A Review of Experimental Methylmercury Toxicity in Rats: Neuropathology and Evidence for Apoptosis*

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About the Author

Kazuo Nagashima was born in 1941 on Sado Island in Japan. After graduating from the Gunma University School of Medicine in 1967, he pursued further study at the Department of Pathology, Faculty of Medicine, University of Tokyo. He joined the Faculty of Medicine upon completion of his studies there and taught from 1972 to 1986. Since 1986, Dr. Nagashima has been a professor in the Department of Pathology at Hokkaido University School of Medicine; he presently chairs that department. Dr. Nagashima has studied virology at Wuerzburg University in Germany and immunology at Rockefeller University in New York. He is particularly interested in neurovirology, neurotoxicology, and general diagnostic pathology.

ABSTRACT

As an animal model for examining the pathogenicity of human organic mercury intoxication, rats have been used for the reproduction of human neurologic diseases. Rats experimentally exposed to methylmercury chloride showed clinical signs of neurologic dysfunction characterized by ataxic behavior. Neuropathology of the diseased animals consisted of lesions such as: (a) degeneration of the peripheral nerve and sensory root nerve with preservation of the motor root nerve; (b) degeneration of the posterior funiculus of the spinal cord; and (c) degeneration of cerebellar granule cells with preservation of Purkinje cells. These findings suggest the human neuropathology of this toxicity. The degeneration was characterized by nerve fiber damage or neuronal cell death accompanied by astrocytic gliosis and activated macrophages or microglias. For the cerebellar granule cells, the mechanism of neuronal cell death was shown to be apoptosis. This fact was verified by histologic and ultrastructural findings as well as by in situ nick-end labeling and electrophoretic methods. Evidence of apoptosis involvement in cerebellar degeneration would provide a new viewpoint from which to analyze the selected degeneration of the nervous system in neurotoxicology.

Keywords. Cerebellum; granular cell; methylmercury chloride

INTRODUCTION

Organic mercury intoxication has been reported in humans since the publication of Hunter and Russell’s landmark description of the neuropathology of the disease (13). In Japan, an endemic occurrence of organic mercury intoxication, Minamata disease, was first reported in 1956. Minamata disease occurred mainly among people living along the shore of Minamata Bay who had repeatedly consumed fish and shellfish contaminated with methylmercury discharged from a local chemical plant (14). Clinically, the disease is characterized by sensory impairment of the extremities (glove and stocking type) with occasional perioral dysesthesia, cerebellar ataxia, and visual field constriction. Pathologic lesions are characterized by loss of neurons from the second through fourth layers of the visual area of the calcarine cortex, disappearance of granule cells of the cerebellum with Purkinje-cell sparing, and axon degeneration accompanied by secondary myelin disruption of the sensory branch of the peripheral nerve with preservation of the motor branch (13, 14, 33).

In 1969, an American family that consumed pork containing methylmercury developed neurologic symptoms consisting of cortical blindness or constricted visual field, diminished hand proprioception, choreoathetosis, and attentional deficits, all of which are indicative of Minamata disease. Neuropathologic findings reported in 1994 were identical to those associated with Minamata disease (6), which indicates that organic mercury intoxication is a condition with specific clinicopathologic features.

Experimental administration of organic mercury has been shown to induce various neurologic deficits in laboratory animals. Rats are most commonly used to study the mechanism behind induced organic mercury neurotoxicity because of the resemblance between the pathologic lesions in rats and humans. Recent reviews on the neurotoxicology of mercury and its compounds provide more detailed information (4, 32, 36).
The mechanism by which methylmercury chloride–induced neuronal damage occurs has been extensively analyzed, and possible nervous system targets of methylmercury include: (a) the membrane systems that involve receptors, transport, and metabolism of neurotransmitters; (b) systems involving axonal transport, cytoskeletons, and the blood–brain or blood–nerve barrier; (c) the signal transduction system, including protein and nucleic acid metabolisms; (d) the respiratory and energy-generating system; and (e) other metabolic systems which provide substances needed to maintain neural activities (23). Recently, the apoptotic process has been shown to be a pivotal mechanism in cerebellar granule cell degeneration (21). This discovery has created a new field of research in the area of organic mercury intoxication. In this review, we will introduce the neuropathology of experimental methylmercury chloride intoxication in the rat, demonstrate the apoptotic process of cerebellar degeneration, and propose an approach for future toxicologic studies of organic mercury poisoning.

