many studies related to transport pathway to the CNS by inorganic mercury, but studies which related to the transport pathway in the CNS are not performed. Therefore, this study was performed to examine effects and time dependent mercury distribution in the spinal cord caused by low concentration exposure to Hg^0 vapor by histopathological references, and then, to hypothesis the transport pathway of mercury to and in the spinal cord according to the mercury distributions. Sixteen mice were exposed to Hg^0 vapor (0.040 to 0.100 mg/m^3) all day for 1, 3, 7, 10, 14, 21 and 28 days. Mice were killed under diethylether anesthesia after exposure. The spinal cord was taken from each mouse. Histopathological examinations including autometallography, and double staining for immunohistocemistry (GFAP) and autometallography, for mercury distribution were performed. No histopathological changes were seen in the spinal cord. No mercury were seen in sections obtained from control animals. Mercury deposits, which can be seen by light microscopy as black granules, were found to accumulate within neuronal perikarya of the neurons and astrocytes. Mercury granules were demonstrated in the all spinal cord sections examined. The mercury deposits were first detected in the motor neurons in ventral horn and spread out the neurons in the dorsal horn following to substancia intermedia. No mercury granules were seen in the endothelial cells of blood vessels in any areas of the spinal cord. By electron microscopy, mercury granules like substances were found in lysosome like structures of neurons and astrocytes. In conclusion, this study indicated that the transport pathway of the inorganic mercury to the spinal cord was suspected to retrograde axonal transport as same as studies previously performed. Furthermore, it was suggested that

transneuronal transport was one of the ways of mercury granules spread within the spinal cord.

INTRODUCTION

Elemental (metallic) mercury (Hg^0) is one of the inorganic mercury and a highly toxic metal that can cause serious adverse health effect [12]. Elemental mercury is generally recognized as a heavy, silver liquid at room temperature, and is found in thermometers, fluorescent light bulbs, barometers, manometers and switches in children's shoes that light up [12]. One environmental problem of concern today is occupational exposure to Hg^0 vapor occurs in a variety of industries, but the general population is primarily exposed from continuous off-gassing from dental amalgam fillings [8, 9, 12, 31].

The central nervous system (CNS) is the critical organ for Hg^0 vapor exposure [12]. Over-exposure to mercury vapor gives rise to neurological effects with initially a fine high-frequency intention tremor and neurobehavioral impairment [12]. Peripheral nerve involvement has also been observed [12]. Long-term, low-level exposure has been found to be associated with less pronounced symptoms of erethism, characterized by fatigue, irritability, loss of memory, vivid dreams and depression [12]. Despite these clinical observations, histological changes of low concentration Hg^0 vapor exposure on the CNS are not well understood.

In previous studies, two transport pathways of the inorganic mercury to the CNS have been reported. One is that inorganic mercury is transported by

bloodstream to the CNS [10, 11, 17-19, 28, 32, 33]. The details of system are that elementary mercury (Hg^0) readily crosses the lung alveoli due to its high difusibility and lipid solubility and is taken up by erythrocytes, in which catalases oxidize the elemental mercury to divalent ionic mercury (Hg^{2+}). Although the ionic mercury does not readily pass through the blood-brain barrier, a fraction of the metallic mercury is transferred from the bloodstream into tissues, including the CNS, where it is trapped by oxidation [17, 18, 28]. The other is that inorganic mercury is transported by retrograde axonal transport pathway to the CNS [1-5, 13, 21, 22, 27]. The system is that inorganic mercury (Hg^{2+}) is taken up in nerve terminals in skeletal muscles (neuromuscular junction) and then transported retrogradely along the motor neurons to the cell bodies in the spinal cord and dorsal root ganglia [3-5, 13, 27]. However, the details of the mechanism by which mercury is taken up at the nerve endings are not known [2].

Until now, there are many studies related to transport pathway to the CNS by inorganic mercury, but studies to the transport pathway within the CNS are not performed. Therefore, this study was performed to examine effects and time dependent mercury distribution in the spinal cord caused by low concentration exposure to Hg^0 vapor by histopathological references, and then, to suspect the transport pathway of mercury to and within the spinal cord according to the mercury distribution.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice (4 weeks old) were obtained from Nippon Clea Co.

(Osaka, Japan). The animal facility was maintained under temperature of 23 ±1℃, relative humidity of 55±10%, and negative atmospheric pressure. The mice received mouse chow and filtered tap water *ad libitum*.

Exposure to mercury (Hg^0) vapor (For light microscopy)

Experimental groups were exposed to Hg^0 vapor (0.040 to 0.100 mg/m^3) all day for period of 1, 3, 7, 10, 14, 21,and 28 days (Table. 1). Exposure system is shown in Fig. 1. Mercury concentration in the exposure chamber (20 liter) was measured once at 20 min by the air sampling method [13]. Control mice were exposed to mercury- free room air for 28 days before being sacrifices. Mice were killed under diethylether anesthesia after exposure to mercury vapor. Tissues for histological examination (spinal cord) were fixed in 10% neutral buffered formalin.

