Acute Cytotoxic Effects of Mercuric Compounds in Cultured Astrocytes Prepared from Cerebral Hemisphere and Cerebellum of Newborn Rats

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We investigated acute cytotoxic effects and Hg accumulation after exposure to methylmercury (MeHg) or Hg^{2+} in the presence or absence of serum in cultured astrocytes prepared from the cerebral hemisphere or cerebellum of newborn rats. Dose-related changes in viable cell numbers after exposure to mercuric compounds were not different between astrocytes from both regions under the specified conditions. Accumulation of each compound for 3 h was similar in both astrocytes but that for 24 h became different, especially that of Hg^{2+}. In both astrocytes, susceptibility to the respective compounds was higher in the order of those exposed immediately after, without, and 24 h after changing the serum-containing medium to a serum-free defined medium (SFDM). Accumulation for 3 h was higher in the respective astrocytes exposed to MeHg or Hg^{2+} immediately after being maintained in SFDM than in those exposed 24 h after. These results suggest that accumulation of mercuric compounds up to 3 h strongly correlates with susceptibility, at least when maintained in SFDM. Astrocytic morphology changed to a satellite shape after the medium change to SFDM particularly in cerebellar astrocytes but only a few in cerebral hemisphere astrocytes, and it was reverted to a polygonal shape by MeHg but not Hg^{2+} at 3 μM. The present results suggest that although some properties such as morphological changes and Hg accumulation are different between cerebral hemisphere and cerebellar astrocytes, these differences are not simply reflected by susceptibility to the acute cytotoxicity of mercuric compounds.

Key words astrocyte; methylmercury; inorganic mercury; susceptibility; morphological change

Mercuric compounds such as methylmercury (MeHg) are major hazardous environmental pollutants. MeHg is easily absorbed from the intestine, and transported into the brain across the blood-brain barrier.[1)] It has been demonstrated that MeHg neurotoxicity may be modulated by dysfunction of astrocytes such as disturbing ion homeostasis and the uptake of glutamate [a major excitatory transmitter of the central nervous system (CNS)] in cultures,[2,3)] since glutamate can induce neuronal cell damage.[4—6)] Indeed, a recent study revealed that MK-801, an antagonist of *N*-methyl-D-aspartate receptor, reduced MeHg toxicity, particularly in the cerebral cortex but not in the cerebellum,[7)] suggesting the involvement of glutamate in *in vivo* MeHg neurotoxicity, at least in the cerebral cortex. Not only MeHg but also mercury vapor (Hg^0) absorbed from lung tissue is also taken up into the brain before it is oxidized, and then converted to Hg^{2+}.[8)] Since Hg^{2+} is not easily eliminated, the CNS is critically affected by repeated exposure to Hg^0 vapor.[8)] Interestingly, both MeHg and Hg^{2+} converted from MeHg accumulate preferentially in astrocytes rather than in neurons,[1,9—11)] whereas Hg^{2+} is generally seen in neurons after exposure to mercuric chloride or Hg^0 vapor.[12,13)] Since glutamate uptake in cultured astrocytes is also inhibited by Hg^{2+},[2,14,15)] an astrocyte dysfunction induced by Hg^{2+} may also indirectly contribute MeHg neurotoxicity.

MeHg neurotoxicity has been observed at relatively local regions including granule cells in the cerebellum and neurons in the interstices of the visual cortex.[1)] Although reasons for this are uncertain, it might be possible to reflect brain region-specific susceptibility to mercuric compounds in astrocytes. This assumption might be supported by the facts that there are brain region-dependent differences in glutamate uptake and regulations of its transporters in cultured astrocytes.[16—18)] However, few reports demonstrate the differences in the cytotoxic effects induced by mercuric compounds between the astrocytes from different brain regions.

In the present study, astrocyte cultures prepared from the cerebral hemisphere and cerebellum of newborn rats were exposed to MeHg or Hg^{2+} under several culture conditions, and changes in viable cell numbers and morphology were investigated as indicators of acute toxicity of mercuric compounds. We also examined the relationship between susceptibility and Hg accumulation after exposure to mercuric compounds in these cultured astrocytes.

