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Author(s): Marquea D. King , David S. Lindsay , S. Holladay , and M. Ehrich
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Neurotoxicity and Immunotoxicity Assessment in CBA/J Mice with Chronic *Toxoplasma gondii* Infection and Multiple Oral Exposures to Methylmercury

Marquea D. King*, David S. Lindsay, S. Holladay, and M. Ehrich, Department of Biomedical Sciences and Pathobiology, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia 24061; *Present address: National Center for Environmental Assessment, U.S. Environmental Protection Agency, 1200 Pennsylvania Avenue, NW, MC 8623-D, Washington, D.C. 20460. *e-mail: lindsayd@vt.edu*

ABSTRACT: The present study was conducted to determine the effect of multiple low doses of methylmercury (MeHg) on the course of a chronic *Toxoplasma gondii* infection. Four groups of 6-wk-old female CBA/J mice either were fed 25 *T. gondii* tissue cysts of the ME-49 strain or were vehicle control. Six weeks later, half of each group was orally gavaged with 8-mg/kg body weight doses of MeHg on days 0, 2, 4, 7, 10, and 13, totaling 4 experimental groups. Mice were killed on day 17 or 18 after MeHg exposure. Flow cytometric analysis of lymphocyte subpopulations in the thymus demonstrated a significant increase in the percentage of $CD4^-CD8^+$ T-cells in mice 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 all T-cell subpopulations when compared with 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. These data indicate that multiple low doses of MeHg may not exacerbate chronic toxoplasmosis, but MeHg-induced effects on the immune system were evident.

Toxoplasma gondii is a protozoan parasite that can cause a 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 acquired immune deficiency syndrome patients. In some patients suffering from toxoplasmic encephalitis, the infection may be lethal (Daubener et al., 1997; Belanger et al., 1999). Toxoplasmic encephalitis is caused by the reactivation of latent tissue cysts of *T. gondii*. Once reactivated, the tachyzoites released from tissue cysts destroy nervous tissue and cause progressive meningoencephalitis (Gazzinelli et al., 1993).

Neurotoxicants also damage nervous tissue, but few studies examining interactions of *T. gondii* and neurotoxicants have appeared in the literature (King et al., 2003). For example, methylmercury (MeHg) is a well-documented neurotoxicant that accumulates in the brain and causes severe mental and visual dysfunction, including chronic encephalopathy (U.S. Department of Health and Human Services, 1994; Schaumburg et al., 2000). Contaminated fish, grains, and seeds are a common source of human exposure to MeHg. In addition to being neurotoxic, MeHg is also immunotoxic (Moszczynski, 1997), enhancing the probability of interaction with *T. gondii*. Therefore, it was hypothesized that MeHg accumulation could worsen a chronic *T. gondii* infection. In the present study, multiple dosing with MeHg was used to provide longer exposure to this neurotoxicant than a single dose, with the potential of increasing time for a deleterious MeHg–*T. gondii* interaction.

Six-week-old female CBA/J mice obtained from Jackson Laboratories (Wilmington, Massachusetts) were used for experimental *T. gondii* infection and MeHg exposure (n = 10 per group). These mice were used for this study because they are resistant to acute infection but are prone to develop encephalitis during a chronic infection (Suresh et al., 1991). *Toxoplasma gondii* dosing was as described previously (King et al., 2003). Methylmercury II chloride (Alfa Aesar, Ward Hill, Massachusetts) was dissolved in dimethyl sulfoxide (DMSO) at room temperature and diluted with phosphate buffered saline (PBS; 1:100). Oral doses of 8 mg/kg body weight were given to each mouse on days 0, 2, 4, 7, 10, and 13. Vehicle controls received a DMSO–PBS suspension. The 4 experimental groups of mice used were 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.

