results

Body and lymphoid organ weights

No animals died from chronic toxoplasmosis. Body weights of the mice remained unchanged throughout dosing among all experimental groups when compared to control, with no difference among all groups. Thymus weights were not significantly affected by the exposure (Table 1). A trend toward effect was observed in the MeHg only group, which had a numerical decrease in organ weight ($P = 0.08$). Spleen organ weights showed a marked increase in groups exposed to MeHg. Splenic cell viability showed a significant decrease in mice infected with *T. gondii* with and without MeHg exposure ($P < 0.05$). Viability of cells in the thymus was unchanged (Figure 1).

TABLE 1

Body, Spleen, and Thymus Weights of CBA/J Mice Infected with *T. gondii* and/or *T. gondii* and MeHg*

Treatment	Body weight (g)	Thymus weight (mg)	Spleen weight (mg)
Control	23.4 ± 0.5	43.1 ± 4.4	73.2 ± 3.4
MeHg	23.8 ± 0.5	25.4 ± 5.2	113.9 ± 13.0[a,b]
T. gondii	24.3 ± 0.8	36.0 ± 5.1	78.5 ± 4.8
T. gondii/MeHg	25.1 ± 0.6	31.5 ± 5.6	118.5 ± 8.2[a,b]

[*] Female CBA/J mice exposed every 2-3 days to 8 mg/kg MeHg over 18 days (n=10)

All data are expressed as weight mean ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

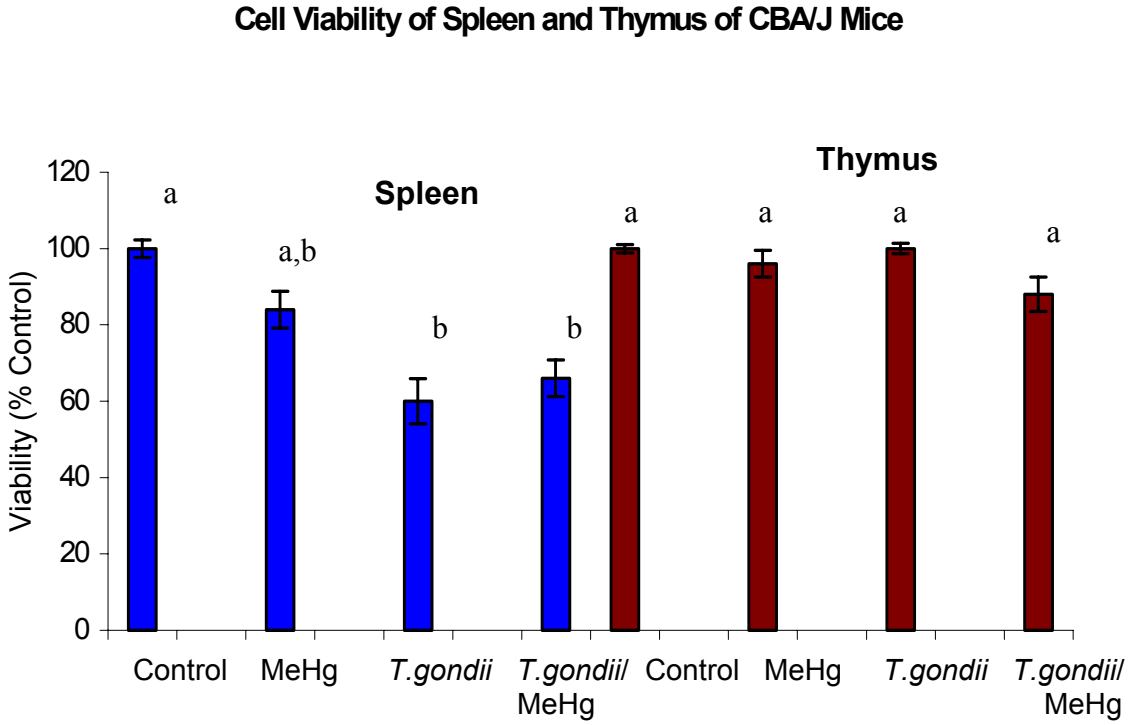

Figure 1 Mice dosed with 8 mg/kg body weight MeHg every 2-3 days for 18 days, after a 6-week *T. gondii* chronic infection. Sacrificing was day 17 or 18 post MeHg exposure. Viability of cells, are expressed as % of total cells present within the spleen and thymus as was determined by flow cytometric analysis. In the spleen, *T.gondii* decreased viability to 60% of control. Each experimental group contained an n=10. Comparisons among groups were performed using a Tukey's non – parametric test. For each organ no shared letters above bars implies groups are significantly different ($a < 0.05$).

Lymphocyte Subpopulations in the Thymus and

The thymus from mice exposure to MeHg, with or without a *T. gondii* infection showed a significant decrease in total numbers of $CD4^+CD8^-$, $CD4^-$ $CD8^+$,and $CD4^+CD8^+$ T-cells ($P < 0.05$) when compared to control and *T. gondii* infected groups (Table 2). The CD4-CD8- cell population of the thymus was numerically decreased, although not significantly. Each of the subpopulations of splenocytes from mice co-exposed to *T. gondii* and MeHg was decreased, although these reductions were not statistically significant (Table 2). When expressed as percent of total cells (Table 3), thymocyte subpopulations of mice exposed to MeHg with a concurrent *T. gondii* infection showed a significant increase in the $CD8^+$ T-cell subpopulation when compared to control or *T. gondii* alone ($P < 0.05$).

T. gondii infected mice had a marked increase in the percentage of non-T cell subpopulation and a decrease in the $CD4^+CD8^-$ T-cells of the spleen when compared to both control and MeHg exposed mice (Table 3). Splenic T-cell absolute numbers (i.e., the product of percentage of cells in a given population and total spleen cellularity) showed no significant changes among any of the experimental groups (Table 2).