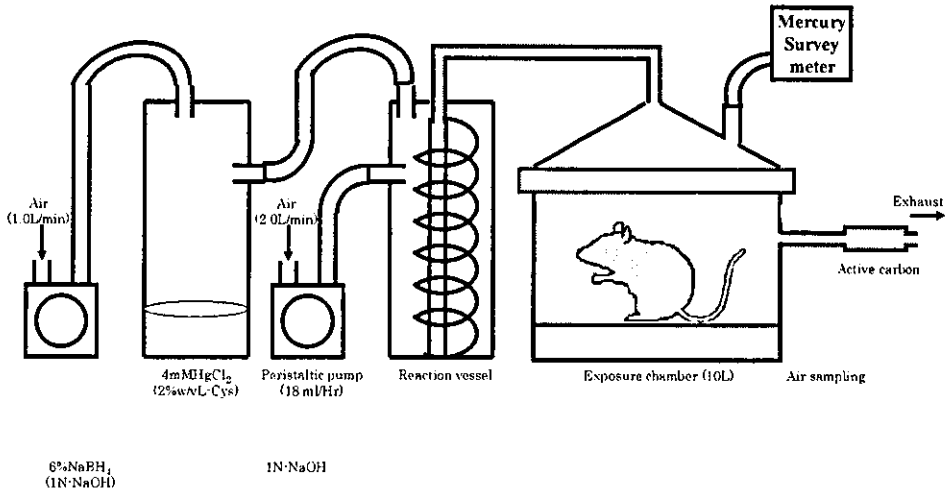


Fig. 1. Mercury vapor exposure system

Exposure to mercury (Hg^0) vapor (For electron microscopy)

Experimental groups were exposed to Hg^0 vapor (0.040 to 0.100 mg/m^3) all day for period of 7, 10 and 14 days. Exposure system is shown in Fig. 1. Mercury concentration in the exposure chamber (20 liter) was measured once at 20 min by the air sampling method [13]. Control mice were exposed to mercury- free room air before being sacrifices. Mice were killed under diethylether anesthesia by transcardial perfusion at 4 % paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) at room temperature. The spinal cords were removed and post-fixed in the same fixative for 12 h.

Table. 1 Experimental procedure

	n	Hg^0 vapor level (mg/m^3)	Exposure time (hour)	Duration of exposure (day)
Exposure	**2**	**0.040-0.100**	**24**	**1**
	2	**0.040-0.100**	**24**	**3**
	2	**0.040-0.100**	**24**	**7**
	2	**0.040-0.100**	**24**	**10**
	2	**0.040-0.100**	**24**	**14**
	2	**0.040-0.100**	**24**	**21**
	2	**0.040-0.100**	**24**	**28**
Control	**2**	**0**	**0**	**0**

Tissues: spinal cord

Histopathology

Tissues fixed in 10% neutral buffered formalin were embedded in paraffin, sectioned at 3 μ m and stained with hematoxylin and eosin (HE).

Autometallography

Paraffin sections were stained for mercury by autometallography [7]. The sections were pretreated with 1% potassium cyanide for 2h to eliminate non-specific staining from silver sulphides or selenides, placed in physical developer containing 50% gum Arabic, citrate buffer, hydroquinone and silver nitrate at 26℃ for 43 min in the dark. Excess silver was removed by 5% sodium thiosulphate. The sections were counterstained with hematoxylin. The reaction product was seen as small black grains of silver surrounding an invisible mercury core within the tissue.

Immunohistochemistry and Autometallography

The primary antibody used in this study was Glial Fibrillery Acidic Protein (GFAP) (N1506): (DakoCytomation, Inc., California, USA). Immunohistochemistry was performed on 3 μm paraffin section which was fixed with 10% neutral buffered formalin. Immunohistochemistry was performed by the avian-biotin peroxidase complex (ABC) method, in which labeled Streptavidin biotin (LSAB) kit (DAKO, Glostrup, Denmark) was included. After deparaffinization of the sections, to improve the binding of several antibodies, the sections were transferred into citric acid buffer and boiled for 20 minutes (min) at 98 ℃ in a micro oven according to a standard microwave treatment protocol. After blocking endogenous peroxidase activity with 3% H_2O_2, sections were preincubated with 10% normal goat serum for 5 min in the micro oven. Thereafter the sections were incubated with the respective primary antibody 10 min in the micro oven. The sections were sequentially incubated with peroxidase-conjugated goat anti-rabbit IgG diluted

1:400 (DAKO, Glostrup, Denmark) for 7 min in the micro oven. The positive reaction resulted in brown staining with the substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB). After immunohistochemistry, autometallography method was performed as previously discribed. Then the sections were counterstained with hematoxylin.

Transmission electron microscopy

The spinal cords fixed in buffered 4% paraformaldehyde were fixed with 2.5% glutaraldehyde, rinsed in 0.1M phosphate buffer (pH: 7.4). Tissues were performed Autometallography method. And tissues were post fixed in 1% osmium tetroxide, dehydrated in alcohols and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-100CX electron microscope (JEOL, Tokyo, Japan).

RESULTS

Gross findings

No significant changes were observed in any organs.

Histological findings

No significant changes were observed in the spinal cord.

Histological localization of mercury

No silver-mercury grains were detected in the control sections.

Spinal cord

After exposure to mercury vapor for 1 day, silver-enhanced mercury was not detected (Fig. 2-A, B). After exposure to mercury vapor for 3, 7 and 10 days, silver-enhanced mercury was detected in the neurons in the nucleus motorius (Fig. 2-C, D and 3). After exposure to mercury vapor for 14 days, silver-enhanced mercury was spread over the neurons in the nucleus intermediolateraris (Fig. 4-A, B). After exposure to mercury vapor for 21 days, silver-enhanced mercury was spread further on the neurons in nucleus proprius dosalis (Fig. 4-C, D). Then after exposure to mercury vapor for 28 days, silver-enhanced mercury was spread over the neurons in nucleus posteromarginalis (Fig. 5-A, B). The amount of mercury granules in the neurons showed time dependent increase (Fig. 7). From 14 days after exposure, silver-enhanced mercury was detected in the astrocytes (Fig. 6).