**Experimental Design and Mercury Concentration**

Various administrative methods have been used in rat experiments. Neurological disease could be induced by any method of methylmercury chloride administration, that is, percutaneous or per os injection using a metallic catheter or per os administration via drinking water. When we first injected methylmercury chloride 10 mg/kg/day subcutaneously, one-half of the animals became moribund within 2 wk. Approximately 10 days after the administration of methylmercury chloride, these rats developed a spastic gait and showed hind-leg crossing when suspended by their tails. Symptoms were reduced in severity when the amount of methylmercury chloride administered was reduced. In the rats that received 10 mg/kg/day subcutaneously for 7 days, methylmercury chloride concentrations in the cerebrum and cerebellum were 20.2 and 22.5 μg/g, respectively, at 10 days, and 30.8 and 27.7 μg/g, respectively, at 14 days. In rats that received 10 mg/kg/day for 10 days, the brain contained 30.4 μg/g after 21 days (24, 26, 37). Neuropathologic findings in these acutely ill animals revealed that neuronal necrosis occurred in the cerebellar cortex and the brain stem nuclei, which is not the case in human subjects. However, when we administered 4 mg/kg/day subcutaneously or orally (using a gastric tube), a milder occurrence of the ataxic disease developed in approximately 3 wk and continued to gradually progress. In this experiment, the concentration of methylmercury chloride was 23.1 ± 4.1 μg/g in the cerebrum and 22.4 ± 4.6 μg/g in the cerebellum. When we administered 4 mg/kg orally every other day, the progression of the disease was slowed; the rats developed neurologic symptoms after approximately 24 days. At 24 days, the concentration of methylmercury chloride was 21.35 ± 3.01 μg/g in the brain and 19.24 ± 3.71 μg/g in the cerebellum. When we administered 4 mg/kg orally every other day, the progression of the disease was slowed; the rats developed neurologic symptoms after approximately 24 days. At 24 days, the concentration of methylmercury chloride was 21.35 ± 3.01 μg/g in the brain and 19.24 ± 3.71 μg/g in the cerebellum. These experiments indicate that neurologic symptoms develop when the methylmercury chloride concentration in the brain exceeds approximately 20 μg/g. Surprisingly, the liver and kidney, which did not show any significant clinical and pathologic findings, contained 3 times and 6 times the methylmercury chloride concentration contained by the cerebrum and the cerebellum, measuring 64.37 ± 15.40 and 122.67 ± 22.55 μg/g, respectively (Table I). In all experiments, the weight growth of the rats receiving methylmercury chloride was gradually arrested in comparison with that of the control animals.

**NEUROPATHOLOGIC FINDINGS**

**Peripheral Nerves**

Consistent with the previous findings, when the initial signs of hind-leg ataxia developed, the prominent pathologic lesions were limited to the peripheral nerves (the sciatic nerves) (20). Nerve fibers were beaded and vesicular in appearance, and numerous myelin balls were observed using Klüver-Barrera stain (Fig. 1c). Immunostaining with an antibody (NF200 kDa, Labsystems, Helsinki), which recognizes the high molecular weight of phosphorylated neurofilament (200 kDa) (7), illustrated that the degenerated nerve fibers had a rounded and segmented appearance and appeared as dark globules when compared with the well-preserved fibers (Fig. 1a). The well-preserved fibers were considered to indicate motor nerves because the fibers of the anterior root were well preserved, as will be described later. When the fibers were stained with the ED1 monoclonal antibody, which
reacts with rat macrophages (5), numerous immunoreactive cells were found in the degenerated nerve fibers. Some of them were shown to contain degenerated myelin, axons, or both (Fig. 1b). Degeneration of the nerve fibers was well demonstrated by teased fiber preparation (Fig. 1d).

**Sensory Ganglia**

In contrast to the well-preserved nerve fibers of the control rats (Fig. 2a), the ganglia of the paralyzed animals were markedly deteriorated, exhibiting interruption of the fibers by clear beaded spaces or bubbles, some of which contained myelin balls (Fig. 2b). Neurons appeared to remain intact. However, type A neurons, large neurons that communicate with the gracile nucleus of the medulla oblongata (12), seemed to have already disappeared, leaving only intact type B neurons (29). Neurofilament (200 kDa) staining demonstrated that the fibers were interrupted, leaving clear spaces, or were aggregated as round bodies that showed increased staining (Fig. 2c). Again, ganglion cells remained unstained. ED1 immunostaining revealed numerous macrophages among the degenerated nerve fibers (Fig. 2d). Some of the bundles of nerve fibers running adjacent to the trigeminal ganglia remained intact. These intact fibers are thought to be the motor branch of the trigeminal nerve. Similar findings were obtained when we examined the dorsal root ganglia or spinal ganglia.