TABLE 2

Absolute Numbers of T-cell Subpopulations in Thymus and Spleen from Mice Infected with *T. gondii* and/or *T. gondii* and MeHg*

Treatment	Total Cellularity	Thymic T-cell subpopulation			
		$CD4^+CD8^-$	$CD4^-CD8^+$	$CD4^-CD8^-$	$CD4^+CD8^+$
Control	86.4 ± 8.4	21.8 ± 7.0	4.1 ± 0.5	2.9 ± 0.7	57.7 ± 4.7
MeHg	25.6 ± 6.6[a,b]	5.1 ± 2.5[a]	1.3 ± 0.3[a,b]	0.8 ± 0.3	18.4 ± 3.8[a,b]
T. gondii	82.5 ± 4.9	11.5 ± 2.3	4.6 ± 0.8	3.5 ± 1.8	64.7 ± 5.9
T. gondii/MeHg	48.3 ± 10.8[a,b]	6.7 ± 2.8	2.7 ± 0.6[a,b]	2.0 ± 0.7	37.7 ± 9.2[b]

Treatment	Total Cellularity	Splenic T-cell subpopulations		
		$CD4^+CD8^-$	$CD4^-CD8^+$	non-T-cells
Control	52.0 ± 6.5	22.7 ± 2.7	14.7 ± 1.6	14.7 ± 2.8
MeHg	76.9 ± 41.7	28.8 ± 13.5	18.7 ± 9.1	28.7 ± 21.1
T. gondii	69.0 ± 6.3	21.3 ± 2.2	16.2 ± 2.0	30.4 ± 4.0
T. gondii/MeHg	31.6 ± 6.0[b]	12.3 ± 2.5	8.4 ± 1.8	10.6 ± 2.8

* Female CBA/J mice exposed every 2-3 days to 8 mg/kg MeHg over 18 days (n=10)

All data are expressed as mean x 10^6 cells ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

TABLE 3

T-cell Subpopulations as a Percent of Total Cells in the Thymus and Spleen of Mice Infected with *T. gondii* and/or *T. gondii* and MeHg*

Treatment	**Percentages of Thymic T-cell subpopulations**			
	$CD4^+CD8^-$	**$CD4^-CD8^+$**	**$CD4^-CD8^-$**	**$CD4^+CD8^+$**
Control	19.9 ± 4.9	5.3 ± 0.7	2.9 ± 0.5	72.0 ± 4.8
MeHg	19.1 ± 3.6	6.3 ± 1.7	4.4 ± 1.5	69.5 ± 4.8
T. gondii	15.1 ± 3.6	5.4 ± 0.7	2.2 ± 0.3	77.3 ± 3.4
T. gondii/MeHg	12.9 ± 2.5	10.3 ± 2.0[a,b]	3.8 ± 0.9	73.0 ± 3.9

Treatment	**Percentages of Splenic T-cell subpopulations**		
	$CD4^+CD8^-$	**$CD4^-CD8^+$**	**Non-T cells**
Control	44.5 ± 1.9	28.8 ± 1.0	26.2 ± 2.8
MeHg	42.1 ± 2.1	28.3 ± 2.0	28.3 ± 3.7
T. gondii	31.2 ± 2.3[a,c]	23.5 ± 2.3	44.0 ± 4.5[a,c]
T. gondii/MeHg	35.2 ± 2.8[a]	24.4 ± 1.7	40.0 ± 4.5

* Female CBA/J mice exposed every 2-3 days to 8 mg/kg MeHg over 18 days (n=10)

All data are expressed as percent mean ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

[c] Statistically significant difference ($P < 0.05$) when compared to MeHg-treated mice

Apoptosis in Thymocytes and Splenocytes

Flow cytometric analysis with a triple color stain allowed determination of apoptosis in individual T-cell subpopulations of the thymus and spleen (Table 4 and 5). The only significant change in thymocytes was noted as an increase in early apoptotic $CD4^+CD8^-$ T-cells in mice exposed to *T. gondii*/MeHg ($P < 0.05$). The $CD4^-CD8^+$ and $CD4^+CD8^+$ T-cell subpopulations did not undergo any significant changes (Table 4).

T. gondii infected mice had more significant changes in apoptosis of spleen cells, with decreases in percentages of live $CD4^+CD8^-$ and non-T cells and increases in late apoptotic/necrotic $CD4^+CD8^-$ and $CD4^-CD8^+$ T-cell subpopulations (Table 5). Not all of these changes were seen in mice exposed to both *T. gondii*/MeHg. Apoptosis staining (TUNEL) of the spleen and thymus revealed no differences among treated versus non-treated groups of mice (data not shown).

TABLE 4

Apoptosis of Thymocytes in mice infected *T. gondii* and/or *T. gondii* and MeHg*

Treatment	**Percentages of $CD4^+CD8^+$ T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	3.7 ± 1.2	80.3 ± 8.9	16.1 ± 8.1
MeHg	1.5 ± 0.4	82.3 ± 4.6	13.9 ± 3.5
T. gondii	3.6 ± 1.7	88.5 ± 1.5	7.9 ± 1.1
T. gondii/MeHg	0.9 ± 0.3	76.3 ± 5.7	17.6 ± 3.1
	Percentages of $CD4^+CD8^-$ T-cells		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	70.4 ± 6.1	24.2 ± 3.9	4.5 ± 3.7
MeHg	67.1 ± 3.7	28.1 ± 3.7	2.8 ± 0.8
T. gondii	70.3 ± 5.5	26.3 ± 4.9	1.6 ± 0.7
T. gondii/MeHg	53.4 ± 5.2	42.3 ± 5.5[a]	3.3 ± 0.8
Treatment	**Percentages of $CD4^-CD8^+$ T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	17.1 ± 2.2	71.9 ± 7.7	10.8 ± 8.0
MeHg	11.2 ± 1.4	80.1 ± 1.2	8.7 ± 1.8
T. gondii	16.1 ± 3.3	79.6 ± 2.4	4.3 ± 1.6
T. gondii/MeHg	18.3 ± 10.3	72.9 ± 9.3	8.8 ± 2.0

[*] Female CBA/J mice exposed every 2-3 days to 8 mg/kg MeHg over 18 days (n=10)

All data are expressed as percent mean ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

[c] Statistically significant difference ($P < 0.05$) when compared to MeHg-treated mice