No mercury granules were detected in the endothelial cells of blood vessels (Fig. 5-C).

In electron microscopy, mercury granules like substances were detected in the lysosome like structures of neurons and astrocytes (Fig. 8).

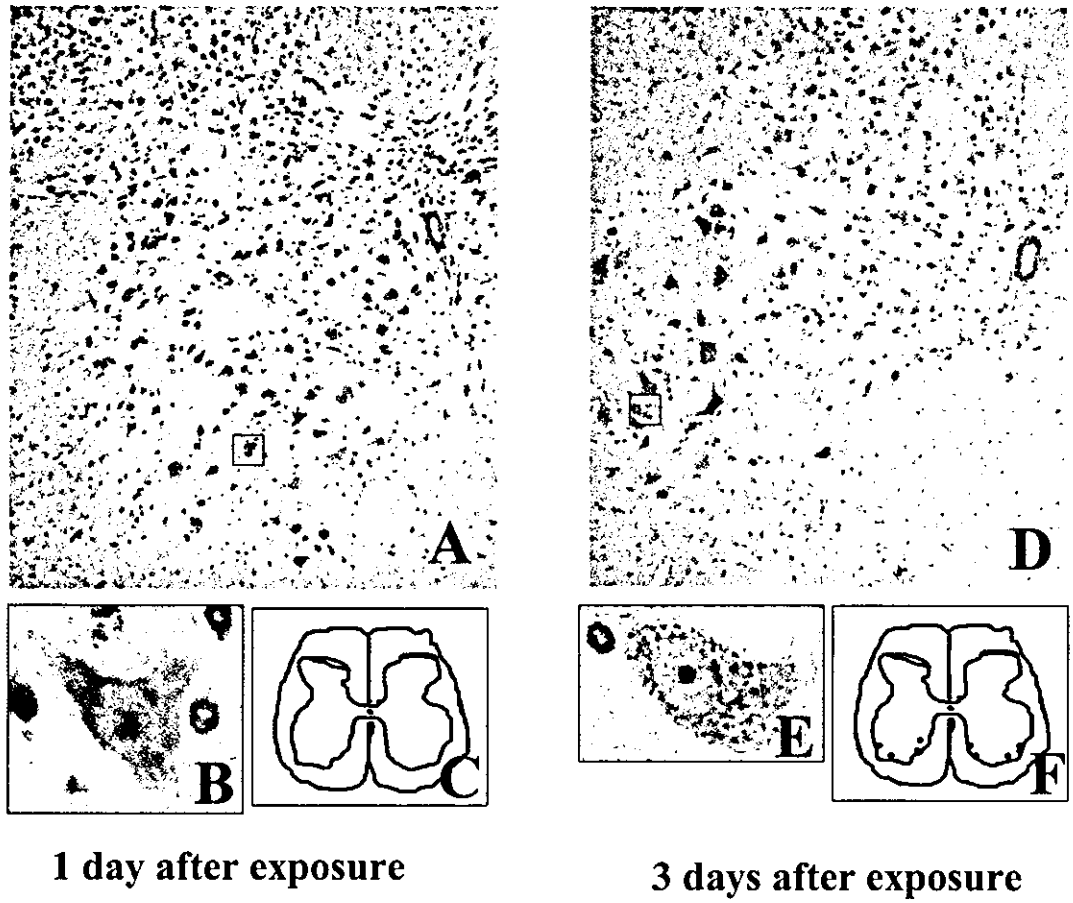


Fig.2

Histological localization of mercury in the spinal cord 1 and 3 days after Hg^0 vapor exposure.

One day after exposure, no mercury granules were detected in the spinal cord. Three days after exposure, silver-enhanced mercury was detected in the neurons in the nucleus motorius. Fig. 2-B and E show the neuron in the nucleus motorius. Fig. 2-C and F show shematic drawing of mercury distribution and amounts of mercury granules. Autometallography. Fig. 2-A : ×72 Fig. 2- B : ×1320 Fig. 2-D : ×120 Fig. 2-E : ×800

