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Effects of Methylmercury and Inorganic Mercury on the Growth of Nerve Fibers in Cultured Chick Dorsal Root Ganglia

KYOKO MIURA,[1,2] SEIICHIRO HIMENO,[2] NORI KOIDE[2] and NOBUMASA IMURA[2]

[1]*Department of Environmental Sciences, Faculty of Economics, Wako University, Tokyo 195-8585, and* [2]*Department of Public Health and Molecular Toxicology, School of Pharmaceutical Sciences, Kitasato University, Tokyo 108-8641*

MIURA, K., HIMENO, S., KOIDE, N. and IMURA, N. *Effects of Methylmercury and Inorganic Mercury on the Growth of Nerve Fibers in Cultured Chick Dorsal Root Ganglia.* Tohoku J. Exp. Med., 2000, **192** (3), 195-210 —— Inhibition of the growth of nerve fibers by mercurials was quantitatively estimated by measuring the length of fibers in the cultured chick dorsal root ganglion. Morphological changes in nonneuronal cells were also evaluated. The growth rates of nerve fibers were constant for 2 to 6 days after the start of incubation. Methylmercury depressed nerve fiber growth dose- and time-dependently by 50% and completely at 3×10^{-6} M and 7×10^{-6} M, respectively. About 10-fold higher concentrations of inorganic mercury were required for the same extent of inhibition. The nerve fibers exposed to inorganic mercury shrank at an early stage of exposure and thereafter grew again within 24 hours. Electron microscopic examination revealed that methylmercury decreased microtubule mass extensively in nerve fibers, while inorganic mercury markedly altered surface membrane structure. These results suggested that microtubule disruption is involved in methylmercury-induced depression of nerve fibers but not in that induced by inorganic mercury. Characteristic effects on the growth of nerve fibers and the proliferation of nonneuronal cells were observed on the treatment with other metals such as cadmium, silver and chromium. Thus, dorsal root ganglion culture seems to be useful for the evaluation of toxic effects of metals in vitro. ———— dorsal root ganglion; nerve fiber growth; methylmercury; inorganic mercury; microtubule

Mercuric compounds have been shown to contribute to environmental pollution. A large number of studies have been published on the difference in pathogenicity between organic and inorganic mercury (WHO 1976). Methylmer-

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Address for reprints: Nobumasa Imura, Ph.D., Department of Public Health and Molecular Toxicology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.

e-mail: imuran@mc2.pharm.kitasato-u.ac.jp

cury poisoning is characterized by neurological disturbances (WHO 1990), whereas the toxicity of inorganic mercury is primarily directed to the kidney (Ganote et al. 1975; Naganuma and Imura 1984; WHO 1991). The difference in pathogenicity of these two mercurials has been ascribed mainly to their organ distribution. The major part of inorganic mercury administered to animals is deposited in the kidney, leading to nephrotoxicity. However, methylmercury is distributed to various organs, but it exerts toxic effects selectively on the brain. The mechanisms underlying the different toxicity between these two mercurials are not fully understood. Using cultured mammalian cells, several investigators reported that alkylmercuric compounds including methylmercury were more toxic than inorganic mercury with regard to inhibitory effects on cell proliferation (Umeda et al. 1969; Nakazawa et al. 1975; Miura and Imura 1987; Miura 2000). It has been shown that methylmercury depressed cell proliferation by specifically disrupting microtubules that compose mitotic spindles (Sakai 1975; Miura et al. 1978; Sager 1988), whereas inorganic mercury inhibited cell proliferation through the damages to various cell organelles (Miura et al. 1979). These observations indicate that different modes of action are concerned in cell proliferation inhibition with methylmercury and inorganic mercury.

On the other hand, neuronal cells differentiate at an early stage of development and do not proliferate thereafter. Methylmercury also damages the differentiated nervous system selectively in children and adults (WHO 1976, 1990). However, mechanisms underlying methylmercury or inorganic mercury toxicity on differentiated neuronal cells are poorly understood.

We attempted to evaluate toxic effects of mercurials at the cellular level by measuring nerve fiber growth using cultured dorsal root ganglia excised from 8 day-old chick embryos. The results obtained here show that methylmercury is more toxic than inorganic mercury with regard to nerve fiber growth. Methylmercury decreased the overall microtubule mass in the fiber, whereas inorganic mercury exhibited more severe damage in membrane structures than in microtubules.

Materials and Methods

Reagents

Methylmercuric chloride (CH_3HgCl), mercuric chloride ($HgCl_2$), cadmium chloride ($CdCl_2$), silver nitrate ($AgNO_3$) and potassium chromate (K_2CrO_4) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo). [^{203}Hg] $HgCl_2$ and [^{203}Hg] CH_3HgCl were obtained from the Radiochemical Center (Buckinghamshire, UK). Fetal bovine serum (FBS; Gibco BRL, Bethesda, MD, USA), equine serum (Hyclone Laboratories, Inc. Logen, UT, USA) and Eagle's minimal essential medium (EMEM, Nissui Pharmaceutical Co., Ltd., Tokyo) were purchased from Wako Pure Chemical Industries Ltd. (Tokyo). Nerve growth factor (NGF, 2.5S subunits) from adult mouse submandibular glands and collagen

(Type I) from rat tail were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

The dorsal root ganglia from 8 day-old chick embryos were excised under a dissecting microscope and cultured on Petri dishes (35×10 mm) coated with reconstituted rat-tail collagen at 37°C in a 5% carbon dioxide and 95% air atmosphere. The excised ganglia were cultured in 1 ml of EMEM medium containing 10% equine serum, 10% chick embryo extracts (CEE), 1 unit/ml NGF, 0.5% glucose, 0.025% glutamine and 0.08% $NaHCO_3$. The growth of nerve fibers from the ganglia was absent when NGF and CEE were removed from the medium. The medium was changed on the 3rd day of incubation. Living cultures of both experimental and control groups were observed daily with a phase contrast microscope. Selected cultures were stained using the Bodian staining method for nervous tissue (Bodian and Mellors 1945).

Quantitative measurement of fiber growth

The ganglia excised from chick embryos were incubated in growth medium for 48 hours in order to allow the ganglia to attach to the dish and start growing. The length of nerve fibers at 8 points of each radial outgrowth was measured as (a) μm as shown in Fig. 1C under a phase contrast microscope equipped with micrometer. After the measurement, the medium was replaced with fresh medium (1 ml) containing various concentrations of chemicals. The length of fibers at the same 8 points of each ganglion was measured as (b) μm as shown in Fig. 1D every day for 4 days without medium change. The growth of fibers was expressed as (b-a) μm. Six ganglia were cultured in each dish and 3 or 4 ganglia in which nerve fibers grew uniformly in each direction, were selected for measuring fiber growth. The density, vacuolization, migrating distance, and the degree of degeneration of nonneuronal (neuroglia and fibroblast) cells that migrated from the ganglion were examined microscopically. The extent of damage by chemicals was scored as follows: −, no effect; +, slight effect; ++, moderate effect; +++, severe effect.