TABLE 5

Apoptosis of Splenocytes in mice infected *T. gondii* and/or *T. gondii* and MeHg*

Treatment	**Percentages of $CD4^+CD8^-$ T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	81.9 ± 6.1	16.5 ± 5.9	1.6 ± 0.4
MeHg	89.4 ± 2.9[b]	9.3 ± 2.7	1.3 ± 0.4
T. gondii	42.7 ± 10.5[a]	32.7 ± 6.3[c]	24.5 ± 8.2[a,c]
T. gondii/MeHg	73.5 ± 4.6[b]	19.8 ± 2.9	6.8 ± 1.9[b]
Treatment	**Percentages of $CD4^-CD8^+$ T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	1.7 ± 0.2	95.2 ± 0.7	3.1 ± 0.8
MeHg	6.3 ± 1.1[a,b]	89.2 ± 1.3	4.5 ± 0.9
T. gondii	1.2 ± 0.1	71.2 ± 9.6[a]	27.6 ± 9.7[a,c]
T. gondii/MeHg	3.6 ± 0.9	84.1 ± 2.7	12.3 ± 3.1
Treatment	**Percentages of Non - T-cells**		
	Live	**Early Apoptotic**	**Late Apoptotic/Necrotic**
Control	75.5 ± 5.4	18.4 ± 4.5	6.9 ± 1.2
MeHg	86.6 ± 2.6[b]	10.5 ± 1.5	3.4 ± 1.3
T. gondii	51.2 ± 6.9[a]	18.1 ± 3.3	31.6 ± 7.9
T. gondii/MeHg	72.6 ± 2.7[b]	20.4 ± 1.9	9.8 ± 2.2[b]

[*] Female CBA/J mice exposed every 2-3 days to 8 mg/kg MeHg over 18 days (n=10)

All data are expressed as percent mean ± standard error

[a] Statistically significant difference ($P < 0.05$) when compared to control

[b] Statistically significant difference ($P < 0.05$) when compared to *T. gondii*-infected mice

[c] Statistically significant difference ($P < 0.05$) when compared to MeHg-treated mice

Behavioral Assessment

Analysis for testing of repeated behavioral responses of mice exhibiting a certain response on each day was performed. Two-day pre-treatment baseline values were used to compare behavioral responses to each day post MeHg exposure based upon effects that deviated from normal responses. Multiple day analysis showed that 4 out of 27 responses showed a change when comparing MeHg only exposed mice to *T. gondii* /MeHg co-exposed mice (Table 6). Out of 10 mice in each group, the number of mice in the MeHg only group that presented symptoms for each response and on what day. Responses that were different between MeHg and *T. gondii* /MeHg exposed mice were only noted on day 10. Mice given MeHg were unable to balance and cling to a wooden rod, did not respond to a clicking noise, and 4 of these mice were vocal whether being handled or not. Mice co-exposed to *T. gondii* /MeHg showed signs of vocalization while being handled only (3 out of 10 mice). Mice infected with *T. gondii* only or vehicle control showed no neurobehavioral abnormalities prior to sacrifice on day 17 or 18.

TABLE 6

Number of Mice Exposed to MeHg Showing Response that Differed from Baseline

Experimental Day MeHg only	0	2	4	7	10	13
Home cage activity	0	0	0	0	0	0
Click response	0	0	0	0	7	0
Menace response	0	0	0	0	1	0
Wooden rod	0	0	0	0	7	0
Posture	0	0	0	0	0	0
Tail condition	0	0	0	0	0	0
Respiration	0	0	0	0	0	0
Vocalization	0	0	0	0	4	0

Tissue cysts, lesion scoring, and MeHg quantification

Tissue cyst counts indicated no differences between mice with *T. gondii*/MeHg versus those that had only a *T. gondii* infection (Table 7). Mean lesion scoring was similar across all treatment groups including vehicle control. Co-exposed mice had significantly lower brain levels of MeHg when compared to the MeHg only exposed mice (Table 7), but all mice exposed to MeHg had quantifiable amounts in the tissues of the brain and kidneys. Levels of MeHg in mice not experimentally treated with MeHg were non-detectable.

TABLE 7

Mean tissue cyst counts and lesion scores for mice given *T. gondii* and/or *T. gondii* and MeHg*

Treatment	**Mean tissue cyst**	**Mean lesion score**
Control	NA	1.0 ± 0
T. gondii	3.5 ± 1.1	1.0 ± 0
MeHg	NA	1.0 ± 0
T. gondii/MeHg	5.6 ± 1.0	1.0 ± 0

* Female CBA/J mice exposed every 2-3 days to 8 mg/kg MeHg over 18 days after a chronic *T. gondii* infection was established (n=10)

All data are given in mean ± standard error of number of tissue cysts or lesions

NA = Not applicable.

TABLE 8

Mercury burden in the Brain and Kidney of CBA/J Mice treated with MeHg only or infected with *T. gondii* and MeHg*

Treatment	**brain mercury burden**	**kidney mercury burden**
MeHg	0.68 ± 0.17	19.2 ± 3.9
MeHg/*T. gondii*	0.31 ± 0.03[a]	22.1 ± 5.2

* Female CBA/J mice exposed every 2-3 days to 8 mg/kg MeHg over 18 days (n=10)

All data are given in mean ± standard error of parts per million

[a] Difference of (*P*=0.08) when compared to MeHg only

Histopathology

Review of hematoxylin – eosin (H&E) stained slides from the brains of mice given multiple 8 mg/kg doses of MeHg over 17 days did not reveal significant lesions in the brain in animals given MeHg, *T. gondii*, or the combination of these two agents or in vehicle controls. In particular, no cysts or inflammatory lesions or cysts typical of Toxoplasmosis as were seen following a single 20 mg/kg dose of MeHg (Chapter 5) were found. In addition, the cerebellar cortical lesions of granule cell necrosis/apoptosis (1) were absent in the MeHg dosed mice regardless of the presence of a *T. gondii* infection. Hence, there were no consistent astrocytic alterations noted in these sections using GFAP immunohistochemical staining nor were there any detectable apoptotic changes using the TUNEL assay on nervous or immune tissue.

