Effects of Methylmercury and Mercuric Chloride on Differentiation and Cell Viability in PC12 Cells

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The effects of methylmercury (CH₃Hg) or mercuric chloride (HgCl₂) on neurite outgrowth and cell viability were quantified using undifferentiated (unprimed) and differentiated (primed) pheochromocytoma (PC12) cells. In unprimed cells, following 24-h exposure, CH₃Hg significantly decreased NGF-stimulated neurite outgrowth at concentrations of 0.3–3 μM. However, HgCl₂ significantly increased both neurite outgrowth and the number of branch points, a component of neurite outgrowth. In primed PC12 cells, following 24-h exposure, both CH₃Hg and HgCl₂ inhibited NGF-stimulated neurite outgrowth with an EC₅₀ of approximately 0.03 μM; however, there was a difference between CH₃Hg and HgCl₂ effects on the subcomponents of total neurite outgrowth. CH₃Hg significantly decreased both the number of branch points (0.3 μM) and fragment length (0.01 μM), while HgCl₂ only decreased fragment length (0.03 μM). Cell viability was assessed in the same cultures by trypan-blue exclusion. In unprimed cells, the EC₅₀ for cytotoxicity of CH₃Hg in the presence and absence of NGF was 0.21 ± 0.04 and 0.87 ± 0.12 μM, respectively, and for HgCl₂ in the presence and absence of NGF was 8.18 ± 1.52 and 5.02 ± 0.74 μM, respectively. In primed cells, the EC₅₀ for cytotoxicity of CH₃Hg in the presence or absence of NGF was 1.17 ± 0.38 and 0.73 ± 0.14 μM, respectively, and for HgCl₂ in the presence or absence of NGF was 3.96 ± 0.82 and 3.81 ± 0.91 μM, respectively. In the primed PC12 model, cytotoxicity occurred at concentrations that were at least 30-fold higher than the EC₅₀ for neurite outgrowth, suggesting that the mercurial compounds can act selectively on the process of differentiation.

Key Words: neurite outgrowth; developmental neurotoxicity; nerve growth factor (NGF).

Methylmercury (CH₃Hg) is a neurotoxic compound that is ubiquitous in the environment. Much of the human data concerning CH₃Hg neurotoxicity has come from tragic events, which include Minamata Bay and Niigata, Japan (Reuhl and Chang, 1979; Takeuchi et al., 1959) and Iraq (Amin-Zaki et al., 1974; Bakir et al., 1973), where large populations were exposed to high-levels of CH₃Hg. More recently, the neurotoxic effects of low-level CH₃Hg contamination has been studied in the Seychelles Islands (Crump et al., 2000; Davidson et al., 1999; Myers et al., 1995), the Faroe Islands (Grandjean et al., 1998, 1999a; Weihe et al., 1996), New Zealand (Crump et al., 1998), and the Amazon Basin (Dolbec et al., 2000; Grandjean et al., 1999b; Lebel et al., 1998). Following high doses of CH₃Hg, it was evident that CH₃Hg is a potent neurotoxicant and that the developing fetus is more susceptible to CH₃Hg poisoning than adults (Choi et al., 1978; Matsumoto et al., 1965) and that subtle neurobehavioral manifestations, which include decreased motor function and visuospatial performance, were evident following low-level exposure (Dolbec et al., 2000; Grandjean et al., 1998; Myers et al., 1995). Evidence for the susceptibility of the developing organism to the neurotoxic effects of CH₃Hg has also been observed in animal studies using primates (Mottet et al., 1987; Rice, 1989, 1996), cats (Khera, 1973; Khera et al., 1973, 1974), hamsters (Reuhl et al., 1981), mice (Chang, 1977; Chang et al., 1977; Sager et al., 1982, 1984) and rats (Nonaka, 1969; Reuhl and Chang, 1979). Following developmental exposure in both humans and animals, cell loss in the cerebellum and cerebrum, atrophic brains, neuronal degeneration, delayed or abnormal development of the cerebellar granule cell layer, and abnormal cortical migration were prominent in the fetus and infant (Burbacher et al., 1990; Chang, 1977). Interestingly, in humans, the earlier the developmental exposure to CH₃Hg, the more widely distributed the neuropathology in the brain (Chang, 1977). Animal studies using low doses of CH₃Hg have also demonstrated neuropathology similar to that observed in humans exposed to high doses of CH₃Hg (Burbacher et al., 1990). For example, gestational exposure of pregnant rats to 2 mg/kg of CH₃Hg resulted in changes in cell density, cell size, and decreases in the widths of the layers of the posterior neocortex in pups on postnatal days 10 and 21 (Barone et al., 1998), suggesting altered cortical differentiation.