**Root Nerve and Spinal Cord**

The root nerves demonstrated a remarkable difference between the anterior and posterior nerves. Anterior nerve roots were very well preserved (Fig. 3a). In contrast, the posterior roots were devastated, leaving irregular-sized round bodies and fragmented nerve fibers (Fig. 3b). The degenerative changes were traced to the posterior funiculus of the spinal cord, where degenerated nerves were identified as darkly stained round bodies with 200-kDa neurofilament immunostaining. Empty vacuoles were also found in the posterior funiculi of diseased animals; these vacuoles were interpreted to be ballooned segments of the degenerated nerves. ED1-positive macrophages were also found along the length of the degenerated nerve fibers. Degeneration of both the posterior root and the spinal ascending pathway could be ascribed to loss of type A neurons in the spinal ganglia (29), and the mechanism of this selective sensory impairment may be a large amount of accumulated methylmercury chloride in the spinal ganglia (Table 1).

**Cerebellum**

The cerebellum was the most prominently degenerated area in the diseased rats (9, 18). Compared with the control rats, granular cells were reduced in number in the diseased animals (Fig. 4a, b). Despite granular cell reduction in the affected cerebellum, Purkinje cells were often spared (Fig. 4b). The remaining cell nuclei were pyknotic and fragmented. There were no inflammatory cell infiltration or exudative changes in spite of the loss of granular cells. The initial degeneration may represent an ultrastructural swelling of the parallel fiber synaptic boutons, the axonal terminals of granule cells (30). When the moribund animals were examined, the granular cells...
had largely disappeared. Even in this terminal stage, the Purkinje cells remained intact (Fig. 4c). An astrocytic reaction was not as prominent in the early stages of degeneration, but it became obvious in the terminal stages, when most granule cells disappeared (Fig. 4c). ED1 immunostaining showed scattered macrophages in the affected areas, and the ED1-positive macrophages were round or oval in shape. However, following trypsinization, we saw round or oval macrophages as well as numerous ramifying or dendritic ED1-positive cells in the degenerated granular layer and in the molecular layer, which probably reflects a microglial reaction (Fig. 4d).

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By applying the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick-end labeling (TUNEL) method (11), which detects nuclei undergoing apoptosis, we were able to see many positively stained nuclei in the cerebellar granular layers (Fig. 4c). Purkinje cell nuclei were unstained. Positive cells were usually prominent in the deeper part of the cerebellar folia. The initial lesions were usually found in the rostral part of the vermis, the lingula (15).

In the animals examined in the beginning stages of neuronal deficit, most granule cells remained ultrastructurally intact, though shrunken, and electron-dense stained nuclei were occasionally encountered (Fig. 5a). Electron density consisted of a condensation of nucleo-chromatin. Both the nuclei and the cytoplasm were almost completely degenerated. In the advanced stages of the disease, when the animals became moribund, numerous electron-dense nuclei were observed, and some of this electron-dense material protruded from the nuclei, showing typical teardrop features that are compatible with an apoptotic body (Fig. 5b). The cytoplasm was also degenerated. The Purkinje cells, however, were well preserved.

Cerebrum and Brain Stem

The cerebral cortex was generally free from lesions, and the visual cortex was not affected. Occasionally, pyknotic nuclei found positive using the TUNEL method were scattered in the deeper layers of the insula and the parieto-occipital regions. Nerve fiber degeneration was noted in the medulla oblongata. This degeneration was probably due to secondary degeneration of the central branches of the trigeminal nerve deduced from localization. However, when animals were exposed to high doses of methylmercury chloride for a short period of time (10 mg/kg/day subcutaneously), a few neurons from brain-stem nuclei, such as red nuclei, reticular formations, and scattered neurons in the deeper cortical layer, showed eosinophilic shrinkage similar to coagulation necrosis of individual cells. White matter and neurons of basal ganglia remained intact.

Analysis of DNA Fragmentation

DNA extracted from the cerebrum, cerebellum, and peripheral nerves of both control and diseased rats were electrophoresed according to the methods described by Rösl (25). DNA from peripheral nerve tissue and cerebral
FIG. 5.—Ultrastructure of the degenerated cerebellar granule cells. (a) In the early stage of degeneration, a few pyknotic nuclei are observed. Note that the intact nuclei of preserved granule cells are larger than the pyknotic nuclei. ×6,400. (b) In the advanced stage of degeneration, numerous nuclei become pyknotic; some show a teardrop appearance, a typical feature of an apoptotic body. ×6,400.