After killing by CO_2 inhalation 17–18 days after the initial MeHg exposure, 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. The thymus and spleen were immediately weighed and then prepared for detection of cell surface markers using flow cytometry (King et al., 2003). These data are presented as mean ± SE of the percentage of cells that express the surface marker of $CD4^+$, $CD4^-CD8^-$ (DN, double negative), $CD4^+CD8^+$ (DP, double positive), and CD8+ along with the uptake of 7-aminoactinomycin D (7-AAD), which shows apoptosis for individual phenotypes. Statistical analysis was performed using the general linear model (GLM) analysis of variance (ANOVA) from the SAS package (SAS Institute Inc., Cary, North Carolina). Significant differences among each experimental group with $P < 0.05$ were reported.

Mice brains were split into right and left halves after removal. The left half was used for tissue cyst enumeration and MeHg quantification as described previously (Lindsay et al., 1998; King et al., 2003). Paraffin blocks were prepared from the right half of the brains, and from them separate slides were stained with hematoxylin and eosin (H&E) and labeled with TdT-mediated deoxyuridine triphosphate (dUTP)–X nick-end marker (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling [TUNEL], Roche Molecular Biochemicals, Mannheim, Germany), and antibody to glial fibrillary acidic protein (GFAP) (Neuen-Jacob et al., 1993) was introduced, as described previously (King et al., 2003).

Mice were examined for clinical deficits indicative of neurotoxicity after MeHg exposure (days 0, 2, 4, 7, 10, and 13). Examinations were done during the dark cycle of the standard 12-hr light–12-hr dark cycle, using a red light lamp for observation. Behavioral response analyses were obtained and modified using an established protocol from the Laboratory for Neurotoxicity Studies at Virginia–Maryland Regional College of Veterinary Medicine (King et al., 2003), derived from original procedures for behavioral studies in rodents by Moser et al. (1988).

Body weights of the mice remained unchanged throughout dosing among all experimental groups when compared with each other or with control (group means 23–25 g). Thymus weights were decreased in the MeHg-only group (25.4 ± 5.2 mg) compared with controls (43.1 ± 4.4 mg; $P = 0.08$) but not in other groups. Spleen weights showed a marked increase over values from control mice (73.2 ± 3.4 mg) and values from mice given *T. gondii* only (78.5 ± 4.8 mg) in the groups exposed to MeHg (113.9 ± 13.0 mg in MeHg-only mice; $P < 0.05$; 118.5 ± 8.2 mg in *T.gondii*–MeHg mice; $P < 0.05$). Splenic cell viability showed a significant decrease in mice infected with *T. gondii* with or without MeHg exposure ($P < 0.05$). Viability of cells in the thymus was unchanged (Fig. 1).

Absolute numbers of cells in the thymus of mice exposed 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 with control and *T. gondii*–infected groups (Table I). The $CD4^-CD8^-$ cell population of the thymus was also reduced, although not significantly. Each of the subpopulations of splenocytes from mice coexposed to *T. gondii* and MeHg was reduced, although these reductions were not statistically significant. When expressed as a percentage of total cells (Table II), flow cytometric analysis of thymocyte subpopulations of mice exposed to MeHg with a concurrent *T. gondii* infection showed a significant increase in the $CD4^-CD8^+$