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There are currently a number of mechanisms that have been proposed to underlie the developmental neurotoxicity of CH$_3$Hg, including brain region-specific changes in neurotrophic factor expression (Lärkforss et al., 1991), inhibition of embryonic NCAM conversion to adult NCAM (Lagunowich et al., 1991), disassembly of microtubules (Graff et al., 1997), increased mRNA expression of Gadd45 and Gadd153 (Ou et al., 1997) and decreased expression and/or activity of proteins involved in neurotrophic factor signaling (Barone et al., 1998; Haykal-Coates et al., 1998; Mundy et al., 2000). Because these targets underlie crucial processes in the development of the nervous system (i.e., proliferation, migration, and differentiation), perturbations of these processes by CH$_3$Hg could explain the developmental neurotoxicity observed following in vivo exposure.

In order to examine the effects of CH$_3$Hg on the developing nervous system, a number of studies have used differentiating-cell cultures as a model. In vitro exposure to CH$_3$Hg has been shown to inhibit nerve growth factor (NGF)-induced neurite outgrowth in chick sympathetic and sensory dorsal root-ganglia cultures (Nakada et al., 1981; Söderström et al., 1995). One question that arises from these studies is whether the inhibition of NGF-induced neurite outgrowth is due to a selective effect of CH$_3$Hg on differentiation, or is simply the result of cytotoxicity.

Previous work has not examined the effects of CH$_3$Hg on differentiation and cytotoxicity in a systematic manner. The present study evaluates the developmental neurotoxicity of CH$_3$Hg by examining neurite outgrowth and cell viability in the pheochromocytoma (PC12) cell line, an in vitro model which has been used extensively for the study of neuronal differentiation (reviewed in Fujita et al., 1989). In the presence of NGF, PC12 cells cease to divide and instead differentiate into a neuronal phenotype characteristic of sympathetic neurons. Morphological characteristics indicative of differentiation include an increase in cell size and the extension of neurites (Fujita et al., 1989; Greene and Tischler, 1976). In contrast to previous studies using qualitative assessments of differentiation, the present work examined the effects of CH$_3$Hg on differentiation quantitatively, using a video-based imaging system. This system allowed for semi-automated quantitation of a number of morphologic measures of differentiation including cell size, neurite branching, and total neurite length (Das and Barone, 1999). To directly compare the effects of CH$_3$Hg on differentiation and cytotoxicity, cell viability was determined in the same cultures. The process of differentiation can be divided into at least two phases: neurite initiation and neurite elaboration, which may be subserved by distinct mechanisms (Burstein et al., 1978). Thus, we examined the effects of CH$_3$Hg on PC12 cells that were not differentiated (no previous exposure to NGF; unprimed) to determine effects on neurite initiation, and in PC12 cells that had been exposed previously to NGF (primed) to determine effects on neurite growth. Finally, in order to examine the specificity of CH$_3$Hg, we compared the effects of CH$_3$Hg to inorganic mercury (mercuric chloride; HgCl$_2$). HgCl$_2$ is a toxicant that does not specifically target the developing nervous system, but affects renal development and function (Bartolome et al., 1985; Daston et al., 1983, 1984, 1986; Kavlock et al., 1983).

MATERIALS AND METHODS

Materials. Rat pheochromocytoma cells (PC12 cells) were a gift from Dr. Gordon Guroff (NIH, Bethesda, MD). Human recombinant nerve growth factor-beta (NGF), rat tail collagen type I, HEPES, and sodium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO); Dulbecco’s Modified Eagle Medium (DMEM), Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), horse serum and trypsin blue solution were purchased from Life Technologies (Grand Island, NY). Twenty-five cm$^2$ tissue culture flasks, 75 cm$^2$ tissue culture flasks and twelve well plates were purchased from Corning Costar Corporation (Cambridge, MA). Mercuric Chloride (HgCl$_2$) was purchased from J.T. Baker Chemical Company (Phillipsburg, NJ). Methylmercury chloride (CH$_3$Hg) was purchased from Pfaltz & Bauer (Waterbury, CT).