FIG. 6.—Agarose gel electrophoresis of DNA extracted from the peripheral nerves (lane 2), cerebral tissue (lane 3), and cerebellar tissue (lane 4). Note DNA ladder in lane 4. Lane 1: size marker (λ DNA-HindIII digestion).

tissue showed only a smear, but DNA from the cerebellum showed a typical ladder pattern consisting of multiples of approximately 200 base pairs (Fig. 6). Electrophoresis of the DNA extracted from the normal animals did not reveal this DNA ladder. Thus, the moribund process was shown to be accompanied by cleavage of genomic DNA into internucleosome-sized fragments, a hallmark of apoptosis. These findings, combined with morphologic features and nick-end labeling, strongly suggest that cerebellar granule cell death in methylmercury chloride intoxication is due to apoptosis.

CEREBELLAR DEGENERATION AND APOPTOSIS

Research on cerebellar apoptosis during neonatal development, indicates that the granule cells that cannot synapse with Purkinje cells during migration undergo apoptosis, regulating the final granule cell to Purkinje-cell stoichiometry (3, 38). In a primary culture of rat cerebellar neurons, methylmercury chloride has also induced apoptosis (17). In vitro, high levels of extracellular potassium (K) help to ensure proper development and prolong the survival of cerebellar granule cells. When switched from a culture medium containing high K⁺ (25 mM) to one containing a low K⁺ concentration (5 mM), granule cells degenerate and die due to apoptosis (8). Apoptosis of cerebellar granule cells in the culture system
is prevented by insulin-like growth factor I and cyclic adenosine monophosphate (cAMP) (8), insulin (35), brain-derived neurotrophic factor (16), and other factors (10). In experiments with mice, neuronal death could occur by p53-dependent or by p53-independent apoptosis (39). In addition, there are many genes activated before apoptosis (3). In particular, a protease called ICE (interleukin-1β converting enzyme) has turned out to be a pivotal protease that is essential for the completion of apoptosis, activating proteins that kill the cell (1, 22). Therefore, the rapidly progressing field of apoptosis studies can contribute to the research on organic mercury intoxication and provide new clues to resolving the mechanism behind specific neuronal cell degeneration in Minamata disease.

**DISTRIBUTION OF MERCURY AND SPECIES DIFFERENCE**

Research and common sense lead us to believe that selective neuronal degeneration is dependent on the concentration of organic mercury in the brain. Dominant degeneration of sensory peripheral nerves could be ascribed to the large deposition of methylmercury chloride in the dorsal root ganglia or spinal ganglia where the neuronal cell bodies of the sensory nerve are present. The dorsal root ganglia contained methylmercury chloride 2 to 3 times more often than did the spinal cord, where neurons of motor nerves exist (24, 37) (Table 1). Quantification assays of accumulated organic mercury in the cerebrum and cerebellum (developed at the National Institute of Minamata disease, Kumamoto, Japan) do not demonstrate such differences. Research has found that at the time of neurological disease onset, the cerebrum contained 21.35 ± 3.01 μg/g and the cerebellum contained 19.24 ± 3.71 μg/g (Table 1). Experiments with monkeys have found no difference in methylmercury levels in the cerebrum and cerebellum (2, 28). Studies have yet to examine methylmercury chloride concentration at the cellular level. Using histochemical methods, Takeuchi et al (34) reported that the Bergmann’s cells of the cerebellum contained relatively large mercury deposits compared with Purkinje cells. But their histochemical method has been proven to estimate inorganic, not organic, mercury (31). Thus, a method for detecting organic mercury at the cellular level is needed.

In the case of Minamata disease, it is well known that the visual cortex is susceptible to organic mercury intoxication. In rats or mice, visual cortices were relatively well preserved (27). In monkeys and cats, visual cortices have been reported to be selectively damaged by organic mercury (19, 28). There have also been no reported differences in the concentration between the vulnerable regions and the well-preserved regions of the brain (19, 28). Thus, the regionally specific damage from organic mercury and the species differences may mean that there may be other unknown mechanisms at the molecular level.

**CONCLUSION**

Because of the species barrier between humans and animals, matching counterparts for toxicologic diseases have not always been observable in both groups, but organic mercury intoxication in rats is an excellent model to use to analyze the mechanism of nervous system degeneration. The exception is the selective degeneration of the visual cortex, a comparison for which cats and monkeys must be used. Sensory dominant peripheral neuropathy was reproduced in rats, and the large accumulation of organic mercury in the dorsal root ganglia suggests a possible explanation for the sensory disturbances. Selective neuronal death induced by methylmercury in cerebellar granule cells in rats appears to be identical in the human form of the disease, and the mechanism of degeneration is created by DNA fragmentation due to apoptosis. Further examination of this apoptotic process will open new windows for research on human organic mercury intoxication.

**REFERENCES**