MATERIALS AND METHODS

Animals Male and female Wistar rats were obtained from CLEA Japan Co. (Osaka, Japan) and housed in cages in ventilated animal rooms at a controlled temperature (23.5±1.5 °C) and a relative humidity (55±10%) under a 12-h light cycle. The animals were maintained on standard laboratory chow and tap water *ad libitum*. Timed-pregnant animals (one male and one female) were mated and housed together in a stainless cage. After a vaginal plug was observed, each pregnant rat was housed in its own plastic cage until birth. The animals received humane care throughout the experiment according to the Guidelines of the National Institute for Environmental Studies (NIES) and those of the National Institute for Minamata Disease (NIMD).

Cell Cultures Astrocyte cultures were prepared from the brains of newborn rats (within 24 h after birth) according to

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the method of McCarthy and de Vellis[19] with minor modifications. The cerebral hemisphere and cerebellum were removed, and the meninges were then carefully dissected off in minimum essential medium (MEM). Each cerebral hemisphere and cerebellum was washed 3 times in Ca^{2+}, Mg^{2+}-free Hanks' balanced salt solution [HBSS (−)], and treated with 0.1% trypsin at 37 °C for 10 min. After enzymatic treatment, the cells were suspended in Basal Medium Eagle's (BME) with Earl's salts supplemented with 15% fetal calf serum (FCS; Invitrogen Co., Carlsbad, CA, U.S.A.), 0.1% L-glutamine, 0.6% D-glucose, antibiotics (Penicillin–Streptomycin; Invitrogen Co.), and an antimycotic (Fungizon; Invitrogen Co.). Cells were then plated into culture flasks (25 cm^2; BD Bioscience; Billerica, MA, U.S.A.) at approximately 8×10^6—10^7 cells/flask. The flasks were incubated at 37 °C in 6% CO_2 in a humidified atmosphere, and the medium was changed every other day. After cells reached confluence, the medium was changed, and the flasks were placed in a CO_2 incubator for a few hours to equilibrate them with CO_2. The flasks were tightly secured with caps, and shaken at 37 °C for 15—18 h. After removing the floating cells, the cultures were vigorously shaken by hand. Then, the medium was removed, and the cultures were rinsed with HBSS (−) 3—4 times. The remained cells on the flasks were collected using 0.25% trypsin, and resuspended with the fresh medium containing 15% FCS. They were then plated at approximately 2.7×10^4 and 1.5×10^5 cells/well on 24-well and 6-well plates (BD Bioscience) for analyses of cell viability and Hg, respectively. When cells were immunocytechemically stained against glial fibrillary acidic protein (GFAP), a specific marker of astrocytes, almost all cells in these cultures were immunoreactive for GFAP (data not shown), according to the method described previously.[20,21]

Treatment with Mercuric Compounds A few days after plating, in some culture plates at 75—85% confluence, mercuric compounds were added without a medium change as described below. In the other plates at 85—95% confluence, the serum-containing medium was changed to a serum-free defined medium (SFDM) consisting of MEM with bovine serum albumin (BSA; 1 mg/ml; Sigma, St. Louis, MO, U.S.A.), bovine apo-transferrin (100 μg/ml; Sigma), insulin (10 μg/ml; Sigma), aprotinin (1 μg/ml; Sigma), thyroxine (T4; 0.1 nM; Sigma), and antibiotics (Penicillin–Streptomycin; Invitrogen Co.). Methylmercuric chloride (Tokyo Chemical Industry Co., Tokyo, Japan) and mercuric chloride (Wako Pure Chemical Industry, Osaka, Japan) were dissolved in ethanol and distilled water, respectively. Without, immediately upon, and 24 h after the medium change to SFDM, a diluted solution of mercuric compounds was added to each well at a final concentration of 10 nM—50 μM. The solvent alone was added as the respective controls.

Cell Viability Analysis Viable cell numbers were assessed by crystal violet staining[22] as previously described.[20,21]

Hg Analysis Astrocytes exposed to 1 μM MeHg or Hg^{2+} for 3 or 24 h under the conditions mentioned above were rinsed 3 times with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline [PBS (−)], and then collected using 0.1% sodium dodecylsulfate (SDS) in PBS (−). Contents of total Hg in the samples were determined by the oxygen combustion-gold amalgamation method[23] using a Rigaku Mercury Analyzer MA-2, MA-1S or SP-3 (Nippon Instruments Co., Tokyo, Japan). Whole protein contents were determined with a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using BSA as a standard.

Statistical Analysis Significant differences between individual means were determined by one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test. Differences were considered significant at $p<0.05$.