Determination of mercury bound to ganglion cultures

The ganglia (5 ganglia/dish) which were cultured for 48 hours in a 3.5 cm Petri dish containing 1 ml of growth medium were incubated with labeled mercury at 37°C for 1 hour. After the incubation, the cells were rinsed three times with the mercury-free medium, and then suspended in 0.5 ml of Triton X-100 (0.5% solution). Radioactivities of the suspension were measured with an Autowell Gamma system (Aloka, Tokyo).

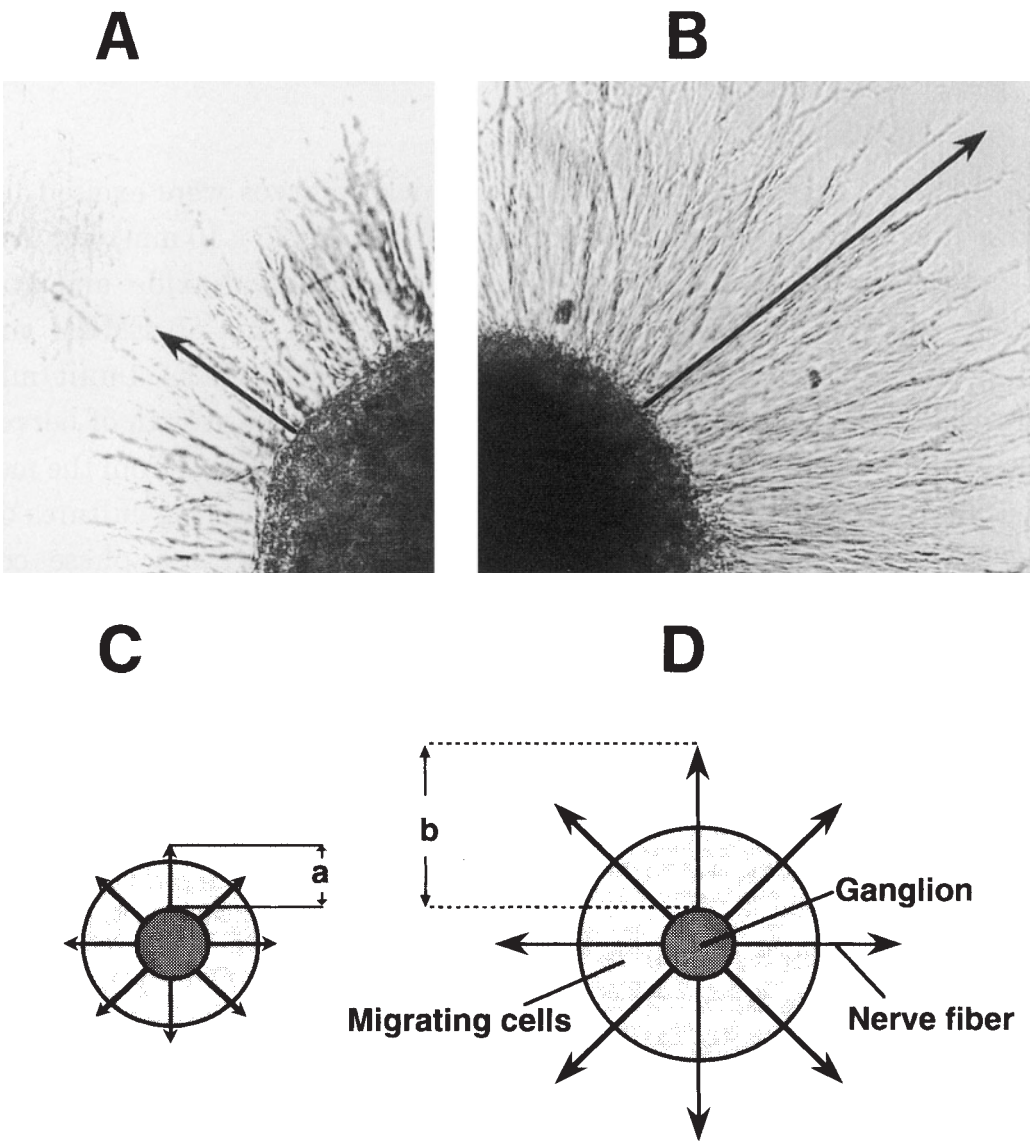

Fig. 1. Measurement of nerve fiber growth from cultured dorsal root ganglia. A and B: Nerve fibers growing from ganglion cultured for 2 (A) or 4 days (B), respectively. C: The length of nerve fibers at 8 points of 2 days-cultured ganglion was measured as (a) μm (usually about 1000 μm). D: After the medium was replaced with fresh medium and incubated for additional 1–4 days, the length of fibers at the same 8 points of the same ganglia was measured as (b) μm. The growth of fibers was expressed as (b-a) μm.

Preparation for electron microscopy

After the incubation for 24 hours with 5×10^{-6} M or 7×10^{-6} M CH_3HgCl, and 3×10^{-5} M or 5×10^{-5} M $HgCl_2$ or without mercurials, the cultured ganglia were rinsed three times with serum-supplemented culture medium, and fixed with 1.8% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) for 1 hour at room temperature and at 4°C for 1 hour in 1% OsO_4 in the same buffer. The fixed cultured ganglia on plastic sheets were dehydrated with ethanol and embedded in Epon 812 with the plastic sheets. Sections produced by a Porter Blume (MT2-B)

ultramicrotome were stained with uranyl acetate and lead citrate and then examined in a Hitachi H-500 electron microscope at 100 kV.

Results

Effects of mercurials on nerve fiber growth from dorsal root ganglia

In control non-treated cultures of ganglia, radial outgrowth of nerve fibers and migrating nonneuronal cells were observed within 24 hours of incubation. Subsequently, nerve fibers formed an extensive fiber network, and spindle-shaped nonneuronal migrating cells formed a dense sheet around the ganglion (Figs. 2A and 2B). Bodian staining confirmed the extension of nerve fibers (Fig. 2C). The length of nerve fibers in control cultures varied from ganglion to ganglion, probably due to the difference in the time required for adhesion to the collagen coated surface of the plates. Once the nerve fibers began sprouting from the ganglion, however, the rate of nerve fiber growth was constant for 4 days throughout the experimental period. Therefore, to avoid variations in nerve fiber growth caused by initial events in explant adhesion, chemicals were added to the medium 48 hours after the start of culture when the growth rates of fibers were in the linear phase.