Discussion

The present study examines the effects of multiple exposures to MeHg on a chronic *T. gondii* infection for comparison to the effects of study previously done in our laboratory using a single higher dose of MeHg (Chapter 5). In this study we tested whether multiple lower dose exposures of an environmental contaminant, MeHg, could induce a relapse of a chronic infection, *Toxoplasma gondii*. Multiple low dose effects in mice were less notable than when a single high dose of MeHg was given. Multiple, lower dose exposure to MeHg did not increase brain cysts of *T. gondii* nor alter morphological effects of the infection. Since both these agents cause detrimental effects on similar systems, nervous

and immune, (48), (77), it was expected that the co – existence of a chronic infection and an environmental contaminant such as those examined here would worsen the initial disease state. However, the results of this study demonstrate that CBA/J mice displayed an immunotoxic response to MeHg exposure but not a relapse of chronic toxoplasmosis.

Immune effects on multiple exposure of MeHg was demonstrated by a marked increase in the splenic weights of mice exposed to MeHg with or without a *T. gondii* infection, while thymus weights were decreased for these same mice. A single MeHg exposure also caused a significant increase in spleen weight (Chapter 5). It was noteworthy that thymus weight was only different in those mice receiving multiple low doses of MeHg without a concurrent *T. gondii* infection. All other experimental groups in both single and multiple dose studies did not show any differences in organ weights. Alterations of organ weights are often primary indicators of potential immunotoxicity (6). Primary lymphoid organs such as the thymus are major targets for toxicity and a number of agents have been shown to induce thymic atrophy (cyclosporine, dioxin). Depletion of lymphoid organs often results in immunosuppression, while expansion and proliferation of various cells are typical responses to immunostimulation (6). The cellularity decrease seen in the thymus of mice exposed to MeHg with or without a *T. gondii* infection is indicative of the depletion seen in lymphoid organs after immunosuppression. Mice co-exposed to *T. gondii* and MeHg had a decreased splenic cellularity when compared to mice infected with *T. gondii* only in the present study. This result was different from the study with a single high dose of

MeHg (Chapter 5). Reasons for the differences seen with the alternate MeHg regimen are unknown.

The interaction of *T. gondii* and MeHg could be associated with effects on the immune system. MeHg in combination with *T. gondii* caused a significant increase in the percentage of mature $CD8^+$ T-cells in the thymus while showing no variation in the number found in the spleen. These single positive cells are the mature T-cells that then migrate to the periphery. These data indicate that there may be a lack of migration of $CD4^-CD8^+$ T-cells and/or the possible increased production of these cells at this subchronic dose of MeHg. These T-cells ($CD4^-CD8^+$) are responsible for killing cells infected by viruses, tumors, or toxicants. Other studies have found that low dosages of MeHg (10 ppm) did not affect memory of lymphocytes and that T rather than B cells are generally affected when a secondary immune response is altered after exposure to subclinical amounts of environmental contaminants (121).

Examination of mice exposed to low, multiple doses of MeHg over an 18 day period showed that mouse body weights remained unchanged throughout the dosing scheme. In experimental MeHg poisoning in rats or mice, the first symptom has been noted as the loss of body weight (115). Results we obtained in this study were not in agreement with those results, although our previous study with a single, higher MeHg dose was (Chapter 5). The responses that indicated alterations in nervous system behavior were negligible in mice co-exposed to *T. gondii*/ MeHg and noted only on day 10 after repeated exposure to MeHg alone. Other studies confirm this same pattern of neurological symptoms

(115). Higher doses would be expected to produce a variety of symptoms in a short time period and then the animal would die, while with exposure to lower doses, such as the present study, the symptoms of MeHg poisoning are delayed (34). It is possible that behavioral abnormalities could appear at time points beyond the 17 or 18 days we examined, but sacrifice time was determined by the need to gather endpoints before the mice succumbed to the chronic *T. gondii* infection. This usually occurs within between 8-9 weeks post infection (96).

Histopathological examination of brain tissue did not show any lesions, tissue cyst enumeration, or glial scarring that would have been indicative of MeHg poisoning. Again, more time may be needed before these events occur (115). Brains from mice exposed to both MeHg with *T. gondii* contained less mercury than those given only MeHg. The amount in the kidneys was higher than that found in the brain, indicating the animals' ability to continue to excrete mercury. The quantity of mercury in the brain in this study (0.68 ppm) was, however, significantly higher than the 0.35 ppm seen 7 days following a 20 mg/kg MeHg dose (Chapter 5). Also notable was the difference in MeHg burden in the brains of mice co-exposed to *T. gondii* and MeHg – 0.31 ppm in the present study, and 0.04 ppm with a single MeHg exposure (Chapter 5).

A plausible relationship between chronic parasitic infections and environmental contaminant exposure has not been previously explored due to the lack of relevant epidemiology studies (122) (34). This study is novel in that it evaluates *in vivo* responses of both the immune and nervous systems following multiple exposures to a relatively low dose of an environmental contaminant that

can cause neurological impairment that has the potential to be made worse over time with concurrent parasitic infections (123). The results of this study demonstrate that in CBA/J mice undergo immunotoxicity due to MeHg exposure but not a relapse of chronic toxoplasmosis.

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PART V. DISCUSSION

Chapter 7. General Discussion and Conclusions

The present study is the first time that the effects of both acute and subchronic levels of MeHg were determined along with a chronic parasitic infection in an *in vivo* model. Our hypothesis was that suppression of the immune system to MeHg with a single high dose could cause a relapse of a chronic *T. gondii* infection. The results proved that a single high dose of MeHg caused immunotoxic effects that lead to the reactivation of *T. gondii* tissue cysts in the brain of co-exposed mice. We then further tested whether exposure to multiple low doses of MeHg would cause similar effects on the immune and more notable effects on the nervous system of mice. Results showed that a chronic *T. gondii* infection was not exacerbated by multiple low doses of MeHg over a period of 18 days. A lack of neurological symptoms was noted although immune dysfunction occurred. The length of exposure of the low doses of MeHg may still not have been enough for neurological effects. Doses may not have been high enough in the multiple dose study to affect susceptibility to *T. gondii* infection. Several environmental pollutants may affect the susceptibility to disease as well as a decreased resistance to pathogens, thereby exacerbating the disease process and furthering the development of complications (106) (124) while most do so over periods of time greater than 30 days. Previous studies demonstrated that as a ubiquitous contaminant, acute exposure to MeHg leads to critical immune dysfunction (34), which supports the results found in our single dose (20 mg/kg) MeHg exposure.