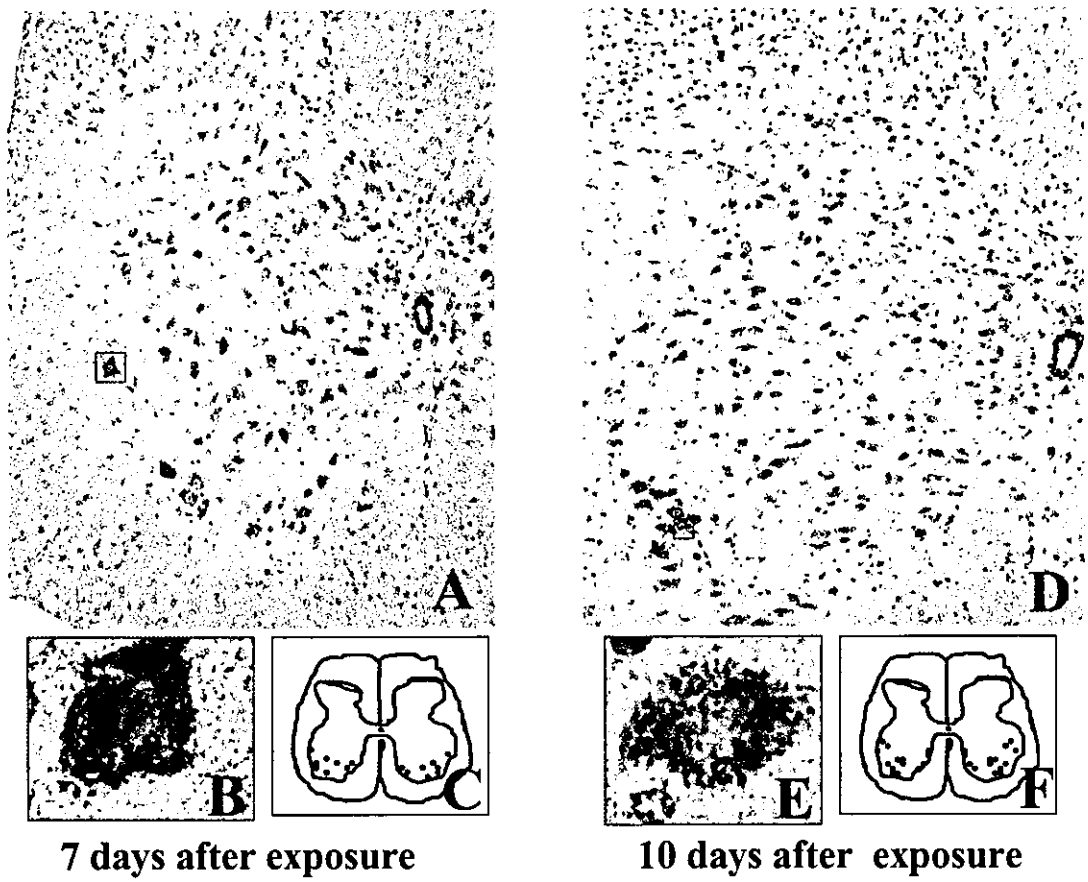


Fig. 3
Histological localization of mercury in the spinal cord 7 and 10 days after Hg^0 vapor exposure.

Seven and ten days after exposure, silver-enhanced mercury was detected in the neurons in the nucleus motorius. Fig. 3-B and E show the neuron in the nucleus motorius. Fig. 3-C and F show schematic drawing of mercury distribution and amounts of mercury granules. Autometallography. Fig. 3-A and D : ×96 Fig. 3-B : ×800 Fig. 3-E : ×1200

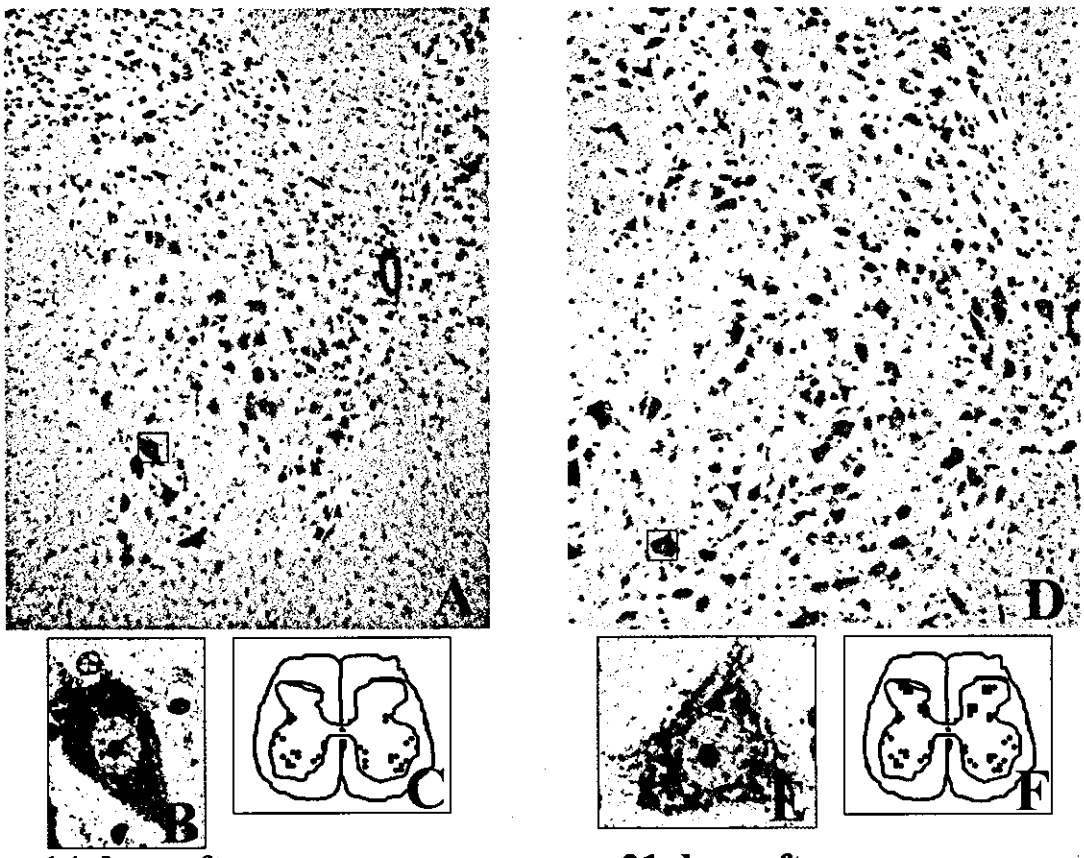


Fig. 4

Histological localization of mercury in the spinal cord 14 and 21 days after Hg^0 vapor exposure.