Methylmercury inhibited the growth of nerve fibers in a dose-dependent manner (Fig. 3A). Fifty percent growth inhibition was induced by 3×10^{-6} M and complete inhibition by 7×10^{-6} M methylmercury. On the other hand, inorganic mercury inhibited nerve fiber growth temporarily within 24 hours after the treatment (Fig. 3B). At 24 hours, the lengths of nerve fibers exposed to inorganic mercury at the concentrations over 5×10^{-5} M were reduced to less than

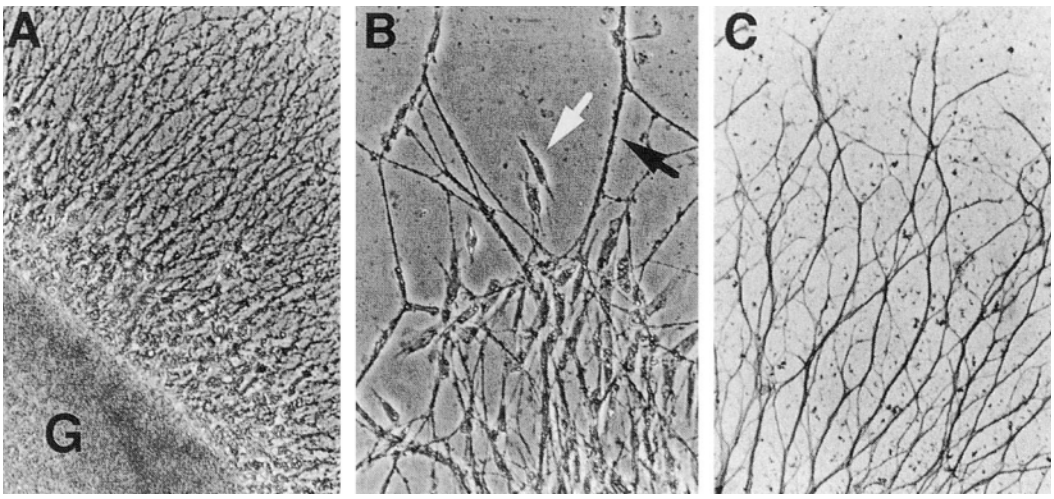

Fig. 2. Photomicrograph of nerve fibers and migrating nonneuronal cells in control culture.
Dorsal root ganglia were cultured at 37°C for 48 hours. A: Phase contrast photomicrograph of nerve fibers sprouting out from the dorsal root ganglion explant (×80). B: Nerve fibers (arrow) and migrating nonneuronal cells (white arrow) (×152). C: Networks of nerve fibers stained by the Bodian staining method (×80). G, ganglion.

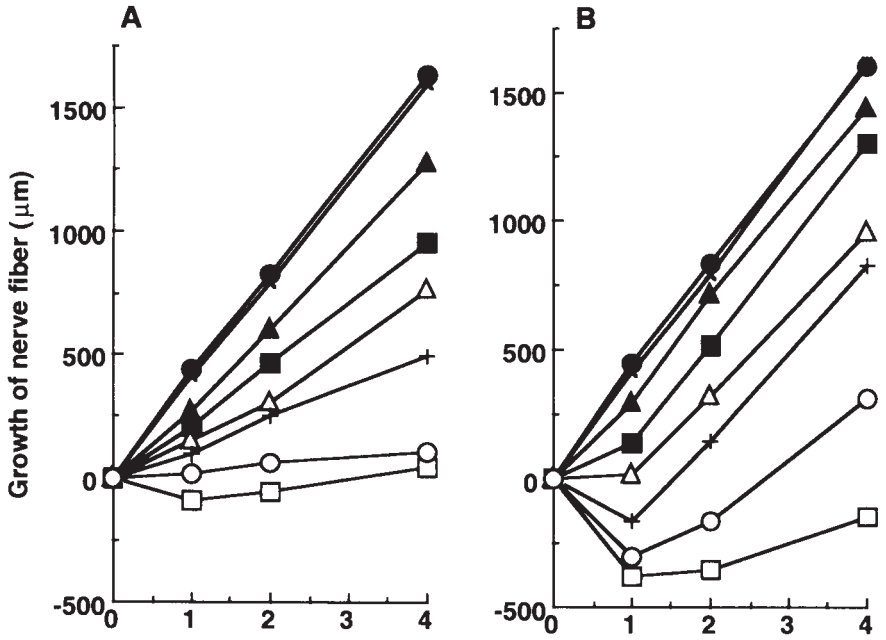

Fig. 3. Effects of methylmercury and inorganic mercury on nerve fiber growth of dorsal root ganglia.
The length of the nerve fiber sprouting out from the ganglia cultured for 48 hours in the control medium was measured. Then the cultures were incubated with fresh medium (1 ml) containing mercurials for 4 days without medium change.
Growth was shown as the increase of fiber length (μm) after replacement of the medium with fresh one with or without mercury. Each value represents the mean of 32 measurements. SDs were less than 36 μm.
A: control (●), 10^{-7} (×), 10^{-6} (▲), 2×10^{-6} (■), 3×10^{-6} (△), 5×10^{-6} (+), 7×10^{-6} (○), 10^{-5} (□) M CH_3Hg^+. B: control (●), 10^{-6} (×), 10^{-5} (▲), 2×10^{-5} (■), 3×10^{-5} (△), 5×10^{-5} (+), 7×10^{-5} (○), 10^{-4} (−) M Hg^{2+}.

those observed at the start of exposure (48 hours after the start of culture), suggesting that the nerve fibers were retracted by inorganic mercury exposure. However, the growth rates of nerve fibers returned to near the control level after an additional 24 hours cuture except for the groups exposed to inorganic mercury at concentrations over 7×10^{-5} M. This result apparently indicates a different mode of action by inorganic mercury on the growth inhibition of nerve fibers compared with methylmercury.

The effects of inorganic mercury on nerve fiber growth at the initial phase of exposure were further examined (Fig. 4). When nerve fibers that had grown to about 1000 μm were treated with inorganic mercury at 7×10^{-5} M, they apparently disappeared within 1 hour as shown in Figs. 4A and 4B. However, the fibers sprouted again thereafter (Fig. 4C). In the ganglion exposed to 2×10^{-5} M inorganic mercury for 2 hours, the nerve fibers did not shrink but almost ceased to grow for 24 hours, and then started to grow again (Fig. 4A).