Cell culture. Experiments were performed on pheochromocytoma cells from passage 10 or 11 (passage number after receipt in our laboratory) to minimize interassay variability. The cells were grown in 25 cm$^2$ tissue culture flasks with complete DMEM medium containing 7.5% heat-inactivated FBS, 7.5% horse serum, 2 mM HEPES and 44 mM sodium bicarbonate. Cultures were maintained according to standard protocols (Greene and Tischler, 1976) at 37°C in a 95% humidified incubator with 5% CO$_2$. A frozen stock of PC12 cells from passage 8 cells were thawed every week and grown for four days. On the fifth day in culture, the cells were split (passage 9) and replated for the unprimed-cell or primed-cell assay.

Neurite differentiation and morphology. Passage 9 PC12 cells (1 × 10$^4$ cells/well) were replated in 12-well collagen-coated tissue culture plates, in complete DMEM/F12 medium containing 10% FBS and 44 mM sodium bicarbonate. After 2 h, this medium was removed and replaced with DMEM/F12 without FBS, in the presence or absence of NGF and in varying concentrations of CH$_3$Hg or HgCl$_2$. In all these in vitro experiments, cells were exposed and examined from either passage 10 (unprimed) or 11 (primed) only in serum-free DMEM/F12 medium.

In unprimed cells (undifferentiated PC12 cells not exposed to NGF), determinations of the effects of CH$_3$Hg and HgCl$_2$ on NGF-stimulated neurite outgrowth were performed during the initiation phase of neurite outgrowth. These PC12 cells were cultured in serum-free medium for 24 h in the presence or absence of NGF (50 ng/ml) and in combination with either CH$_3$Hg or HgCl$_2$ at doses ranging from 0 to 3 μM. After 24 h of exposure, images from cells were digitized and saved for later analysis to quantify neurite outgrowth (see details below).

In addition to examination of the effects of CH$_3$Hg and HgCl$_2$ on neurite outgrowth, a primed-cell assay, modified from protocols previously published (Greene and Tischler, 1976; Shaughnessy and Barone, 1997), was utilized to examine the acute effects of toxicants on NGF-induced neurite outgrowth. Primed PC12 cells are cells that have been exposed to 50 ng/ml of NGF for 7 days. The cells were cultured for one week in serum-free DMEM/F12 medium and fed with fresh NGF (50 ng/ml) on days 3, 5, and 7 following the plating of passage 10. On day 8, primed cells were harvested, washed with NGF-free complete DMEM/F12 medium and centrifuged at 500 rpm for 5 min at 4°C. The collected cells were finally replated (1 × 10$^5$ cells/well) in twelve-well collagen-coated tissue culture plates. Fifty ng/ml of NGF, with or without various concentrations of mercury compounds in DMEM/F12 media, was added. Primed PC12 cells were incubated for 24 h at which time neurite outgrowth was measured.

After 24 h of incubation, images of the PC12 cells were captured with phase contrast video microscopy (nominal 200× magnification) and digitized with a
MTI-DAGE81 high-resolution black and white camera interfaced with a personal computer. Imaging software (Presage, Advanced Imaging Concepts, Inc., Princeton, NJ) was used to quantify the means of cell size and neurite outgrowth and constituent measures of neurite outgrowth (branch points per cell, fragments per cell, average fragment length per cell, and total neurite outgrowth per cell). This was performed by defining the phase-bright perimeter of the cell bodies based upon threshold values and subtracting that from subsequent images of the total neurite network. The image of the total neurite network was skeletonized to one pixel in width and the total length of neurite outgrowth calculated per cell. The point at which any fibers crossed or branched was determined from this skeletonized image and recorded as the number of branch points. These branch points were subtracted from the image of total neurite outgrowth to derive fiber fragments that were uniquely labeled to determine both number and length of fiber fragments. These measures of neurite outgrowth were quantified for cell clumps ranging in number from 3 to 9 cells. The number of cells/clump was counterbalanced across each experimental condition such that no experimental condition was skewed with only small- or large-cell clumps.