Fourteen days after exposure, silver-enhanced mercury was spread over the neurons in the nucleus intermediolateraris. Twenty-one days after exposure, silver-enhanced mercury was spread over the neurons in nucleus proprius dosalis. Fig. 4-B and E show the neuron in the nucleus motorius. Fig. 4-C and F show shematic drawing of mercury distribution and amounts of mercury granules. Autometallography. Fig. 4-A : ×96 Fig. 4-B : ×780 Fig. 4-D : ×138 Fig.4-E : ×800

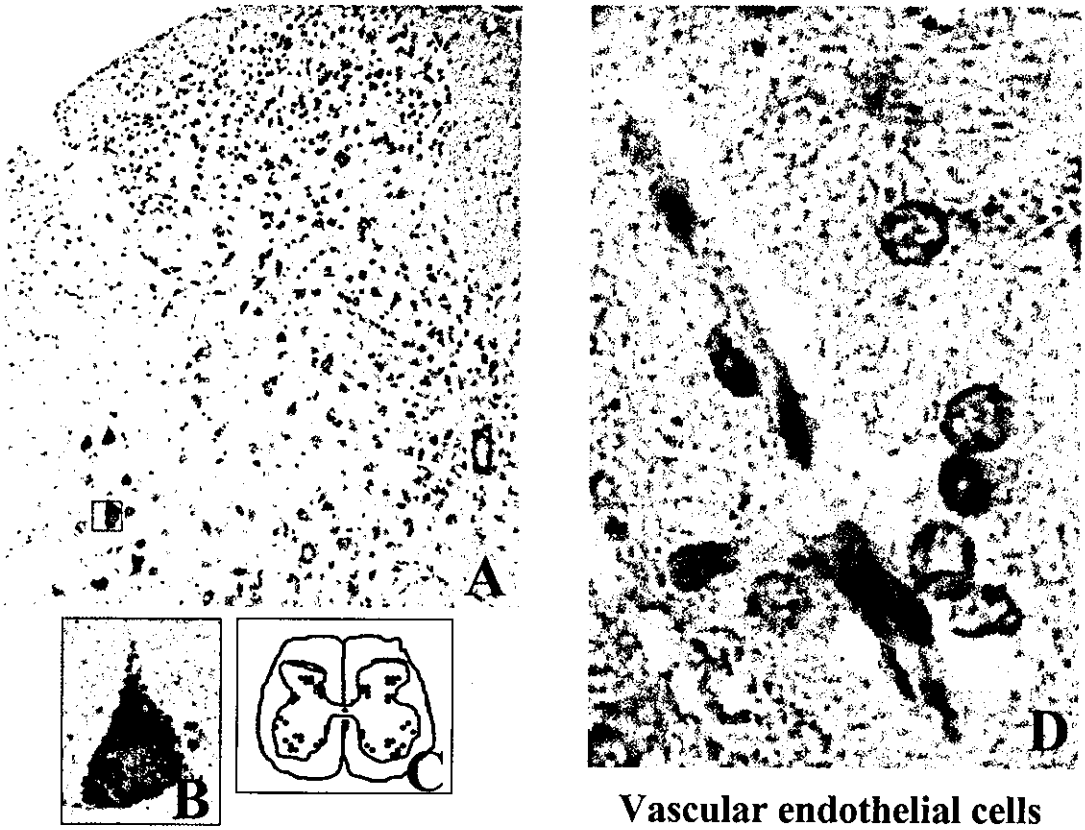


Fig. 5

Histological localization of mercury in the spinal cord 28 days after Hg^0 vapor exposure.

Twenty-eight days after exposure, silver-enhanced mercury was spread over the neurons in nucleus posteromarginalis. Fig. 5-B shows the neuron in the nucleus motorius. Fig. 5-C shows shematic drawing of mercury distribution and amounts of mercury granules.

Fig. 5-D : Vascular endothelial cell did not have mercury granules. Autometallography. Fig. 5-A : ×84 Fig. 5-B : ×720 Fig. 5-D : ×1600