In the recovery phase, the growth rates of nerve fibers cultured in the inorganic mercury-free media were higher than those cultured in inorganic mercury-containing media at both concentrations of inorganic mercury. These

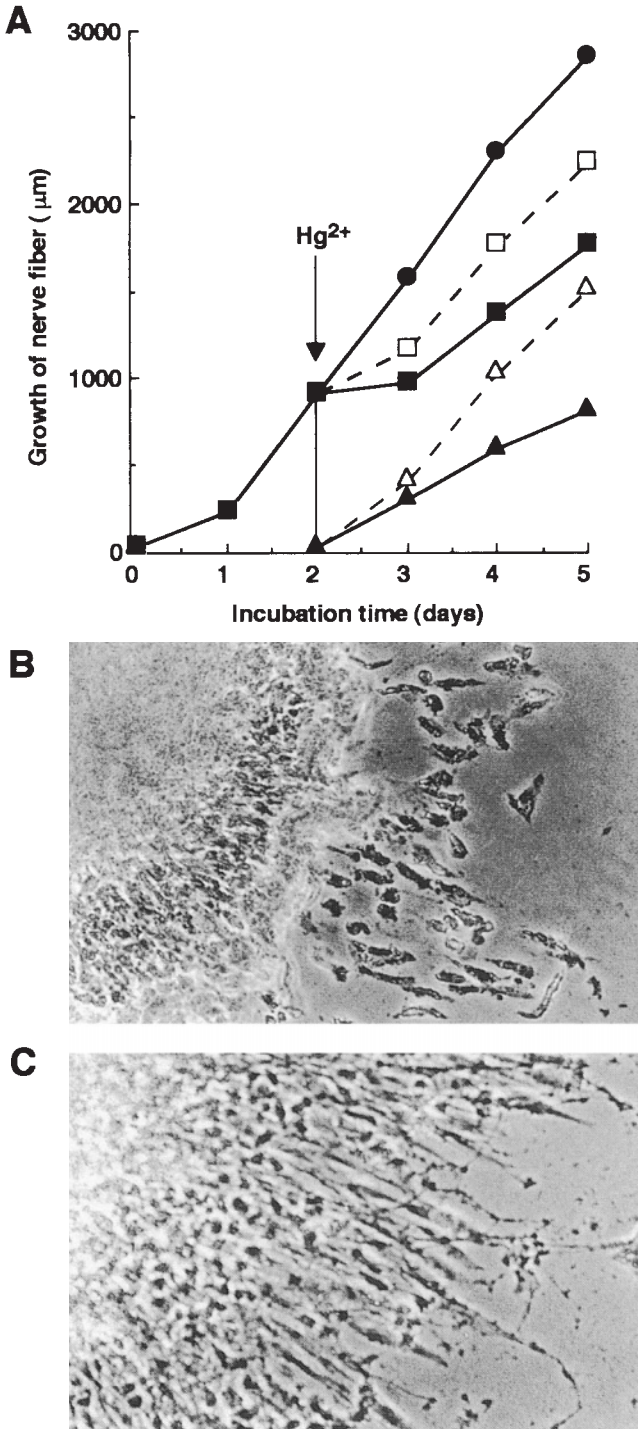

Fig. 4. Recovery of nerve fiber growth in the presence or absence of inorganic mercury.

Dorsal root ganglion explants were cultured for 48 hours in control medium. Thereafter, the medium was replaced with fresh medium (1 ml) containing Hg^{2+} (arrow). A: Growth curves of Hg^{2+}-treated nerve fibers. control (●), 2×10^{-5} M for 3 days (■), 7×10^{-5} M for 3 days (▲), 2×10^{-5} M for 2 hours, then control medium for 3 days (⌐), 7×10^{-5} M for 1 hour, then control medium for 3 days (▲). Each value represents the mean of 24 measurements. SDs were less than 19 μm. B and C: Phase contrast photomicrographs of cultured ganglia exposed to 7×10^{-5} M Hg^{2+}. Dorsal root ganglia were cultured for 48 hours, then incubated with 7×10^{-5} M Hg^{2+} for 1 hour (B) and 24 hours (C). ($\times$120).

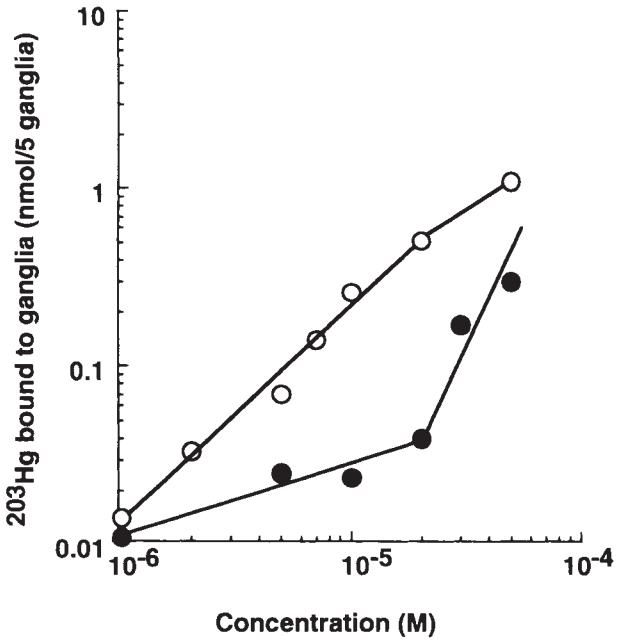

Fig. 5. Amounts of mercury bound to dorsal root ganglion cultures incubated in methylmercury or inorganic mercury for 1 hour.
The ganglia cultured in control medium for 48 hours were incubated with $CH_3{}^{203}HgCl$ or $^{203}HgCl_2$ for 1 hour at 37°C. Radioactivities bound to ganglion cultures were determined as described in Materials and Methods. Each value represents the mean of duplicate measurements. ○, CH_3Hg^+, ●, Hg^{2+}.

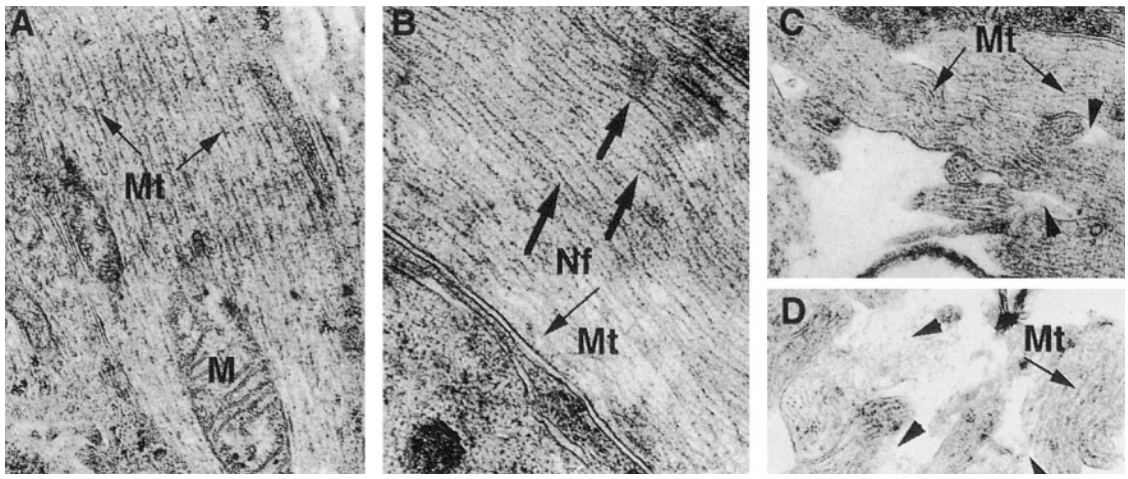

Fig. 6. Electron micrographs of nerve fibers exposed to mercurials for 24 hours at the growth inhibiting concentrations of nerve fibers.
Dorsal root ganglia cultured for 48 hours were incubated with mercurials for 24 hours. A: Nerve fiber in control (×18 125), B: Nerve fiber exposed to 7×10^{-6} M CH_3Hg^+ for 24 hours (×35 960), C and D: Nerve fiber exposed to 5×10^{-5} M Hg^{2+} for 24 hours (×9062), M: mitochondria, Mt: microtubules (arrows), Nf: neurofilaments (broad arrows), Damaged surface membrane (arrow-heads).

results indicate that fiber growth inhibition was caused by at least two events, i.e., retraction of fibers or cessation of growth in the initial phase and depression of growth rate in the late phase.