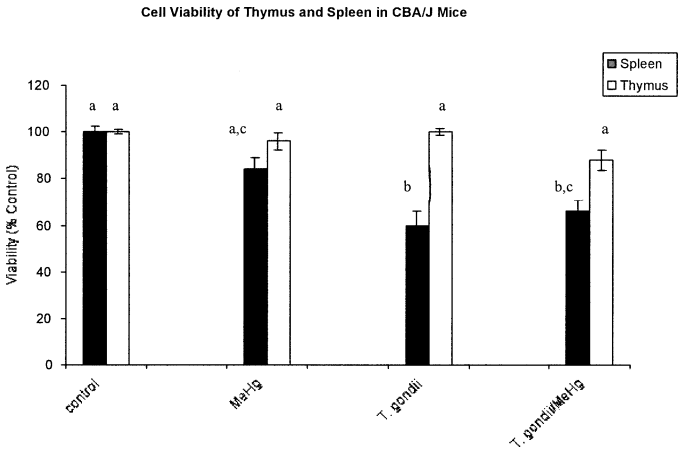

FIGURE 1. Mice dosed with 8 mg/kg body weight methylmercury (MeHg) every 2–3 days for 18 days, after a 6-wk *Toxoplasma gondii* chronic infection. Sacrificing was day 17 or 18 post MeHg exposure. Viability of cells, expressed as percentage of total cells present within the spleen and thymus, was determined by flow cytometric analysis. In the spleen, *T. gondii* decreased viability to 60% of control. Viability in spleen cells from this group of mice was also different from MeHg-treated mice ($P < 0.05$). Decreases were seen in the spleen of mice *T. gondii*/MeHg mice. 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 that groups are significantly different ($P < 0.05$).

T-cell subpopulation when compared with control or *T. gondii* alone ($P < 0.05$). *Toxoplasma 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 with both control and MeHg-exposed mice (Table II). Splenic T-cell absolute numbers showed no significant change among any of the experimental groups.

A review of H&E–stained slides from the brains of mice that were given multiple 8-mg/kg doses of MeHg for 17 days did not reveal significant lesions in the brain in animals that were given MeHg, *T. gondii*, or the combination of these 2 agents, or in vehicle controls. In addition, the cerebellar cortical lesions of granule cell necrosis–apoptosis (Nagashima et al., 1996) were absent in the MeHg-dosed mice regardless of the presence of a *T. gondii* infection. There were neither consistent astrocytic alterations noted in these sections using GFAP immunohistochemical staining nor any detectable apoptotic changes using the TUNEL assay on nervous or immune tissue.

Flow cytometric analysis with a triple color stain allowed for determination of apoptosis in individual T-cell subpopulations of the thymus and spleen. The only significant change in thymocytes was an increase in early apoptotic $CD4^+CD8^-$ T cells in mice exposed to *T. gondii*–MeHg (from 24.1 ± 3.9% in controls to 42.3 ± 5.5%; $P < 0.05$). The $CD4^-CD8^+$ and $CD4^+CD8^+$ T-cell subpopulations did not undergo any significant change.

Toxoplasma 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 $CD4^+CD8^-$ and $CD4^-CD8^+$ T-cell subpopulations. Not all these changes were observed in mice exposed to both *T. gondii* and MeHg. Apoptosis staining (TUNEL) of the spleen and thymus revealed no difference between treated and nontreated groups of mice (data not shown).

Analysis for testing of repeated behavioral responses of mice exhibiting a certain response on each day was performed. Multiple-day analysis indicated that 4 of 27 responses showed a change when comparing MeHg-only–exposed mice to *T. gondii*-MeHg–coexposed mice (click response, menace response, agility on a wooden rod, and vocalization). Of the 10 mice in each group, 70%, 10%, 70%, and 40% given only MeHg demonstrated differences in the above-listed responses, respectively, but only on day 10. Mice coexposed to *T. gondii*–MeHg showed signs of vocalization when handled (3 of the 10 mice) on day 10. Mice infected with *T. gondii*–only or vehicle control showed no neurobehavioral abnormality before sacrifice on day 17 or 18.

Although the number of tissue cysts was 3.5 ± 1.1 in mice exposed to *T. gondii* only and 5.6 ± 1.1 in mice given *T. gondii*–MeHg, tissue cyst counts were not significantly different between the groups. Lesion scoring was similar for all groups. Coexposed mice had significantly lower brain levels of MeHg than the MeHg-only–exposed mice (0.31 ± 0.03 vs. 0.68 ± 0.17 ppm, respectively). Kidney levels were 19.2 ± 3.9 ppm in mice treated with MeHg only and 22.1 ± 5.2 ppm in mice treated with *T. gondii*–MeHg. Levels of MeHg in mice not experimentally treated with MeHg were undetectable.