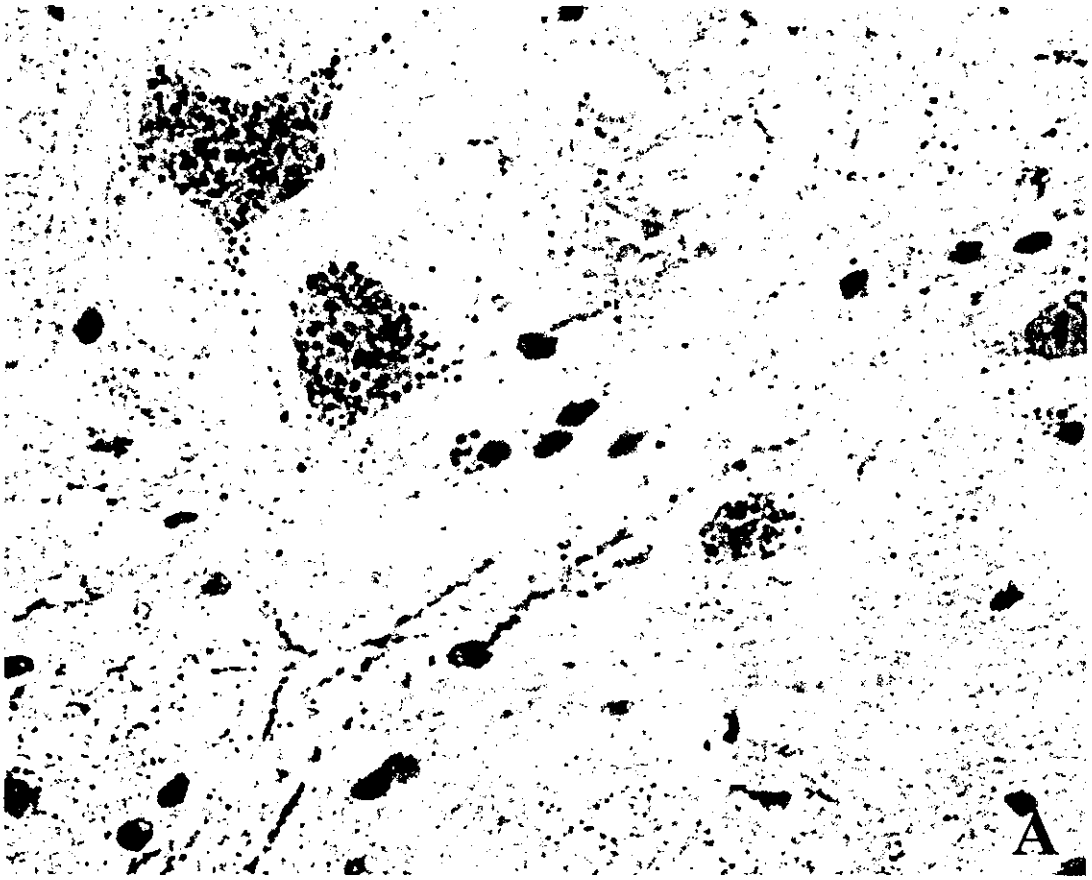


Fig. 6

Double staining for immunohistochemistry (GFAP) and autometallography in the spinal cord after Hg^0 vapor exposure.

From fourteen days after exposure, silver-enhanced mercury was detected in the cytosol and dendrites of astrocytes. Immunohistochemistry (GFAP) and Autometallography. Fig. 6-A : × 1000

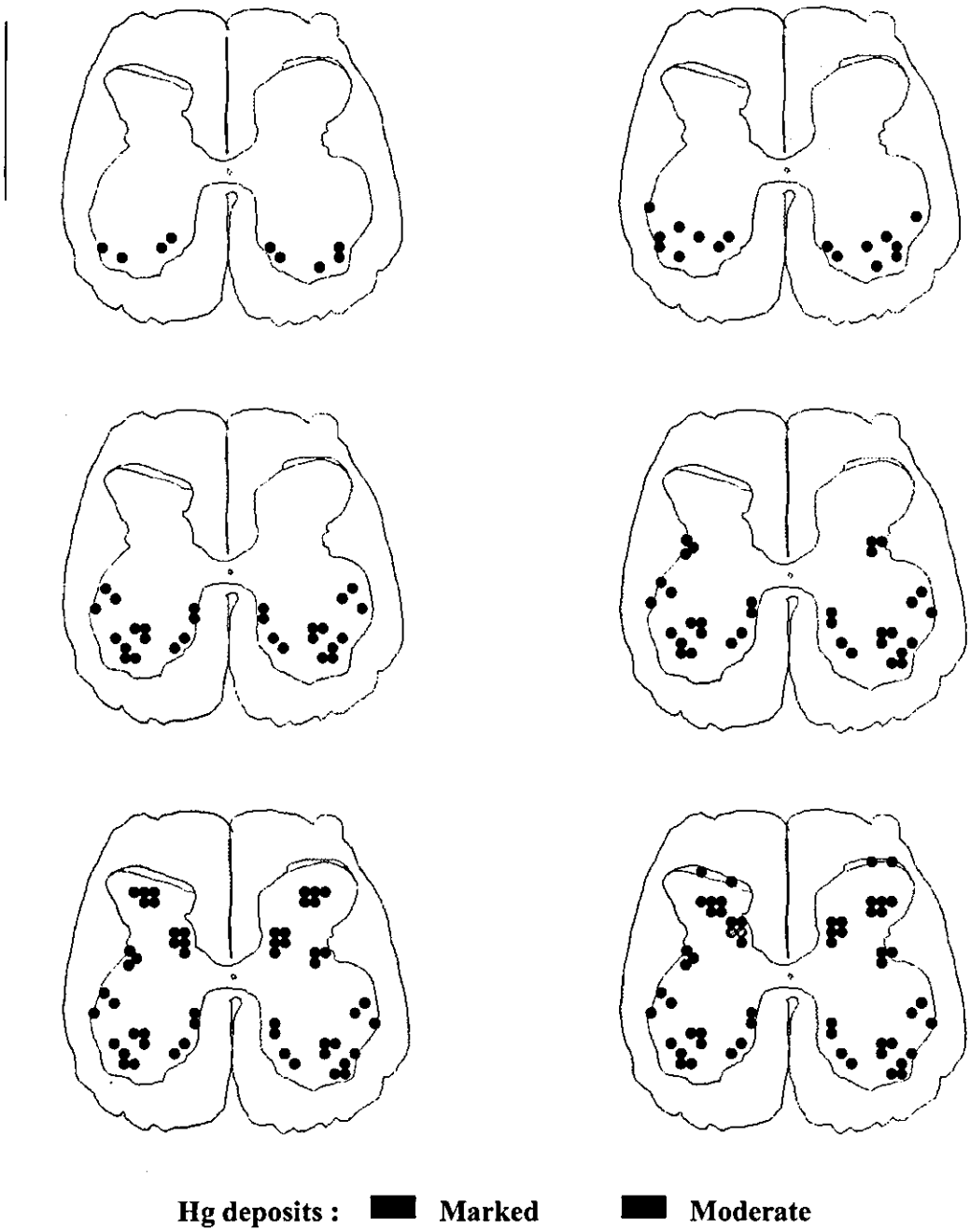
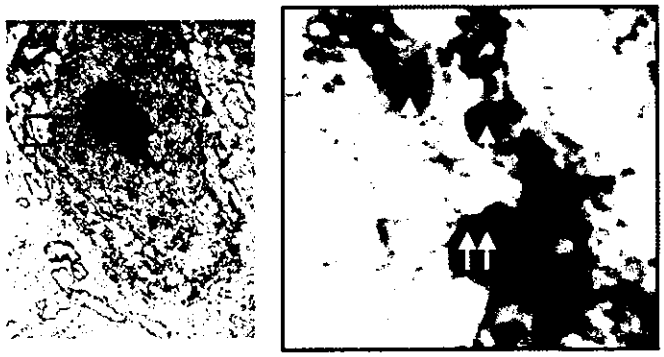