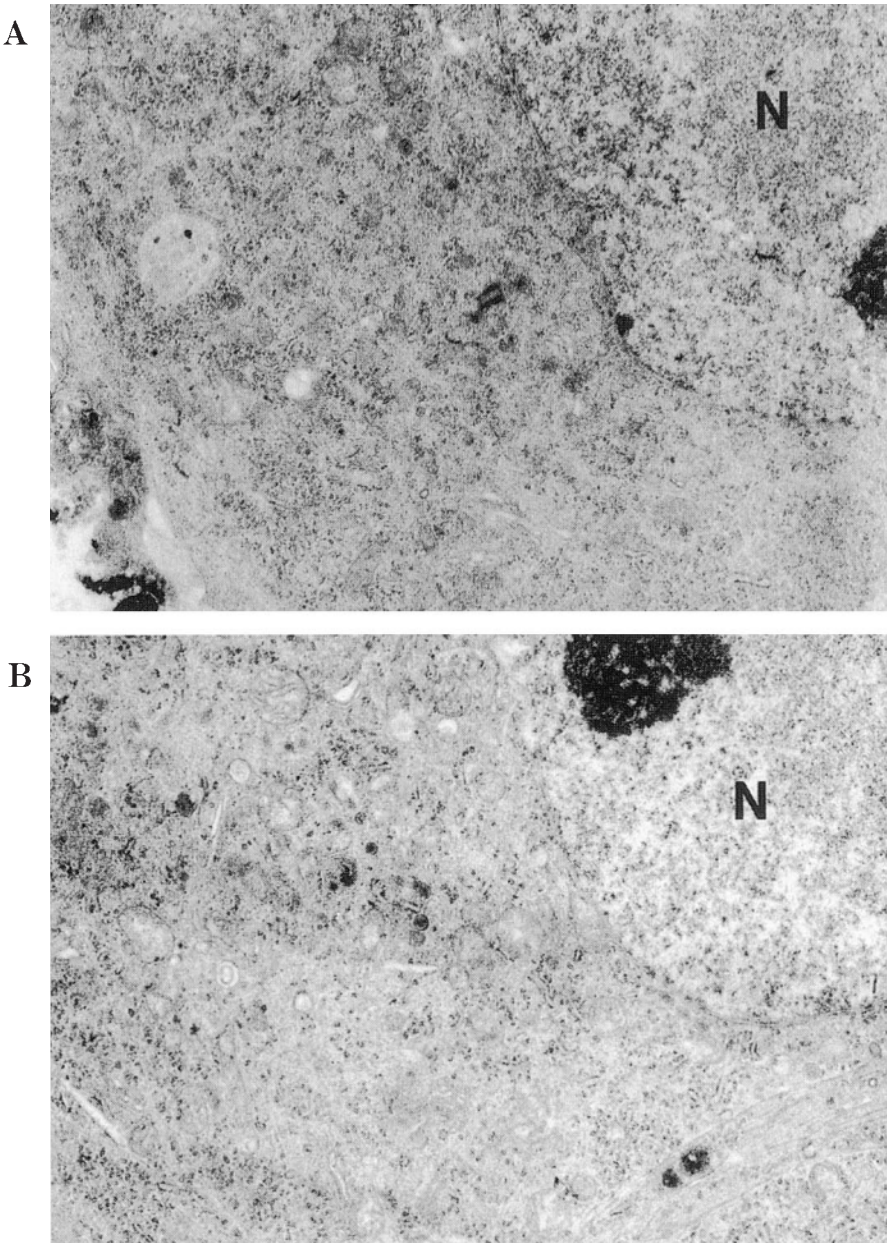

Fig. 7. Electron micrographs of neuronal cell body exposed to methylmercury for 24 hours at the growth inhibiting concentrations of nerve fibers.
Dorsal root ganglia were treated with methylmercury as described in Fig. 6.
A: control ($\times 6750$), B: 7×10^{-6} M CH_3Hg^+ for 24 hours ($\times 6750$), N: nucleus

Next, the uptake of the two mercurials into the ganglia was examined to investigate the difference in the inhibitory effects on nerve fiber growth between methylmercury and inorganic mercury. As shown in Fig. 5, methylmercury was taken up more readily by cultured ganglia than inorganic mercury. The amount of ^{203}Hg incorporated into the ganglia exposed to methylmercury increased gradually as methylmercury concentration in the medium increased, and was about 10 times that of inorganic mercury at 10^{-5} M. Conversely, the uptake of ^{203}Hg into the ganglia exposed to inorganic mercury increased slowly when the inorganic mercury concentration was less than 2×10^{-5} M. However, a steep

increase of ^{203}Hg uptake into ganglia was observed at the concentrations over 2×10^{-5} M of inorganic mercury.

Ultrastructural changes in nerve fibers exposed to mercurials

Ultrastructural changes in the nerve fibers of ganglia, which were exposed to growth inhibiting concentrations of methylmercury (7×10^{-6} M) or inorganic mercury (5×10^{-5} M) for 24 hours, are presented in Fig. 6. In the control nerve fiber, numerous microtubules oriented parallel to the axis were observed (Fig. 6A), while the mass of microtubules decreased markedly in the fiber treated with methylmercury (Fig. 6B). In this fiber (Fig. 6B), a large number of neurofilaments occupied most part of axoplasm. The nerve fibers treated with inorganic mercury (Fig. 6C) exhibited irregularly bending structures indicative of fiber retraction, but microtubules were still observed. Damaged membrane structures were also observed in the surface areas of nerve fibers (Fig. 6D). On the other hand, in neuronal cell body morphological changes in cell organelles were not evident by the treatment with 7×10^{-6} M methylmercury for 24 hours (Fig. 7B).