Cytotoxicity assays. Following 24-h exposure to CH₃Hg or HgCl₂, and assessment of neurite outgrowth, cytotoxicity was determined for similar concentration-response assessments by the trypan blue-exclusion method. Cells were stained by adding 10 μL of trypan blue (1:100 dilution of 0.4%) into each well and allowed to distribute throughout the well for 5 min at 37°C. Cells with disturbed plasma membrane permeability stained blue, whereas undamaged (viable) cells appeared translucent. Both attached and detached cells were measured for trypan blue staining. Cells were counted with an IMT-2 inverted light microscope at 10× magnification with a mean total cell count of 50 cells per field. Viability results were expressed as a percent of the number of total cells.

Exposure. CH₃Hg and HgCl₂ were each dissolved in sterile, distilled water at a stock concentration of 10 mM. Concentrations of 1–1000 μM were prepared by serial dilutions of the stock mercurial solutions. Cells were exposed to either of the mercury solutions, which were added in a volume of 10 μL (1:100 dilution) per well, and exposure lasted for 24 h. Control wells were dosed with an equivalent volume (10 μL) of the vehicle (sterile, distilled water), which did not cause any adverse effects on neurite outgrowth.

Statistical analysis. Neurite outgrowth and cytotoxicity were examined using a split-plot design with subsampling. The main plots were the plates, subplots were the wells, and subsampling occurred with the sampling of 2 images per well. Each well was considered the minimum unit of measure (n) and subsampling of a well was considered a replicate and averaged. Each experiment had at least an “n” of 3 data points per condition and analysis was performed on data compiled from 3 experiments, resulting in at least an n of 9 per condition. The main factors included in the global analysis of variance (ANOVA) were assay condition (unprimed versus primed), acute presence or absence of NGF, concentration of mercury species, and species of mercury (CH₃Hg versus HgCl₂). The endpoints included in the ANOVA were total neurite outgrowth/cell, branch points/cell, fiber number/cell, fiber length/cell, cell size, and cell viability. The percent viability data was transformed with an arc sin transformation. Following determination of significant interactions between each experimental factor and step-down ANOVAs were performed on each experimental factor and differences between the group means were compared using Tukey’s Studentized Range Test. Statistically significant differences are reported when p ≤ 0.05. For simplicity’s sake, the focus of the description of the experimental results was based on the step-down ANOVA and post-hoc comparisons of group means. EC₅₀’s for neurite outgrowth and cytotoxicity were determined from concentration-response curves for individual experiments using non-linear regression (GraphPad Software, Inc., San Diego, CA) followed by ANOVA of the respective EC₅₀ determined from each experimental condition.

RESULTS

Characterization of the PC12 Cell Model

Initially, a quantitative assessment of morphological measures of differentiation was determined by examining cell-body size, number of branch points, fragment length, and total neurite outgrowth per cell using an image-analysis program. Unprimed PC12 cells were plated at a low density (1 × 10⁴ cells/ml) which allowed for the measurement of total neurite outgrowth of individual groups of cells (groups of 3–9 cells) before the cell culture becomes confluent (Das and Barone, 1999; Shaughnessy and Barone, 1997). During this differentiation period (7 days exposure to 50 ng/ml NGF), total neurite outgrowth per cell was measured every 24 h. Consistent with observations in previous studies on NGF-induced differentiation (Das and Barone, 1999; Greene and Tischler, 1976; Shaughnessy and Barone, 1997), there was a time-dependent increase in neurite outgrowth, number of branch points, fragment length, and cell size. After 7 days of exposure to NGF, a primed-cell condition is obtained where cells are in the same phase of the cell cycle and a more differentiated state. Upon harvest and replating of these cells, neurites are removed. The time course of new neurite elaboration following replating of primed PC12 cells was determined to establish the progression of neurite outgrowth during an acute exposure period. Total neurite outgrowth, cell body area, branch number, fragment number and fragment length per cell was measured at 0, 3, 6, 12 or 24 h after exposure to 50 ng/ml of NGF. A time-dependent increase in total neurite outgrowth was observed, with a measurable increase in neurite outgrowth occurring as early as 6 h after exposure to NGF, a time when normally no neurite outgrowth is present in unprimed cells. The mean cell size, number of branch points, and fragment lengths also increased in a time-dependent fashion in primed PC12 cells.