Data from the study performed with a single dose of 20 mg/kg MeHg as part of this dissertation support our hypothesis of immune system effects. The increase seen in the percentage of thymic $CD4^{-}CD8^{+}$ T-cells possibly blocked the export of this T-cell subset to the periphery, thereby, leading to the significant decrease in the number of splenic $CD8^{+}$ cells. We further showed that most of these T-cells in the spleen were undergoing apoptosis, as were $CD4^{-}CD8^{+}$ T-cells in control mice. Increases in percentage of thymic $CD4^{+}CD8^{-}$ cells could indicate a compensatory mechanism in response to immunotoxicity (48). $CD4^{-}$ $CD8^{+}$ T-cell – mediated protection of *T. gondii* infected cells could possibly occur through a MHC I class pathway. *T. gondii* infects most if not all nucleated cells (3), and these cells have the ability to present MHC I antigens to cytotoxic T-cells which in turn undergo programmed cell death so that they do not disseminate the infection further within the host. A substantial inflammatory response in the brain of *T. gondii* alone infected animals was lacking, an effect confirmed by a decrease in thymic $CD4^{+}CD8^{-}$ and an increase in splenic $CD4^{-}$ $CD8^{+}$T-cells of these infected mice (7).

A chronic *T. gondii* infection that causes a depletion of absolute $CD4^{+}$ T-cell numbers in the thymus or the activation of such cells results in the lack of differentiation into two distinct subsets of T-helper cells (Th1 and Th2). T-helper 1 (Th1) and T-helper 2 (Th2) are identified based on the profile of expression of cytokines, which are inflammatory mediators. The inflammatory mediators are primarily secreted by Th-1 cells are interferon gamma (INF-γ), IL-2, and IL-12 while Th2 type cells primarily secrete IL-4, IL-3, 5, 6, and 10. It is now well

established that Th1 type immune response provides effective immunity against intracellular infections, cancer, and allergies. The suggestion that lack of sufficient anti-inflammatory response contributed to the increase in *T. gondii* tissue cysts in the brains of mice co-exposed to MeHg may be supported by the lack of differentiation to T helper cells. The Th2 type immune responses provide help to B cells for promotion of an antibody response against extracellular organisms and the induction of allergies (58) (48) (58). Studies carried out in continuation of this work should assess the cytokine secretion from both T and B cell types. These mediators of T-cell function would provide a possible indicator of a mechanism for protection during a chronic *T. gondii* infection in future studies (125).

Upon initial encounter with the immune system, *T. gondii* rapidly induces production of the Th-1 promoting cytokine IL-12 that most likely emerges from a subpopulation of dendritic cells (57) (58). Natural killer (NK) and T cells are then activated and triggered to synthesize IFN-γ, the major mediator of host resistance during the acute and chronic phases of a *T. gondii* infection. Cytokine (IFN-γ und TNF-α) rather than cytotoxicity-based effector functions are more critical for protective immunity both during the acute and chronic phases of *T. gondii* infection (57). These T-cell memory responses help to prevent lethal immunopathology (57). This mechanism by which a memory response induces protection of the immune system was enhanced with multiple low doses of MeHg. Mice were more responsive to an immune insult after multiple low doses of MeHg since their T-cells had been previously stimulated from each previous

dose. This could result in mercury immunomodulation of co-exposed mice by promoting a Th2-type response at low doses over long periods of time (34).

Nervous system alterations were apparent with a significant increase of tissue cysts in the brains of mice exposed to *T. gondii* and a single dose of MeHg. This was greater than the number of tissue cysts in mice with chronic *T. gondii* infection alone and greater than the number of tissue cysts present in mice with multiple low doses of MeHg with a chronic *T. gondii* infection. These data support the hypothesis that MeHg exacerbates a *T. gondii* infection. The overall decrease in body weight after MeHg exposure is also a hallmark of immunotoxicity (115).

The present study confirms the hypothesis that MeHg causes immunotoxicity and can cause a relapse of a chronic *T. gondii* infection. A single dose of MeHg had immunotoxic effects that were observed over a seven day period. Even though histopathological lesions, including evidence of apoptosis that were expected from MeHg poisoning were not found to be present in brain, tissue cyst numbers did increase. Furthermore, the differences seen in immune cells along with increased apoptosis of some lymphocytes cell types in *T. gondii*/MeHg exposed mice demonstrate the possibility of further immunodepression in an already immune compromised host with a latent infection. This characteristic has the potential for have a devastating impact on the health status of the host. This study presents novel data on the early effects of a single acute dose of MeHg on a chronic *T. gondii* infection.

MeHg is well known for inducing lesions in the nervous system, but we saw none 7 days after a single dose of 20 mg/kg neither MeHg nor 17-18 days after multiple 8 mg/kg doses of MeHg were given. Timing of MeHg, as well as doses used may have contributed to our observation. Another study, which administered a single dose of 10 mg/kg MeHg in rats found that after 7 days the granule cell layer of the brain appeared to have returned to normal, while on day 3 after dosing, MeHg did produce microscopic changes in cerebellar neurons (120). This further supports our conclusion that the time of observation following MeHg dosing is an aspect of studies with *T. gondii*/MeHg that could potentially be modified for future studies. A combination of an increase in dosage and/or a prolonged exposure period may be more appropriate since behavioral and neuronal symptoms are often found under these conditions (18) (114).