Effects of other metals on nerve fiber growth

Growth inhibition of nerve fibers by other metal compounds was examined

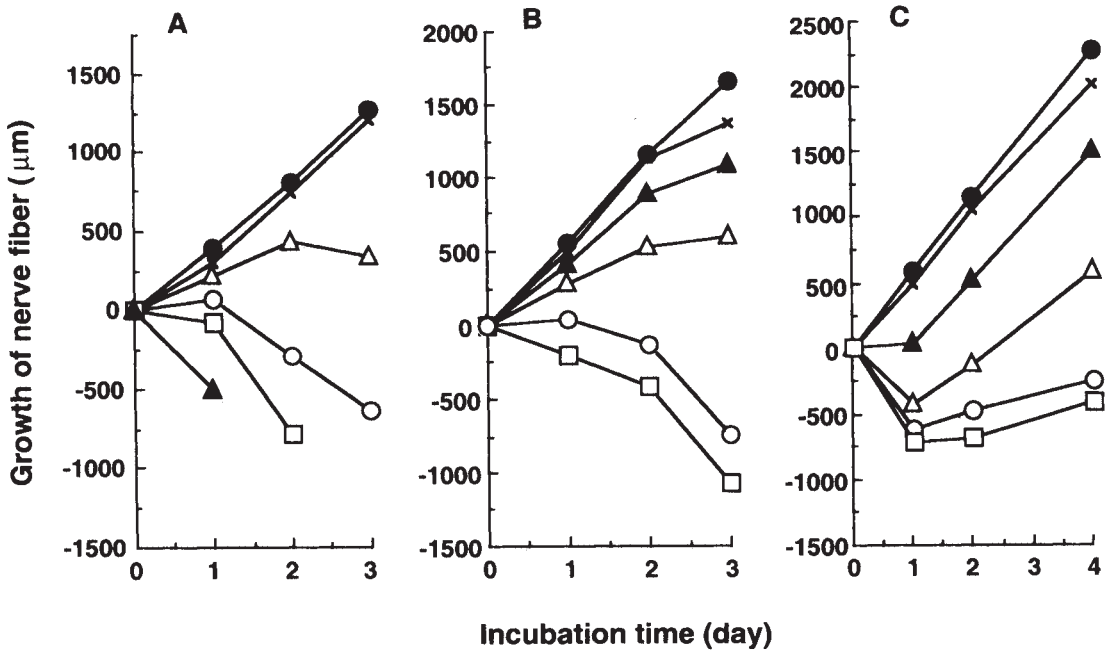

Fig. 8. Effects of Cd^{2+}, Cr^{6+} and Ag^{+} on nerve fiber growth.
Growth of nerve fibers was measured as described in Fig. 3. Each value represents the mean of 24 measurements. SDs were less than 50 μm.
A: control (●), 10^{-6} (×), 5×10^{-6} (△), 10^{-5} (○), 5×10^{-5} (□), 10^{-4} (▲) M $CdCl_2$.
B: control (●), 10^{-5} (×), 5×10^{-5} (▲), 10^{-4} (△), 5×10^{-4} (○), 10^{-3} (□) M K_2CrO_4. C: control (●), 10^{-6} (×), 10^{-5} (▲), 3×10^{-5} (△), 5×10^{-5} (○), 7×10^{-5} (□) M $AgNO_3$.

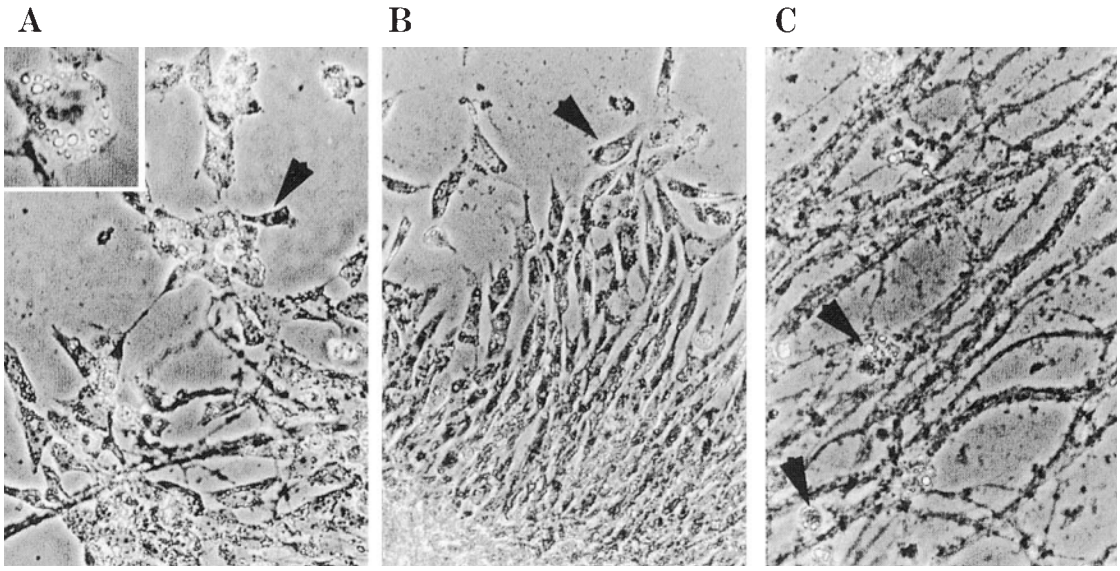

Fig. 9. Phase contrast photomicrographs of migrating nonneuronal cells from ganglia exposed to CH_3Hg^+, Cd^{2+} or Cr^{6+}.
Dorsal root ganglia were cultured for 48 hours in control medium and then incubated with CH_3Hg^+, Cd^{2+} or Cr^{6+} for additional 24-48 hours.
A: 7×10^{-6} M CH_3Hg^+ for 24 hours, B: 2×10^{-5} M Cd^{2+} for 48 hours, C: 10^{-4} M Cr^{6+} for 24 hours. Arrow-heads show nonneuronal cells ($\times$170). A vacuolated cell is enlarged in the inset ($\times$510).

(Fig. 8). Cadmium (Cd^{2+}, Fig. 8A) exhibited a growth inhibitory effect at concentrations higher than 5×10^{-6} M, while much higher concentration of Cr^{6+} was required to exert an inhibitory effect (Fig. 8B). In the case of Ag^+, the growth of nerve fibers was temporarily inhibited within 24 hours, and thereafter it recovered (Fig. 8C) as in the case of inorganic mercury (Fig. 3B). The patterns of growth inhibition by Cd^{2+} and Cr^{6+} closely resembled those observed in methylmercury-treated fibers (Fig. 3A).

Effects of metals on nonneuronal cells migrating from ganglia

The cultured ganglia were exposed to each metal compound for 24 to 48 hours at fiber-growth inhibitory concentrations, and the morphology of migrating nonneuronal cells was examined. Approximately 50% of nonneuronal cells exposed to methylmercury at 7×10^{-6} M for 24 hours became round, and almost all cells exhibited vacuolation (Fig. 9A). Nonneuronal cells exposed to 2×10^{-5} M Cd^{2+} for 48 hours remained intact, although nerve fibers disappeared completely (Fig. 9B). Contrary to Cd^{2+}-exposed cells, the nonneuronal cells exposed to Cr^{6+} at 10^{-4} M for 24 hours were injured severely (Fig. 9C), while only 50% inhibition of nerve fiber growth was observed in the same ganglion (Fig. 8B). As shown in Fig. 4B, exposure to inorganic mercury at 7×10^{-5} M for 1 hour damaged the nonneuronal cells severely, but intact cells appeared again within 24 hours even in the presence of the same concentration of inorganic mercury.