Effects of CH₃Hg and HgCl₂ on the Differentiation of Unprimed PC12 Cells

Unprimed cells, which are naïve to NGF and had not differentiated into a neuronal phenotype, were used to examine the effects of CH₃Hg and HgCl₂ on the initiation of neurites in the presence or absence of NGF. Neurite outgrowth in unprimed cells in the first 24-h period is limited. Analysis of total neurite outgrowth per cell, measured at 24 h in the presence or absence of both NGF and CH₃Hg, indicated that there was an effect of acute NGF exposure and an effect of the concentration of CH₃Hg, but no interaction of these 2 exposures. Post-hoc analysis of the data showed that NGF increased neurite outgrowth, and that CH₃Hg produced a small but significant inhibition of neurite outgrowth at high concentrations (0.3–3 μM, Fig. 1A). Thus, CH₃Hg appears to inhibit the early growth (initiation) of neurites at higher concentrations. For other characteristics of neurite outgrowth including number of branch points, there was a main effect of NGF but no effect of
**CH₃Hg** (Fig. 1C). Neither NGF nor CH₃Hg affected fragment length/cell in unprimed cells (Fig. 1E). As mentioned above, NGF increased cell size. CH₃Hg significantly decreased cell size at concentrations of 0.3 to 3 μM both in the presence and absence of NGF (Fig. 1G).

Examination of the total neurite outgrowth per cell in unprimed cells at 24 h, in the presence of absence of NGF and HgCl₂ indicated that there was an interaction between NGF exposure and HgCl₂. In the presence of NGF, HgCl₂ increased neurite outgrowth and *post hoc* analysis revealed that the effect was significant at 0.1 to 3 μM. In the absence of NGF, a significant increase in neurite outgrowth was observed at 3 μM of HgCl₂ (Figs. 1B, 2A–2D). HgCl₂ also increased the number of branch points (Fig. 1D). Neither NGF nor HgCl₂ affected fragment length/cell in unprimed cells (Fig. 1F). Analysis of cell size revealed that NGF increased cell size while HgCl₂ had no effect, and there was no interaction (Fig. 1H).

**Effects of CH₃Hg and HgCl₂ on the Differentiation of Primed PC12 Cells**

In order to assess the effects of CH₃Hg and HgCl₂ on NGF-induced neurite elongation, we used PC12 cells that had been previously exposed to NGF prior to replating (primed).
Acute exposure of primed PC12 cells to NGF resulted in approximately a 20-fold increase in neurite outgrowth over a 24-h period, which is in contrast to the 7 days it takes unprimed cells to reach this level of elaboration. Analysis of results for total neurite outgrowth per cell in primed cells revealed an interaction NGF and CH₃Hg exposure (Fig. 3A). Examination of the data indicates that NGF increased neurite outgrowth dramatically in primed cells, and that CH₃Hg inhibited NGF-stimulated neurite outgrowth in a concentration-dependent fashion (Figs. 3A, 4A, 4C, 4E) with a significant decrease at concentrations ranging from 0.01 to 3 μM. The EC₅₀ for the inhibition of neurite outgrowth by CH₃Hg was 0.033 ± 0.009 μM. In the absence of NGF, there was very little neurite outgrowth, and no significant effect of CH₃Hg (Fig. 3A). There was also an interaction of NGF and CH₃Hg exposure on the number of branch points (Fig. 3C). In the absence of NGF, there were fewer branch points than in the presence of NGF. CH₃Hg caused a concentration-dependent decrease in branch points in both the presence and absence of NGF. There was an interaction of NGF and CH₃Hg exposure on fragment length/cell in primed cells with CH₃Hg, causing a concentration-dependent decrease in fragment length in the presence of NGF (Fig. 3E). Analysis of cell size revealed an effect of NGF and CH₃Hg, but no interaction between the 2 treatments (Fig. 3G). Thus, overall NGF increased cell size, while CH₃Hg decreased cell size at the higher end of the concentration response curve (0.1–3 μM) in both the presence and absence of NGF.