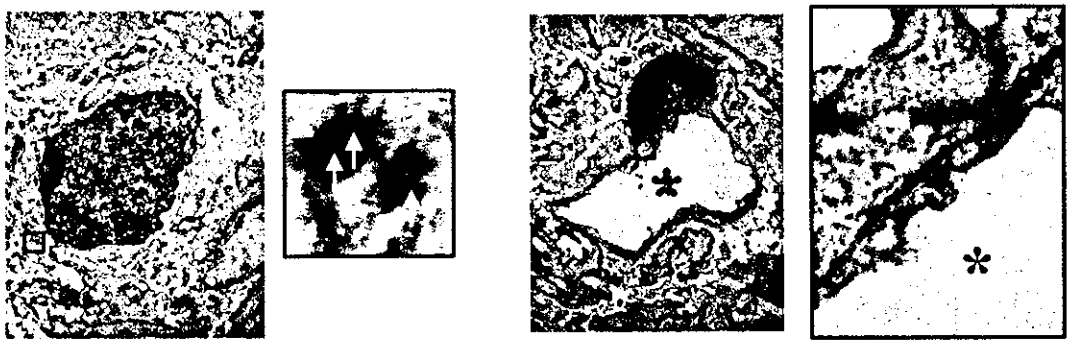
Fig. 7

Shematic drawings of transverse section of spinal cord of mouse after Hg^0 vapor exposure.

Mercury distributions were first detected in the motor neurons in ventral horn and spread out neurons in the dorsal horn following to substancia intermedia. The amount of mercury granules in the neurons showed time dependent increase.



Neuron



Astrocyte

Vascular endothelial cell

Fig. 8
Histological localization of mercury in the spinal cord after Hg^0 vapor exposure for electron microscopy.
Mercury like substances were detected in the lysosome like structures in the neurons and astrocytes.
No mercury granules were detected in the vascular endothelial cell.

DISCUSSION

In previous studies, two transport pathways of the inorganic mercury to the CNS were reported. One is that inorganic mercury is transported by bloodstream to the CNS [10, 11, 17-19, 28, 32, 33]. The details of system are that elementary mercury (Hg^0) readily crosses the lung alveoli due to its high difusibility and lipid solubility and is taken up by erythrocytes, in which catalases oxidize the elemental mercury to divalent ionic mercury (Hg^{2+}) [17,18,28]. Although the ionic mercury does not readily pass through the

blood-brain barrier, a fraction of the metallic mercury is transferred from the bloodstream into tissues, including the CNS, where it is trapped by oxidation [17, 18, 28]. The other is that inorganic mercury (Hg^{2+}) is taken up in nerve terminals in skeletal muscles (neuromuscular junction) and then transported retrogradely along the motor neurons to the cell bodies in the spinal cord and dorsal root ganglia [3-5, 13, 27]. However, the details of the mechanism by which mercury is taken up at the nerve endings are not known [2]. In this study, mercury granules were first detected in motor neurons of the spinal cord after 3 days exposure. These results suggest that the mercury was taken up in nerve terminals in skeletal muscles (neuromuscular junction) and then transported retrogradely along the motor neurons to the cell bodies in the spinal cord as same as studies previously performed.

We hypothesized to two transport pathways of mercury granules within the spinal cord. One is transneuronal transport, the other is transport through the blood brain barrier. Structurally, nerve tissue consists of two cell types: neurons and glial cells [25]. Most neurons consist of three parts: cell body, dendrites and axon [25]. The cytoplasm-filled dendrites, which are multiple, elongated processes, receive and carry stimuli from other neurons to cell [25]. The axon, which is a single cytoplasm-filled process, is specialized for generating and conducting nerve impulses away from the cell body to other nerve cells [25]. In this study, the mercury deposits were first detected in the motor neurons in ventral horn and spread out the neurons in the dorsal horn following to substancia intermedia. No mercury granules were detected in the endothelial cells of blood vessels in the all sections examined. According to these findings and basic neuronal function, transport pathways of mercury

granules within the spinal cord were suggested that transneuronal transport rather than the pathway through the blood brain barrier (Fig. 9).