Discussion

In the present study, toxic effects of methylmercury and inorganic mercury on an isolated nervous cell system were investigated using dorsal root ganglia excised from 8 day-old chick embryos.

A few studies have examined the effects of mercurials on the growth of nerve fibers in in vitro systems. Using dorsal root ganglia excised from 8–10 day-old chick embryos, Kasuya (1972) scored the changes (0 to 3) based on both nerve fiber growth and the morphology of Schwann cells. Nakada et al. (1981) also used dorsal root ganglia of chick embryos and evaluated the changes (0 to +4) based on the length of fiber and the density of fiber networks. These holistic and semi-quantitative evaluations have been useful to determine the effective concentrations of mercurials or to compare the effects between each mercuric compounds. In fact, these experiments demonstrated that methylmercury completely inhibited the fiber outgrowth at 5×10^{-6} M, while about 10-20 fold higher concentrations of mercuric chloride were required for similar inhibition (Kasuya 1972; Nakada et al. 1981).

In order to verify the mechanism underlying neuronal damage by mercurials, it is necessary to determine the precise dose-response relationship between mercury concentrations and nerve fiber growth. Therefore, in the present study, the fiber growth was measured by the quantitative methods as described in Materials and Methods. Since the nerve fibers grow linearly for 6 days after the start of incubation of ganglia, the effect of mercurials on nerve fiber growth was determined during the linear growth period.

By this method, the effects of mercurials primarily on the length of nerve fiber, i.e., the efficiency of fiber elongation, was evaluated. Methylmercury concentrations required for 50% and complete inhibition of nerve fiber growth were determined to be 3×10^{-6} M and 7×10^{-6} M, respectively. These concentrations were similar to those required for inhibiting the proliferation of neuronal cells. We have already reported that the proliferation of mouse neuroblastoma and rat pheochromocytoma cells (PC12) was depressed by methylmercury at $3\text{–}5\times10^{-6}$ M (Miura and Clarkson 1993; Miura et al. 1999). These results suggest that a common cellular event caused by methylmercury may be involved in the depression of both cell proliferation and nerve fiber growth.

Nerve fibers contain dense arrays of microtubules that are involved in axonal transport (Baas 1997). In addition, microtubules participate directly in the growth and maintenance of fibers (Bamburg et al. 1986; Tanaka et al. 1995). Anti-microtubule drugs, such as colcemid and nocodazole, are known to deplete the microtubules in axons, thereby leading to the inhibition of axon growth in various neuronal cells (Bamburg et al. 1986; Yu and Baas 1995).

The disruption of microtubules by methylmercury has been well documented in cell free systems (Abe et al. 1975; Imura et al. 1980; Miura et al. 1984; Vogel

et al. 1985) and in cultured cells (Miura et al. 1978, 1984; Imura et al. 1980; Sager et al. 1983). In light of these findings, it was postulated that microtubules in nerve fibers are a target of methylmercury. In fact, in the present study, the mass of microtubules decreased in fibers treated with methylmercury at the fiber growth inhibiting concentration (7×10^{-6} M), although morphology of cell organelles in neuronal cell bodies was not affected.

As shown in Fig. 3, the rate of fiber growth was inhibited dose-dependently in a range of methylmercury concentration from 10^{-6} to 7×10^{-6} M, suggesting that the disruption of nerve fiber microtubules by methylmercury is the cause of inhibition of nerve fiber elongation. In support of this notion, a dose-dependent disruption of microtubules by methylmercury has been observed in both a cell-free system and cultured cells (Abe et al. 1975; Imura et al. 1980; Sager et al. 1983; Miura et al. 1984; Vogel et al. 1985).

It has been reported that microtubules in axons are sensitive to methylmercury and its disruption alters fast axonal transport (Abe et al. 1975) and electrical excitability of axons (Matsumoto and Sakai 1979). In addition to these reports, the present study indicated that growth inhibition of nerve fibers by methylmercury is also ascribed to microtubule disruption.

Individual microtubule in the axon consists of detyrosinated domains and newly assembled tyrosinated domains (Baas and Black 1990). Rochlin et al. (1996) indicated that the tyrosinated domain was easily depleted by the treatment with anti-microtubule drugs, whereas the detyrosinated domain tended to remain in the axon after the treatment (Baas and Black 1990). In the present study, trace levels of microtubules remained in the fibers treated with the growth inhibitory concentration of methylmercury, as shown in Fig. 6B. The remaining microtubules might be composed of detyrosinated domain although this is not yet verified.

Since axons are incapable of locally synthesizing the tubulin subunits that compose microtubules, tubulins have to be transported down the axon from its site of synthesis within the cell body of the neuron. We have previously demonstrated that methylmercury not only depolymerizes microtubules but also inhibits tubulin synthesis at the growth inhibiting concentration (Miura et al. 1998). Thus, nerve fiber growth may be extremely susceptible to methylmercury since such growth strictly depends on microtubule functions.

The mode of action of inorganic mercury on nerve fiber growth appeared to be quite different from that of methylmercury. This discrepancy between methylmercury and inorganic mercury is mainly due to the different membrane permeability of both compounds. The amounts of inorganic mercury incorporated into cultured ganglia increased abruptly at concentrations higher than 2×10^{-5} M, suggesting that the membrane barrier function of nerve fibers was destroyed by inorganic mercury. In studies using mouse neuroblastoma and glioma cells we found that inorganic mercury at $2\text{-}3\times10^{-5}$ M alters membrane barrier

function and is then incorporated into the cells (Nakada and Imura 1982; Miura and Imura 1987).

As shown in Fig. 6, altered membrane structure was observed in nerve fibers exposed to 5×10^{-5} M inorganic mercury, whereas numerous microtubules were evident in the same fiber. Thus, the disruption of microtubules may not be the primary cause of inorganic mercury-induced depression of nerve fiber growth.

Inorganic mercury severely damaged nerve fibers and depressed fiber growth at concentrations higher than 3×10^{-5} M but signs of recovery were apparent within 24 hours after exposure. The recovery of fiber growth was slightly affected by mercury concentrations. These results indicated the existence of viable nerve cells in the inner part of the ganglion. This also means that mercuric chloride interfered with surface areas directly exposed to the medium.