The present study examined the effects of multiple exposures to MeHg on a chronic *T. gondii* infection. Multiple low-dose effects in mice were less notable than when a single high dose of MeHg was given (King et al., 2003). For example, multiple, lower-dose exposure to MeHg did not increase brain tissue cysts of *T. gondii* or alter morphological effects of the infection. Because both these agents have detrimental effects on similar systems, nervous and immune (M. Aschner and J. L. Aschner, 1990; Daubener and Hadding, 1997), it was expected that the coexistence of a chronic infection and an environmental contaminant such as those examined here would worsen the initial disease state. However, the results of the present study showed that CBA/J mice demonstrated immunotoxic response to MeHg exposure but not a relapse of chronic toxoplasmosis.

Immune effects after multiple exposure of MeHg were demonstrated by a marked increase in the splenic weights of mice exposed to MeHg with or without a *T. gondii* infection, whereas thymus weights were decreased for the same mice. It was noteworthy that thymus weight was only different in those mice receiving multiple low doses of MeHg without a concurrent *T. gondii* infection. Alterations of organ weights are often primary indicators of potential immunotoxicity (Descotes, 1999). The cellularity decrease observed in the thymus of mice exposed to MeHg with or without a *T. gondii* infection is indicative of the depletion observed in lymphoid organs after immunosuppression. Mice coexposed to *T. gondii* and MeHg had a decreased splenic cellularity when compared with mice infected with only *T. gondii* in the present study. This result was different from the results of a previous study with a single high dose of MeHg (King et al., 2003).

The interaction of *T. gondii* and MeHg could be associated with effects on the immune system. Methylmercury in combination with *T.*

TABLE I. Absolute numbers of T-cell subpopulations in thymus from mice infected with *Toxoplasma gondii* or *T. gondii* and MeHg.* All data are expressed as mean × 10^6 cells ± SE.

Treatment	Total cellularity	Thymic T-cell subpopulation			
		$CD4^+$	$CD8^+$	$CD4^-CD8^-$	$CD4^+CD8^+$
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†‡	5.1 ± 2.5†	1.3 ± 0.3†‡	0.8 ± 0.3	18.4 ± 3.8†‡
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†‡	6.7 ± 2.8	2.7 ± 0.6†‡	2.0 ± 0.7	37.7 ± 9.2‡

* Female CBA/J mice exposed every 2–3 days to 8 mg/kg MeHg for 18 days (n = 10).
† Statistically significant difference ($P < 0.05$) when compared with control.
‡ Statistically significant difference ($P < 0.05$) when compared with *T. gondii*–infected mice.

TABLE II. T-cell subpopulations as a percentage of total cells in the thymus and spleen of mice infected with *Toxoplasma gondii* or *T. gondii* and MeHg.* All data are expressed as percent mean ± SE.

	Percentages of T-cell subpopulations				
Treatment	$CD4^+$	$CD8^+$	$CD4^-CD8^-$	$CD4^+CD8^+$	Non–T cells
Thymus					
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†‡	3.8 ± 0.9	73.0 ± 3.9	
Spleen					
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†	23.5 ± 2.3			44.0 ± 4.5†§
T. gondii–MeHg	35.2 ± 2.8†	24.4 ± 1.7			40.0 ± 4.5

* Female CBA/J mice exposed every 2–3 days to 8 mg/kg MeHg for 18 days (n = 10). Total cellularity of splenic cells was 52 ± 6.5, 76.9 ± 41.7, 69.0 ± 6.3, and 31.6 ± 6.0 in control, MeHg, *T. gondii*, and *T. gondii*-MeHg–treated mice, respectively.
† Statistically significant difference ($P < 0.05$) when compared with control.
‡ Statistically significant difference ($P < 0.05$) when compared with *T. gondii*–infected mice.
§ Statistically significant difference ($P < 0.05$) when compared with MeHg-treated mice.

gondii caused a significant increase ($P < 0.05$) in the percentage of mature $CD4^-CD8^+$ T cells in the thymus, 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 is either a lack of migration of $CD4^-CD8^+$ T cells or a possible increased production of these cells at this subchronic dose of MeHg, or both. 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 (Koller et al., 1980).