The present study confirmed that single as well as multiple doses of MeHg were detrimental to the immune system. Results with multiple doses were similar to those seen with single dose MeHg exposure. Under either exposure paradigm, however, nervous system effects were minor, probably relating to the timing of MeHg dosing, as noted above. The lack of inflammation, lesions, and increased tissue cysts of mice chronically infected with *T. gondii* and exposed to MeHg may be due in part to the memory response of the T-cell populations. A previously compromised system that has not undergone complete depletion of its immune system components will respond to an insult or an exposure to a potentially immune damaging agent more swiftly than that of a first time exposure (6) (47). Multiple doses of MeHg over time have been proven to have deleterious

effects if given sufficient time to do so (1) (126). Previous studies have neglected to assess the influence of different environmental parameters on the toxic action of metals on chronic parasitic infection (127). Many effects caused by pollutants may influence the subsequent prevalence and intensity of parasites in their target hosts.

U. S. Government regulations heavily influence the exposure limits of several pollutants, thereby, attempting to lessen their prevalence within the areas where individuals are most at risk for contact. Policies, laws, and guidelines have been implemented as to protect the general public from the harmful effects of mercury, its derivatives, and various other pollutants (128) (129) (10, 130). Although the bases for many of these guidelines are from examples of highly toxic acute accidental exposures, these exposure settings may not be realistic for the common everyday contact that one may encounter. For instance, restrictive fish consumption advisories have been called into question since they do not take into consideration the social and economic ramifications of restricting fish and seafood (131). Even though MeHg exposure in the United States occurs primarily through fish and seafood consumption, the Environmental Protection Agency's (EPA) reference dose (RfD) was generated by analysis of acute poisonings in Iraq (1971-1972). Data from this exposure may not be appropriate for calculating the low level chronic exposures through daily fish consumption. Moreover, it has been proposed that dietary MeHg is not a significant source of mercury exposure and does not likely act as a confounder in association with dental amalgam exposure (132). Accidental and industrial MeHg exposures are

most likely to have serious potential health effects on humans and animals (68). These facts make our study extremely relevant due to the nature of the health problems within areas that are endemic with high environmental mercury levels (sub-Sahara Africa and Amazon River Basin) as well as being geographically overlapping with *T. gondii* prevalence. Differences exist in tissue distribution between humans and animals, but whole-body elimination rates are similar. Both humans and animals appear to have similar sensitivity of the central nervous system (CNS) therefore, extrapolation of data may have usefulness for protection of human health (35).

A plausible relationship between chronic parasitic infections and environmental contaminant exposure has not been previously explored due to the lack of relevant epidemiology studies (34). One likely reason for the insufficient epidemiological evidence is that early developmental exposure may produce undetected, latent damage (133) that surfaces only when functional capacities are challenged by other conditions, such as aging (116), drugs or chemicals (134). The first health complaints associated with most metals are usually nonspecific. The complex social, political, and legal issues indicate the need for objective tests for health effects. Most important is the identification and measurement of the critical effect, i.e., an effect that alerts the public health authorities that further exposure should cease. Unfortunately, there are no early tests for exposure to mercury as there are for lead and cadmium (122). Given the above data, the serious nature of immune impairment on chronically infected individuals who experience or who are at risk of experiencing a secondary co –

exposure to heavy metal toxicity, should be further studied. The results of our study corroborate two studies that implicate mercury or its derivatives as a serious health risk for immunosuppression, and potentially worsened neurological impairment with concurrent parasitic infections (34), (123). Further studies from our laboratory also implicate immune system effects of low dose multiple exposures to MeHg with a concurrent *T. gondii* infection over a 18 day time period.

Future studies should include variable time frames with likely exposure limits that are commonly encountered in populations endemic with mercury pollution and increased susceptibility to *T. gondii* infection. Endpoints to be analyzed should include those that observe the differences in genetic background of mice infected with the parasite, which could result in a resistant or susceptible phenotype (44) (61). Reviewing the development of a T-helper type 1 or 2 response and the cytokines secreted as the result of an immune response are of particular importance for both a parasitic infection (51) and exposure to an environmental contaminant (79) . Mouse strains that possess MHC class II molecules that are exposed to low subtoxic doses of heavy metals such as MeHg are known to induce immune dysfunction. It is believed that MeHg treated mice exhibit activation of the Th2 cell type, which in turn produce IL-4, this cytokine has been linked to the resistance of Leishmania (common model for study of immune deviation and T-helper response characteristics) (34). Mercury was found to be ineffective as an anti-infectious agent and toxic to those exposed to it, concurrently, it may also be responsible for putting forth detrimental effects on

immunosuppressed hosts, possibly with a chronic parasitic infection by promoting unprotective Th2 responses. The results of our study in corroboration with additional investigations (34) (123) at this time validate that destruction of a host resistance to a chronic *T. gondii* infection indicates that concurrent MeHg exposure is a possible immunomodulator of parasitic infection.

Conclusions

- MeHg caused detrimental effects on the immune system of CBA/J mice when given as a single oral dose of 20 mg/kg and when given 6 times over a 2-week period at 8 mg/kg orally.
- Single high dose exposure to MeHg caused a relapse of *T. gondii* infection as indicated by increased numbers of tissue cysts and decreased inflammation in the brain of mice co-exposed to both *T. gondii* and MeHg. This effect did not occur following multiple low doses of MeHg.
- Neurotoxic effects attenuating behavior of mice exposed to MeHg at the dosages used for these experiments were not notable nor markedly increased in the presence of a chronic *T. gondii* infection with either a 20 mg/kg or 8 mg/kg dose of MeHg.
- Apoptosis was not demonstrated to be a common mechanism for neurotoxic or immunotoxic effects of *T. gondii* and MeHg co-exposure.

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Part VI. REFERENCES

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PART VII. APPENDIX

Dose Response Range for Multiple Dose Study

Purpose and Methods of Experiment:

A dose response range was examined to determine doses of MeHg which could be administered over a defined period to observe both behavioral alterations and toxicity in CBA/J mice. Previous studies showed that a single acute dose of 20 mg/kg body weight of MeHg resulted in visible behavioral responses as well as observed morbidity. These data were created to find an appropriate dosage for our multiple low dose study. Six different doses of MeHg were examined based upon literature data (1). In the published study, histopathological effects using both H&E and GFAP stained nervous tissue were observed. However, the study was carried out in rats and was therefore modified for the present multiple low dose study performed.