RESULTS

Susceptibility to MeHg and Hg^{2+} in Cultured Astrocytes Astrocyte cultures obtained from the cerebral hemisphere and cerebellum were exposed to MeHg or Hg^{2+} in the presence or absence of serum, and changes in the viable cell numbers were determined. Dose-related changes in those numbers 24 h after exposure to each mercuric compound were similar in cerebral hemisphere and cerebellar astrocytes under the specified exposure conditions (Figs. 1A—F). In the presence of serum, viable cell numbers decreased by more than 5 μM of MeHg and by more than 30 μM of Hg^{2+} (Figs. 1A, B). When the exposures were made immediately after changing the serum-containing medium to SFDM, astrocytes from both regions died upon exposure to the respective compounds at the lower doses (more than 3 μM of MeHg, and more than 10 μM of Hg^{2+}) (Figs. 1C, D). Interestingly, both astrocytes exposed to the respective compounds 24 h after the medium change to SFDM were more resistant to them than those exposed immediately after (Figs. 1C—F). The viable

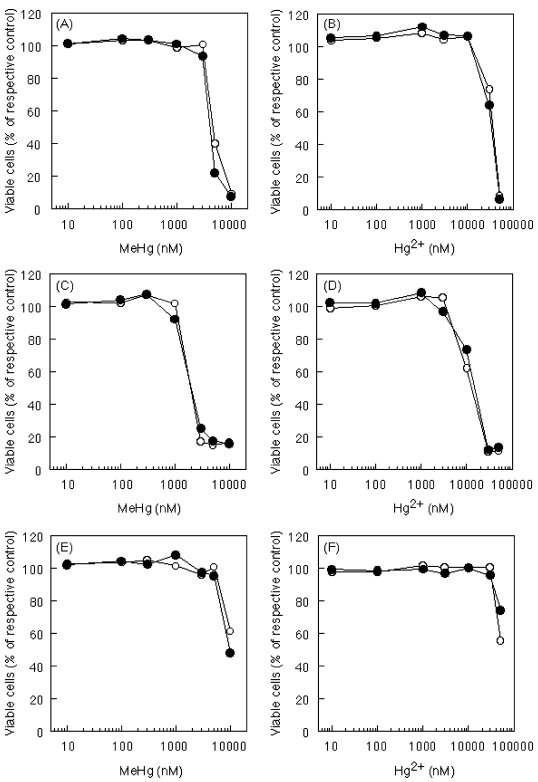

Fig. 1. Dose-Related Changes in Viable Cell Numbers 24 h after Exposure to Mercuric Compounds in Astrocyte Cultures Prepared from Cerebral Hemisphere (○) or Cerebellum (●)

Astrocyte cultures were exposed to either MeHg (A, C, E) or Hg^{2+} (B, D, F) without (A, B), immediately upon (C, D), or 24 h after (E, F) changing the serum-containing medium to SFDM. Values represent the mean obtained from 3 determinations.

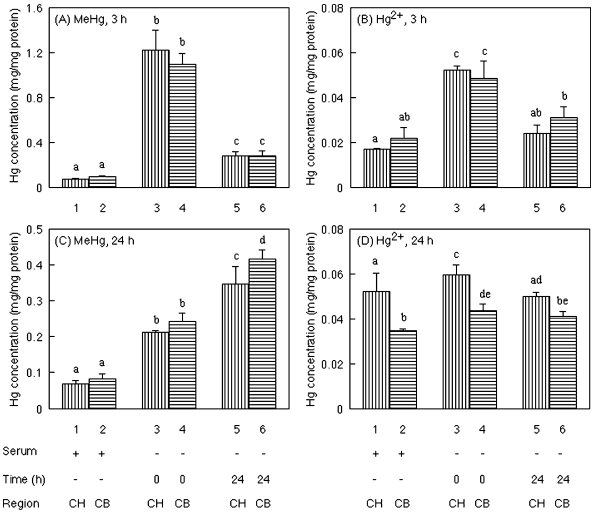

Fig. 2. Hg Accumulation in Cultured Astrocytes Prepared from Cerebral Hemisphere or Cerebellum after Exposure to Mercuric Compounds

Astrocytes prepared from cerebral hemisphere (CH; nos. 1, 3, 5) or cerebellum (CB; nos. 2, 4, 6) were exposed to each of MeHg or Hg^{2+} at a concentration of 1 μM for 3 or 24 h without (nos. 1, 2), immediately upon (nos. 3, 4), or 24 h after (nos. 5, 6) changing the serum-containing medium to SFDM. Values represent the mean±S.D. obtained from 3 determinations. Values with different letters (a—e) are significantly different ($p<0.05$).