Analysis of the effects of HgCl₂ on total neurite outgrowth in primed cells showed similar results to those obtained with CH₃Hg. There was a significant interaction of NGF with HgCl₂ exposure (Fig. 3B). NGF increased neurite outgrowth in primed cells, and HgCl₂ inhibited NGF-stimulated neurite outgrowth in a concentration-dependent fashion (Figs. 3B, 4B, 4D, 4F) with significant decreases at concentrations of 0.03–3 μM. The EC₅₀ for the inhibition of neurite outgrowth by HgCl₂ was 0.026 ± 0.006 μM. In the absence of NGF, there was little neurite outgrowth, and no effect of HgCl₂ (Fig. 3B). For number of branch points, there was a significant increase produced by NGF exposure, which was not altered by HgCl₂, unlike CH₃Hg (Fig. 3D). There was an interaction of NGF with
HgCl₂ for fragment length/cell in primed cells (Fig. 3F), with HgCl₂ causing a concentration-dependent decrease in fragment length only in the presence of NGF. Analysis of cell size revealed an effect of NGF and HgCl₂ but no interaction between these two treatments (Fig. 3H). NGF increased cell size, and post hoc analysis revealed that HgCl₂ decreased cell size only at 3 μM, and in both the presence and absence of NGF.

Effects of CH₃Hg and HgCl₂ on Cell Viability

To determine the effects of mercury compounds on cell survival, unprimed PC12 cells were exposed for 24 h to various concentrations of either CH₃Hg or HgCl₂ in the presence or absence of NGF, as described above, and cell viability was examined using trypan-blue exclusion. CH₃Hg exposure resulted in a concentration-dependent decrease in cell viability (Fig. 5A) with a significant interaction of NGF and CH₃Hg. The EC₅₀ for cytotoxicity was lower in the absence of NGF (EC₅₀ = 0.21 ± 0.04 μM) than in the presence of NGF (EC₅₀ = 0.87 ± 0.12 μM) (Table 1). HgCl₂ produced a concentration-dependent decrease in cell viability (Fig. 5B), and statistical analysis revealed a significant effect of HgCl₂ and no interaction with NGF. EC₅₀ values of 5.02 ± 0.74 and 8.18 ± 1.52 μM were obtained in the presence or absence of NGF, respectively (Table 1). In total, the cell viability data
indicate that CH₃Hg is more cytotoxic than HgCl₂, and that cytotoxicity was greatest following CH₃Hg exposure in the absence of NGF (Table 1).

The results of the trypan-blue exclusion cell viability assay in primed PC12 cells revealed an interaction of exposure to NGF and CH₃Hg. There was a concentration-dependent decrease in cell viability (Fig. 5C). EC₅₀ values of 1.17 ± 0.38 and 0.73 ± 0.14 μM were obtained in the presence or absence of NGF, respectively (Table 1). Analysis of the concentration-response curves for HgCl₂ revealed a significant effect of HgCl₂ only. Regardless of NGF exposure, HgCl₂ significantly affected cell viability above 0.3 μM in primed PC12 cells.
(Figs. 5B, 5D). EC_{50} values of 3.91 ± 0.91 μM in the presence of NGF and 3.96 ± 0.82 μM in the absence of NGF were obtained (Table 1). As observed with unprimed cells, CH_3 Hg is more cytotoxic than HgCl_2 in primed cells and CH_3 Hg-induced cytotoxicity was greatest in the absence of NGF (Table 1).

**DISCUSSION**

In the present study, we used an in vitro culture system as a model to examine the effects of CH_3 Hg and HgCl_2 on neurotrophin-mediated neuronal differentiation and survival. The differentiation of PC12 cells was examined by measuring a number of endpoints of neurite outgrowth with a video-based imaging system, which allowed for semi-automated quantitation of differentiation and cell viability in the same cultures. The results indicated that decreased neurite outgrowth was a more sensitive index of toxicity for both mercury species than overt cytotoxicity.