A range of six doses were examined, including a vehicle control (PBS with DMSO) and dilutions of MeHg from a 20 mg/kg stock solution (see Methods and Materials of Dissertation, Toxicant Dosing – MeHg dosing). Mice were dosed by oral gavage every other day. Doses included: 0, 4, 8, 12, 16, and 20 mg/kg body weight per mouse. There were two mice in each experimental group. All groups were observed every other day from the day of MeHg exposure. Behavioral assessments and body weights were taken as well.

Results and Discussion of Experiment:

The results from these experiments demonstrated that 8 mg/kg body weight dose of MeHg provided behavioral alterations without morbidity in a period of less than 14 days. The 14 day time period was of importance because

periods of greater lengths could result in death of those mice concurrently infected with *T. gondii* (125). This time frame as well as this dose was thought to be acceptable when reviewing data obtained from literature based studies as well as from our preliminary dose range response experiment. The data below denote observed changes and mortality of mice for 11 days. Mice that survived MeHg exposure were euthanized. Each mouse weight is given in grams and mice that were euthanized or that succumbed to exposure are denoted as E or S respectively. Mice with behavioral alterations are highlighted in bold.

TABLE 1 Dose Range Response for Body Weights (g) of CBA/J Mice

DAY	0	2	4	6	8	10	11
Dose (mg/kg)							
0	26 / 30	27 / 30	26 / 30	26 / 30	27 / 30	27 / 30	E / E
4	28 / 23	27 / 24	27 / 22	27 / 22	26 / 22	27 / 22	E / E
8	29 / 26	30 / **21**	29 /**23**	29 / S	**28** / -	**28** / -	E
12	24 / 22	**20** / **19**	**19** /**16**	**20** / E	**18** / -	**17** / -	E
16	24 / 25	**20** / S	**19** / -	S / -			
20	24	21	**20**	**19**	**20**	**20**	E

n= 2/group, weights given for both mice

Behavioral alterations for mice whose weights are noted in bold include: body weight, cage viability, cage activity, ataxia, posture, coat condition, tail condition, respiration, wooden rod test, menace test, vocalization, and click response

In Vitro Effects of Methylmercury on *T. gondii*

Purpose and Methods of Experiment:

The obligate intracellular protozoan parasite, *T. gondii*, infects numerous hosts. Infection occurs through oral or congenital exposure. The kidney is a major organ for methylmercury (MeHg) accumulation in the body. An *in vitro* microliter plate assay was performed to determine the direct effects of MeHg on developing *T. gondii* (tachyzoites) growing in kidney cells (135).

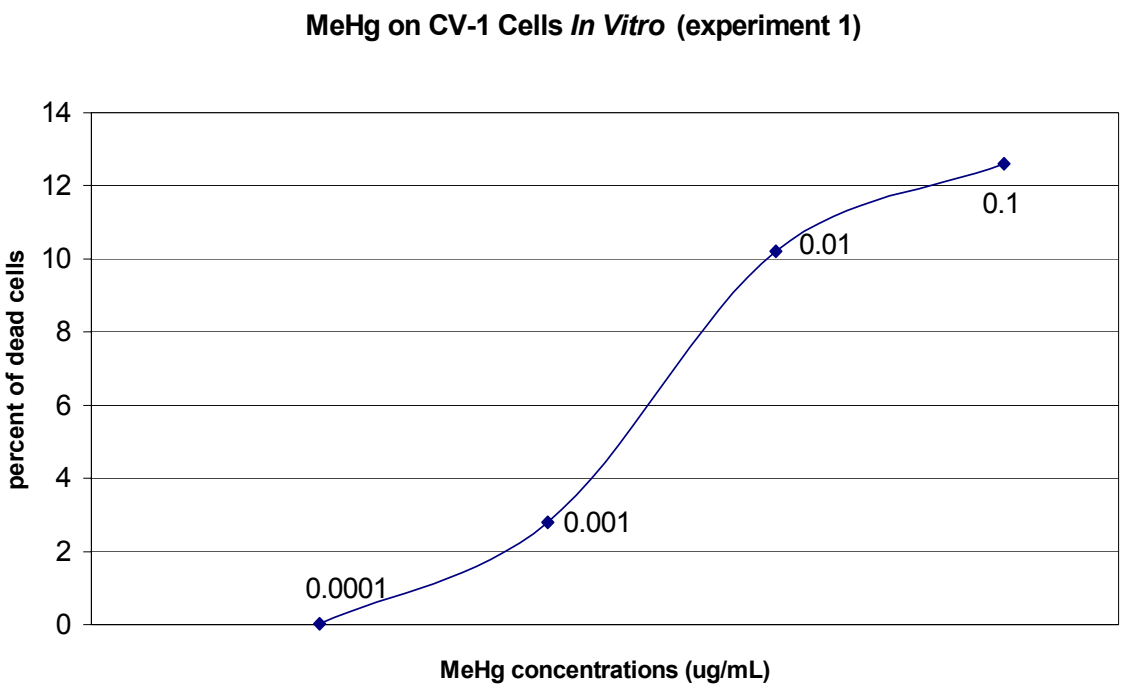
For these studies, flat bottom 96-well microliter plates were inoculated with CV-1 cells (African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Manassas, Virginia)) and the resulting monolayers were used to determine the effects of MeHg as measured by monolayer destruction (plaque formation). MeHg was examined at 0.0001, 0.001, 0.01, and 0.1 μg/ml. Monolayers were treated with test agents for 2 hours on designated columns of wells on each plate and then inoculated with 2 x 10^4 *T. gondii* tachyzoites. Experimental and control wells were included on the same plate. Untreated and uninfected monolayers served as controls for the parasite and uninfected but MeHg treated CV-1 cells served as toxicity controls. Each treatment was repeated in replicates of at least 36 wells. Plates were visually monitored daily and the assay was terminated when 90-100% of the tachyzoite infected cells not exposed to MeHg had lysed (2 to 4 days post infection). The plates were rinsed in PBS, fixed in 100% methanol for 5 minutes, and the cells stained with gram crystal violet solution for 5 minutes. Areas of tachyzoite induced destruction or CV-1 cell death due to toxicity did not take up

the crystal violet. An ELISA plate reader, (Molecular Devices, Sunnyvale, CA) at 600 nm was used to quantitate the amount of crystal violet present. The percent reduction for each treatment was calculated using the optical densities (OD) of the means of the control and treatment wells.