The patterns of toxic effects on nerve fiber growth and proliferation of nonneuronal cells differed among the metals examined. Methylmercury was the most toxic. The efficiency of metals depressing the growth of the nerve fibers as judged by the complete inhibition at 24 hours exposure was in the order of CH_3Hg^+ (7×10^{-6} M) > $Cd^{2+}=Ag^+$ (10^{-5} M) > Hg^{2+} (3×10^{-5} M) > Cr^{6+} (5×10^{-5} M). Methylmercury seems to damage both nerve fiber growth and nonneuronal cells. In the case of Cd^{2+}, nerve fiber growth is more vulnerable than cell proliferation of nonneuronal cells. In contrast, in the case of Cr^{6+}, nerve fiber growth inhibition was clearly preceded by damage to migrating nonneuronal cells.

Thus, it appears that the modes of toxic actions of metals on dorsal root ganglion cells are different. This culture system seems to be useful for comparing and characterizing the effects of chemicals on neuronal and nonneuronal cells in vitro.

Acknowledgments

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References

1) Abe, T., Haga, T. & Kurokawa, M. (1975) Blockage of axoplasmic transport and depolymerization of reassembled microtubules by methylmercury. *Brain Res.*, **86**, 504–508.
2) Baas, P.W. & Black, M.M. (1990) Individual microtubules in the axon consist of domains that differ in both composition and stability. *J. Cell Biol.*, **111**, 495–509.
3) Baas, P.W. (1997) Microtubules and axonal growth. *Curr. Opin. Cell Biol.*, **9**, 29–36.
4) Bamburg, J.R., Bray, D. & Chapman, K. (1986) Assembly of microtubules at the tip of growing axons. *Nature*, **321**, 788–790.
5) Bodian, D. & Mellors, R.C. (1945) The regenerative cycle of motorneurons. *J. Exp. Med.*, **81**, 469–488.
6) Ganote, C.E., Reimer, K.A. & Jennings, R.B. (1975) Acute mercuric chloride nephrotoxicity. An electron microscopic and metabolic study. *Lab. Invest.*, **31**, 633–647.

7) Imura, N., Miura, K., Inokawa, M. & Nakada, S. (1980) Mechanism of methylmercury cytotoxicity: By biochemical and morphological experiments using cultured cells. *Toxicology*, **17**, 767–771.
8) Kasuya, M. (1972) Effects of inorganic, aryl, alkyl and other mercury compounds on the outgrowth of cells and fibers from dorsal root ganglia in tissue culture. *Toxicol. Appl. Pharmacol.*, **23**, 136–146.
9) Matsumoto, G. & Sakai, H. (1979) Microtubules inside the plasma membrane of squid giant axons and their possible physiological function. *J. Membr. Biol.*, **50**, 1–14.
10) Miura, K., Suzuki, K. & Imura, N. (1978) Effects of methylmercury on mitotic mouse glioma cells. *Environ. Res.*, **17**, 453–471.
11) Miura, K., Nakada, S., Suzuki, K. & Imura, N. (1979) Ultrastructual studies on the cytotoxic effects of mercuric chloride on mouse glioma. *Ecotoxicol. Environ. Safety*, **3**, 352–361.
12) Miura, K., Inokawa, M. & Imura, N. (1984) Effects of methylmercury and some metal ions on microtubule networks in mouse glioma cells and in vitro tubulin polymerization. *Toxicol. Appl. Pharmacol.*, **73**, 218–231.
13) Miura, K. & Imura, N. (1987) Mechanism of methylmercury cytotoxicity. *CRC Crit. Rev.*, **18**, 161–188.
14) Miura, K. & Clarkson, T.W. (1993) Reduced methylmercury accumulation in a methylmercury-resistant pheochromocytoma PC12 cell line. *Toxicol. Appl. Pharmacol.*, **118**, 39–45.
15) Miura, K., Kobayashi, H., Toyoda, H. & Imura, N. (1998) Methylmercury-induced microtubule depolymerization leads to inhibition of tubulin synthesis. *J. Toxicol. Sci.*, **23**, 379–388.
16) Miura, K., Koide, N., Himeno, S., Nakagawa, I. & Imura, N. (1999) The involvement of microtubular disruption in methylmercury-induced apoptosis in neuronal and nonneuronal cell lines. *Toxicol. Appl. Pharmacol.*, **160**, 279–288.
17) Miura, K. (2000) Methylmercury toxicity at cellular levels. *J. Health Science*, **46**, 182–186.
18) Naganuma, A. & Imura, N. (1984) Effect of time intervals of selenium administration after injection of mercuric chloride on toxicity and renal concentration of mercury in mice. *Ind. Health*, **22**, 91–96.
19) Nakada, S., Saito, H. & Imura, N. (1981) Effect of methylmercury and inorganic mercury on the nerve growth factor-induced neurite outgrowh in chick embryonic sensory ganglia. *Toxicol. Lett.*, **8**, 23–28.
20) Nakada, S. & Imura, N. (1982) Uptake of methylmercury and inorganic mercury by mouse glioma and mouse neuroblastoma cells. *Neurotoxicology*, **3**, 249–258.
21) Nakazawa, N., Makino, F. & Okada, S. (1975) Acute effects of mercuric compounds on cultured mammalian cells. *Biochem. Pharmacol.*, **24**, 489–493.
22) Rochlin, M.W., Wickline, K.M. & Bridgman, P.C. (1996) Microtubule stability decreases axon elongation but not axoplasm production. *J. Neurosci.*, **16**, 3236–3246.
23) Sager, P.R., Doherty, R.A. & Olmsted, J.B. (1983) Interaction of methylmercury with microtubules in cultured cells and in vitro. *Exp. Cell Res.*, **146**, 127–137.
24) Sager, P.R. (1988) Selectivity of methylmercury effects on cytoskeleton and mitotic progression in cultured cells. *Toxicol. Appl. Pharmacol.*, **94**, 474–486.
25) Sakai, K. (1975) Effects of methylmercuric chloride on ascites hepatoma AH13 cells, especially on cell growth and nucleic acid sysnthesis. *Kumamoto Med. J.*, **28**, 124–134.
26) Tanaka, E., Ho, T. & Kirschner, M.W. (1995) The role of microtubule dynamics in growth cone motility and axonal growth. *J. Cell Biol.*, **128**, 139–155.
27) Umeda, M., Saito, K., Hirose, K. & Saito, M. (1969) Cytotoxic effect of inorganic, phenyl and alkyl mercuric compounds on HeLa cells. *Jpn. J. Exp. Med.*, **39**, 47–58.

28) Vogel, D.G., Margolis, R.L. & Mottet, N. (1985) The effects of methylmercury binding to microtubules. *Toxicol. Appl. Pharmacol.*, **80**, 473–486.
29) WHO (1976) *Environmental Health Criteria 1: Mercury*. World Health Organization, Geneva.
30) WHO (1990) *Environmental Health Criteria 101: Methylmercury*. World Health Organization, Geneva.
31) WHO (1991) *Environmental Health Criteria 118: Inorganic Mercury*. World Health Organization, Geneva.
32) Yu, W. & Baas, P.W. (1995) The growth of the axon is not dependent upon net microtubule assembly at its distal tip. *J. Neurosci.*, **15**, 6827–6833.