Examination of mice exposed to low, multiple doses of MeHg for 18 days showed that mouse body weights remained unchanged throughout dosing. In experimental MeHg poisoning in rats and mice, the first adverse symptom noted is the loss of body weight (Suzuki and Miyama, 1971). Results obtained in the present study were not in agreement with the results of Suzuki and Miyama (1971), although our previous study with a single, higher MeHg dose was (King et al., 2003). The responses that indicated alterations in nervous system behavior were negligible in mice coexposed to *T. gondii*–MeHg and were noted only on day 10 after repeated exposure to MeHg alone. Other studies confirm the same pattern of neurological symptoms (Suzuki and Miyama, 1971). Massive doses would be expected to produce a variety of symptoms in a short time, and then the animal would die, whereas on exposure to lower doses, such as ours, the symptoms of MeHg poisoning are delayed (Bagenstose et al., 2001). It is possible that behavioral abnormalities could appear at time points beyond the 17 or 18 days of our experiment, but time of death was determined by the need to gather endpoints before the mice succumbed to the chronic *T. gondii* infection. This usually occurs within between 8 and 9 wk PI (Suresh et al., 1991).

Histological examination of brain tissue did not show any lesion, tissue cyst enumeration, or glial scarring that would have been indicative of MeHg poisoning. Again, more time may be needed before these events occur (Suzuki and Miyama, 1971). Brains of mice exposed to MeHg with a concurrent *T. gondii* contained less mercury than those given only MeHg. The level in the kidneys was higher than that found in the brain, indicating the animals' ability to continue to excrete mercury. In this study, the quantity of mercury in the brain (0.68 ppm) was, however, significantly higher than the 0.35 ppm observed 7 days after a 20-mg/kg body weight MeHg dose. Also notable was the difference in MeHg level in the brains of mice coexposed to *T. gondii* and MeHg, i.e., 0.31 ppm in the present study and 0.04 ppm with a single MeHg exposure (King et al., 2003).

A plausible relationship between chronic parasitic infections and environmental contaminant exposure has not been explored previously because of the lack of relevant epidemiology studies (Clarkson et al., 1983; Bagenstose et al., 2001). However, an earlier study in an animal model demonstrated enhanced immunotoxic effects when mice were concurrently exposed to dioxin and *T. gondii* (King et al., 2000). The present study is novel in that it evaluates in vivo responses of both the immune and the nervous systems after multiple exposures to a relatively low dose of an environmental contaminant that can cause neurological impairment, which has the potential to worsen over time with concurrent parasitic infections (Silbergeld et al., 2000). The results of the present study demonstrate that CBA/J mice undergo immunotoxicity because of MeHg exposure but not a relapse of chronic toxoplasmosis.

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Meningoencephalitis Associated with an Unidentified Apicomplexan Protozoan in a Pacific Harbor Seal

J. M. Lapointe*, P. J. Duignan†, B. C. Barr‡, A. K. Petrich§, D. W. MacPherson‖, F. M. Gulland#, and J. P. Dubey¶, University of California–Davis, Davis, California 95616; *Present address: 393 Pequot Avenue, New London, Connecticut 06320; †Institute of Veterinary, Animal, and Biomedical Sciences, Massey University, New Zealand; ‡California Animal Health and Food Safety Laboratory System, Davis, California 95616; §Microbiology Department, St. Joseph's Healthcare, Hamilton, Ontario, Canada L8N 4A6; ‖Centre for Emergency Preparedness and Response, Health Canada, Ottawa, Ontario, Canada K1A 0K9; #The Marine Mammal Center, Marin Headlands, Sausalito, California 94965; ¶Parasite Biology, Epidemiology, and Systematics Laboratory, USDA-ARS, Beltsville, Maryland 20705. *e-mail: jean-martin_lapointe@groton.pfizer.com*