Results and Discussion of Experiment:

The results from initial experiments demonstrated that exposure of tachyzoites to 0.1 mg/ml of MeHg inhibited their subsequent development in cell culture, while concentrations of 0.01, 0.001, and 0.0001 μg/ml of MeHg did not cause as high a percentage of cell death. The effects of MeHg on cell death were concentration related in the experiment one. We found that 0.1 and 0.01 μg/ml of MeHg killed more host cells whereas treatments at lower levels, 0.001, and 0.0001μg/ml, killed less. Repeated experiments showed considerable variability for each concentration of MeHg tested. This made it particularly difficult to reproduce our initial results. We believe the required rinsing of MeHg from each plate caused an abnormal loss of tachyzoites. Percent of dead cells were calculated from the tachyzoites remaining, which in turn led to the variability seen in Figure 1.

Figure 1. Exposure of MeHg on CV-1 Cells in Culture



Methylmercury levels in Brain and Kidney of CBA/J Mice

Purpose and Methods of Experiment:

Methylmercury exposure to both humans and animals is quantifiable in both the brain and kidney. The use of cold vapor generation accessory method of atomic absorption accurately determines the concentration of methylmercury in tissues after exposure (Illinois Department of Agriculture Animal Disease Laboratory). The left kidney and the left half of the brain were removed and placed in 2 ml of HBSS and quantified by atomic absorption in the Toxicology Laboratory of the VMRCVM, using a standard procedure for mercury analysis. This assay determines the concentration of mercury on the Varian SpectraAA-220FS atomic absorption. Sample preparation included: weighing tissue in a beaker, adding 5 ml of concentrated nitric acid, then gently heating until approximately 1 ml of liquid remained. The beaker was then removed and allowed to cool, diluted to 10-20 ml and the volume was recorded. 100µL of antifoam emulsion were added to end the digestion process. Trained technical staff of the Toxicology Laboratory performed the atomic absorption (AA) spectrophotometry analysis. A standard of 30 ppb was used to calibrate the instrument. The wavelength of the instrument was 253.7 λ. Using 20, 30, 40, and 50 ppb Hg standards provided the standard curve for mercury determination. Results of total mercury in ppb were determined by calculating the concentration of mercury (ppb) multiplied by the digestion dilution. The instrument automatically calculated the correct result, with the exception of missing values

or additional dilutions performed during the analysis, in which case additional calculations were manually performed.

Results and Discussion of Experiment:

Mice with a six week *T. gondii* infection and a one time dose of 20 mg/kg body weight of MeHg were found to have greater accumulations of mercury in their kidneys than in their brains. The brains of mice with a *T. gondii* infection and exposure to MeHg contained to have only 0.04 ppm while brains from those treated only with MeHg had an increase of almost ten times as much (0.35 ppm). Mice with a six-week *T. gondii* infection and multiple low doses of 8 mg/kg of MeHg had greater accumulations of MeHg in their kidneys than mice given MeHg only once, with a mean of 22.14 ppm. Mice with multiple MeHg exposure that had no *T. gondii* infection had an average of 19.18 ppm. Mice dosed with *T. gondii* and multiple doses of 8 mg/kg body weight MeHg had an average brain deposition of 0.31 ppm; those mice exposed to MeHg alone had almost 3 times as much mercury accumulation with 0.68 ppm. Results clearly demonstrate that dose as well as time are primary factors of the MeHg burden found in either the brain or the kidney in mice (18).

Table 1

Mercury burden in the brain and kidney of CBA/J mice treated with MeHg only or exposed to *T. gondii* and MeHg

20 mg/kg MeHg in a single dose *		
Treatment	**brain mercury burden**	**kidney mercury burden**
MeHg	0.35 ± 0.30	8.25 ± 1.9
T. gondii / MeHg	0.04 ± 0.01[a]	5.39 ± 1.8

8 mg/kg MeHg in multiple doses **		
Treatment	**brain mercury burden**	**kidney mercury burden**
MeHg	0.68 ± 0.17	19.2 ± 3.9
T. gondii / MeHg	0.31 ± 0.03[b]	22.1 ± 5.2

All mice were female CBA/J, 6-weeks of age chronically infected with *T. gondii* six weeks prior to MeHg exposure.

* Data are expressed as ppm mean ± standard error (n = 5-9); sacrificed 7 days post 20 mg/kg dose of MeHg

** Data are expressed as ppm mean ± standard error (n = 10); sacrificed 18 days post multiple 8 mg/kg doses of MeHg

[a] Statistical difference of P=0.08 when compared to MeHg only exposure was present.

[b] Statistical difference of P=0.07 when compared to MeHg only exposure was present.

PART VIII. VITA

Vita

Marquea Damona King is the second oldest of four girls and was born August 5, 1975 in Philadelphia, Pennsylvania. After moving from Philadelphia, PA to Clayton, Delaware, she obtained her diploma at Smyrna High School, Smyrna, DE in 1993. She immediately began her undergraduate studies at Delaware State University (DSU), Dover, DE, majoring in Chemistry. She was accepted into the prestigious Minority Access to Research Careers (MARC) Program implemented by the National Institutes of Health (NIH). Upon completion of her chemistry degree with 5 summer research experiences under her belt, in May of 1997 she went directly to graduate school at the Virginia-Maryland Regional College of Veterinary Medicine to pursue her doctoral degree in Toxicology with an emphasis in Immunology and Parasitology. During her tenure while at VMRCVM she also was awarded a National Research Service Award (NRSA) from the NIH, which covered her stipend, tuition, fees, and partial monies for supplies. Marquea was also actively involved in University governance while at Virginia Tech and was the first African-American female President of the Graduate Student Assembly (2001-2002). For her many great deeds she was awarded the 2002 Outstanding Graduate Student Service Award at Virginia Tech. Marquea has plans to pursue a career in government with the Environmental Protection Agency in Washington, DC.