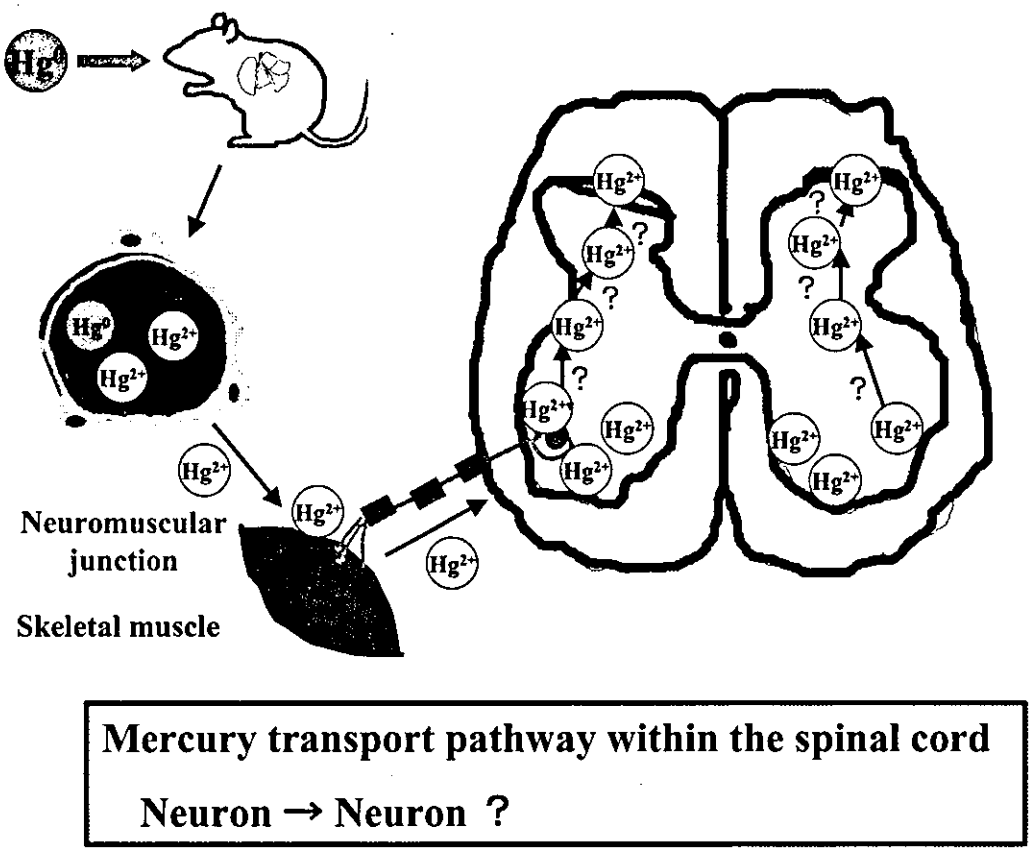


Fig. 9

Hypothesis of mercury transport pathway to and within the spinal cord.
The transport pathway of the inorganic mercury to the spinal cord was suspected to retrograde axonal transport as same as studies previously performed according to mercury distribution. Furthermore, it was suggested that transneuronal transport was one of the ways of mercury granules spread within the spinal cord.

Generally, astrocytes have many processes, some reaching to the surface of the neurons, and still others filling most of the intercellular space of the CNS

[25]. The astrocytic linkage between the blood vessels and the neurons may provide a transport mechanism for the exchange of oxygen, carbon dioxide and metabolites [25]. Astrocytes together with the tightly joined endothelial cells of the capillaries in the CNS, contribute to what is called the blood brain barrier [25]. Blood brain barrier is used to emphasize the impermeability of the nervous system to large or potentially harmful molecules [25]. Double staining for immunohistochemistry (GFAP) and autometallography showed that mercury granules were detected in cytoplasm and dendrites in the astrocytes from 14 days after exposure in this study. Mercury depositions of astrocytes were following to its depositions in neurons. Although, the functions of astrocytes in mercury toxicity are not known, these results suggest that astrocytes passively receive excessive mercury (Hg^{2+}) from neurons. However, we cannot deny the mercury transport through the blood brain barrier in the present study.

Investigations with electron microscopy have demonstrated that these granules correspond to lysosomes and prelysosomal structures [3, 4, 15, 24, 28-31]. Exogenous macromolecules that are taken up by nerve endings by unspecific mechanisms will accumulate in lysosomes of neurons after retrograde axonal transport, and as a rule will then undergo enzymatic degradation [15, 30]. Previous studies have shown that mercury can persist in the cell body of the motor neuron for long periods of time where it is stored in lysosomes [7]. Intralysosomal Hg^{2+} is not metabolized or excreted by the lysosomal enzymes and can remain in the organelle indefinitely [2]. Provably the mercury inside lysosomes is relatively inert biologically, perhaps due to its binding to selenium, a binding known to reduce the toxicity of Hg^{2+} in animals

[20]. And excessive accumulation of mercury in lysosomes of nerve cells could possibly damage the neuron because [12, 24]. In this study, mercury granules like substances were detected in lysosome like structures of neurons and astrocytes in the spinal cord. So it was suspected that the same phenomenon had been occurred in this study.

In conclusion, this study indicated that the transport pathway of the inorganic mercury to the spinal cord was retrograde axonal transport as same as studies previously performed. Furthermore, it was suggested that transneuronal transport was one of the ways of mercury granules spread within the spinal cord.

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