ABSTRACT: A Pacific harbor seal (*Phoca vitulina richardsii*) was found on the central California coast with neurologic signs and labored breathing, which were unresponsive to treatment. Necropsy revealed a nonsuppurative necrotizing meningoencephalitis, a multilocular thymic cyst, and nonsuppurative cystitis and renal pyelitis. Microscopic examination revealed protozoans in the brain, thymic cyst, and bladder mucosa. Ultrastructurally, the protozoal tachyzoites were different from those of *Neospora caninum*, *Toxoplasma gondii*, and *Sarcocystis neurona*; the rhoptries were small and had electron-dense contents, and the organism divided by endodyogeny. Specific antibodies were not detected in serum using agglutination (*N. caninum*, *T. gondii*) and immunoblot assays (*S. neurona*). Immunohistochemistry for these organisms was negative. Polymerase chain reaction on brain tissue using specific primers did not amplify *T. gondii* deoxyribonucleic acid. The meningoencephalitis in this seal thus appears to have been caused by a novel protozoan.

Infections of wild mammals by apicomplexan protozoans, including *Toxoplasma gondii*, *Neospora caninum*, and *Sarcocystis neurona*, have been reported regularly over the past 20 yr. Several pinniped species have been found to be susceptible to *T. gondii* or *S. neurona*, including harbor seals (*Phoca vitulina richardsii*) (Van Pelt and Dietrich, 1973; Lapointe et al., 1998), northern fur seals (*Callorhinus ursinus*) (Holshuh et al., 1985), and sea otters (*Enhydra lutris*) (Lindsay et al., 2001). The target organs vary, but the central nervous system is often severely affected. Simultaneous infections by *T. gondii* and *S. neurona* have also been observed (Lindsay et al., 2001; Miller et al., 2001). The present report describes a harbor seal with meningoencephalitis associated with an apicomplexan protozoan different from *T. gondii*, *N. caninum*, and *S. neurona*.

In June 1994, a juvenile female Pacific harbor seal was found alive but weak on the seashore near Bolinas, Marin County, California, and was brought to the Marine Mammal Center in Sausalito, California. It was 74 cm in length and weighed 21.6 kg; the umbilical stump was not visible. On the basis of appearance and stranding location, its age was estimated at 2–4 wk. It was given supportive treatment for 5 days, including nasogastric intubation and feeding, but on the last day its breathing was labored and it had dilated pupils and suffered seizures. It was given 0.5 mg of diazepam intravenously (i.v.) and 20 mg dexamethasone i.v. but remained unresponsive and was killed shortly thereafter with 3 ml pentobarbital i.v.

On necropsy, a 10-cm diameter mass was found within the cranial mediastinum, containing multiple cystic cavities filled with purulent exudate. The cerebral cortex appeared swollen, pale, and edematous.

For histology, sections of brain, spinal cord, mediastinal mass, liver, lung, kidney, bladder, heart, spleen, stomach, intestine, lymph nodes, adrenals, and pancreas were fixed in 10% buffered formalin and were embedded in paraffin; 5-μm sections were stained with hematoxylin–eosin (HE) and examined using light microscopy.

Sections of cerebral cortex were also examined using transmission electron microscopy. Formalin-fixed tissue was postfixed in 1% osmium tetroxide, dehydrated through graded alcohols and propylene oxide, and embedded in epon–araldite resin. Sections 600 to 900 Å thick were stained with uranyl acetate–lead citrate and examined with a transmission electron microscope at 80 kV.