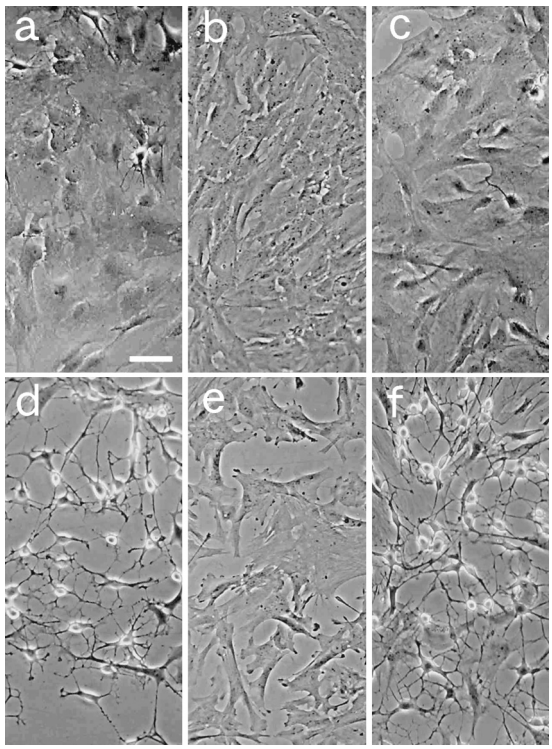

Fig. 3. Influence of Mercuric Compounds on Morphology of Cultured Astrocytes Prepared from Cerebral Hemisphere (a—c) or Cerebellum (d—f)

Astrocytes cultured on 24-well plates were exposed to MeHg or Hg^{2+} for 3 h immediately after changing the serum-containing medium to SFDM, and then fixed with glutaraldehyde. (a, d) Controls, (b, e) 3 μM MeHg, (c, f) 3 μM Hg^{2+}. Bar=50 μm.

cell numbers decreased with exposure to 10 μM MeHg and 50 μM Hg^{2+} in both astrocytes (Figs. 1E, F). After 3-h exposure to the respective mercuric compounds, no differences in the numbers were also observed between astrocytes from both regions under the specified conditions, although doses for decreasing the numbers tend to be higher after 3-h exposure than 24-h exposure (data not shown).

Hg Accumulation in Cultured Astrocytes Figure 2 shows Hg accumulation in cultured astrocytes from the cerebral hemisphere or cerebellum 3 and 24 h after exposure to MeHg or Hg^{2+} at a concentration of 1 μM, at which both mercuric compounds did not decrease viable cell numbers under all conditions examined (Figs. 1A—F). Three hours after exposure to each compound (Figs. 2A, B), Hg concentration was the highest in astrocytes exposed immediately after changing the serum-containing medium to SFDM (nos. 3, 4), although no difference was observed in Hg concentrations between both astrocytes under the specified conditions (Figs. 2A, B). In addition, Hg concentration at 3 h was higher in astrocytes exposed to MeHg 24 h after the medium change (Fig. 2A, nos. 5, 6) than in those exposed in the presence of serum without the medium change (Fig. 2A, nos. 1, 2), although no significant difference was observed between the respective astrocytes exposed to Hg^{2+} without the medium change and those exposed 24 h after (Fig. 2B, nos. 1, 2, 5, 6). Twenty-four hours after exposure to MeHg (Fig. 2C), Hg concentrations in astrocytes from both regions were higher in the following order: those exposed 24 h after (nos. 5, 6), immediately after (nos. 3, 4), and without (nos. 1, 2) the medium change to SFDM. MeHg concentration at 24 h was lower in the cerebral hemisphere than in cerebellar astrocytes only when exposed 24 h after the medium change (Fig. 2C, nos. 5, 6), although no significant region-dependent difference was observed under other conditions (Fig. 2C, nos. 1—4) as were the cases of 3-h exposure (Fig. 2A). In contrast, similar to the 3-h exposure (Fig. 2B), a 24-h exposure to Hg^{2+} led to the highest Hg concentration in the respective astrocytes exposed immediately after the medium change (nos. 3, 4) (Fig. 2D). Interestingly, the Hg concentration was higher in the cerebral hemisphere than in cerebellar astrocytes 24 h after exposure to Hg^{2+} under all conditions examined (Fig. 2D, nos. 1—6).

Morphology in Cultured Astrocytes Astrocytes from both the cerebral hemisphere and cerebellum showed a flat polygonal morphology when maintained in the serum-containing medium (data not shown). When the medium was changed to SFDM, the morphology in most cerebellar astrocytes drastically changed into a process-bearing satellite shape within 3 h (Fig. 3, d) and reverted to a polygonal one within 24 h (data not shown), whereas only a few cerebral hemisphere astrocytes changed their morphology (Fig. 3, a). Surprisingly, most cerebellar astrocytes showed a polygonal shape when exposed for 3 h to MeHg at 3 μM (Fig. 3, e) but not at 1 μM or less (data not shown) immediately after the medium change to SFDM. In contrast, no marked morphological change was observed after exposure to 3 μM Hg^{2+} (Fig. 3, f). Similar to cerebellar astrocytes, there were few cerebral hemisphere astrocytes with a satellite shape upon exposure to 3 μM MeHg, nor was their cell morphology affected at that dosage of Hg^{2+} (Fig. 3, b, c). In other cases, no remarkable changes in astrocytic morphology due to mercuric compounds were observed, except when the viable cell numbers decreased (data not shown).

DISCUSSION

In the present study, there were no region-dependent dif-

ferences in viable cell numbers after exposure to MeHg and Hg^{2+} under the specified conditions (Figs. 1A—F). In contrast, Hg accumulation was similar in cerebral hemisphere and cerebellar astrocytes 3 h after exposure to each mercuric compound (Figs. 2A, B) but became different 24 h after (Figs. 2C, D). It should be noted that Hg accumulation for 3 h was also higher in both astrocytes exposed to the respective mercuric compounds immediately after changing the serum-containing medium to SFDM than in those exposed 24 h after (Figs. 2A, B), as was the case of susceptibility that was higher in the former than in the latter (Figs. 1C—F). In contrast, although accumulation of MeHg for 3 h was higher in the respective astrocytes exposed 24 h after the medium change than in those exposed in the presence of serum (Fig. 2A), susceptibility to MeHg was higher in the latter than in the former (Figs. 1A, E). In addition, susceptibility to Hg^{2+} was also higher in the latter than in the former (Figs. 1B, F), although accumulation of Hg^{2+} for 3 h was similar (Fig. 2B). Accordingly, Hg accumulation up to 3 h might strongly correlate with susceptibility to mercuric compounds, at least in cultured astrocytes maintained in the same serum-free culture medium.

The present results revealed the region-specific changes in morphology of cultured astrocytes, at least after changing the serum-containing medium to SFDM (Fig. 3, a, d). The morphology of cerebellar astrocytes changed from a flat polygonal to a process-bearing satellite shape within 3 h after being maintained in SFDM (Fig. 3, d) and reverted to a polygonal shape within 24 h (data not shown), whereas only a few cerebral hemisphere astrocytes changed their morphology (Fig. 3, a). Since such a morphological change in cerebellar astrocytes was observed even when they were maintained in MEM alone (data not shown), the change might not depend on medium supplements, but rather would be induced by the elimination of serum. In addition to the medium change to SFDM, mercuric compounds could also change the morphology of cultured astrocytes. Particularly in cerebellar astrocytes, exposure to 3 μM MeHg but not 3 μM Hg^{2+} caused them to revert to a polygonal from the satellite shape induced by being maintained in SFDM (Fig. 3, d—f). There are few reports about these temporary and rapid morphological changes from mercuric compounds as well as serum elimination in cultured astrocytes. It has been reported that changes in morphology from a polygonal to a satellite shape in cultured astrocytes are observed when cAMP levels are increased, accompanied with increases in GFAP expression levels,[24,25] and that these changes are also observed during *in vivo* development.[26] Accordingly, our morphological findings might offer the possibility that MeHg inhibits astrocyte development.

The present results suggest that, between cultured astrocytes prepared from the cerebral hemisphere and cerebellum, no region-dependent differences exist in susceptibility to the acute cytotoxicity of MeHg and Hg^{2+}, although properties of astrocytes such as morphological changes after changing the serum-containing medium to SFDM are different. It is also suggested that changes in astrocytic morphology might be a useful marker of the acute cytotoxicity of MeHg, since they are observed before decreasing viable cell numbers induced by MeHg exposure.

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