The PC12 cell line, derived from a rat adrenal medullary pheochromocytoma tumor, has been used extensively as a model for investigating biomolecular events involved in neuronal differentiation. In the presence of NGF, proliferating

**TABLE 1**

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<th>CH_3 Hg</th>
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<tr>
<td>Unprimed</td>
<td>0.32 ± 0.04 μM</td>
<td>8.18 ± 1.52 μM</td>
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<tr>
<td>Primed</td>
<td>0.73 ± 0.14 μM</td>
<td>3.95 ± 0.82 μM</td>
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*Note.* EC_{50} values for cytotoxicity were determined from concentration-response curves for individual experiments using non-linear regression followed by ANOVA of respective EC_{50}. Data are mean ± SE and representative of at least 6 independent measures.
PC12 cells cease to divide and differentiate into a neuronal phenotype, which includes the elaboration of neurites (Das and Barone, 1999; Greene and Tischler, 1976; Shaughnessy and Barone, 1997). In unprimed PC12 cells, following stimulation with NGF, total neurite outgrowth increased in a time-dependent manner. Other measures of differentiation, such as cell-body area, number of branches, and fragment lengths, also increased in a time-dependent manner. These measures of differentiation were used to determine the effects of mercury compounds on the initial events of differentiation. The increase in NGF-stimulated total neurite outgrowth in unprimed PC12 cells was robust only after 48 h. In contrast, the primed-cell system provided the ability to quantify the acute effects of mercury compounds on neurite outgrowth in a fully differentiated cell.

**Neurite Outgrowth in Unprimed Cells**

Evaluation of neurite outgrowth in unprimed PC12 cells provides a measure of early events in nervous system differentiation after neurotrophic-factor stimulation. The lack of robust effects of 24-h exposure to CH$_3$Hg on neurite outgrowth in unprimed PC12 cells suggests that CH$_3$Hg does not selectively alter early events of differentiation. In the presence of NGF, there was a decrease in total neurite outgrowth at the higher doses of CH$_3$Hg.

In contrast to the effects of CH$_3$Hg on unprimed PC12 cells, there was an increase or induction of neurite outgrowth following HgCl$_2$ exposure, principally in the presence of NGF. At concentrations between 0.1 and 3 μM, there was an increase in total neurite outgrowth in the presence of NGF and an increase at 3 μM in the absence of NGF. This HgCl$_2$-induced increase in total neurite outgrowth was due to an increase in the number of branch points and fiber branches (data not shown) with no significant effect on fragment length. These data suggest that HgCl$_2$ was increasing initiation of neurites versus having an effect on neurite elongation. Similar findings were observed previously using this neurite outgrowth assay after exposure to lead acetate, which also stimulated neurite initiation at low doses (Crumpton et al., 2000). This increase in initiation of neurite outgrowth may be associated with the ability of HgCl$_2$ to stimulate basal diacylglycerol production and activate protein kinase C (Nicotera et al., 1992). During NGF-induced differentiation, there is an increase in calcium flux (Schubert, 1978) and cytosolic calcium concentrations (Pandiella-Alonso et al., 1986). HgCl$_2$ has been shown to decrease intracellular calcium, a process that could be prevented by pretreatment with calcium entry blockers (Rossi et al., 1991). The HgCl$_2$-induced increase in intracellular calcium concentration, along with the increase induced by NGF, may stimulate proteins including PLC-γ (Nicotera et al., 1992; Vignes et al., 1993) which could account for this increase in neurite outgrowth.

**Neurite Outgrowth in Primed Cells**

The primed PC12 cell assay may be considered an *in vitro* approximation of dendritic elaboration following repeated stimulation with neurotrophins *in vivo*. In the present model, total neurite outgrowth is analogous to dendritic elaboration, while the components of neurite outgrowth (number of branch points and fragment length) may be counterparts to the number of branches and branch length *in vivo*. In the primed PC12 cell assay, exposure of cells to either CH$_3$Hg or HgCl$_2$ inhibited NGF-stimulated neurite outgrowth in a concentration-dependent fashion. Significant inhibition was observed at approximately 0.03 μM for both CH$_3$Hg and HgCl$_2$, a concentration that did not result in cytotoxicity (approximately 90% cell viability). The EC$_{50}$’s for the inhibition of neurite outgrowth by either CH$_3$Hg or HgCl$_2$ were approximately 35–150 times lower than the EC$_{50}$’s for cytotoxicity. This suggests that inhibition of neurite outgrowth is a sensitive measure of toxicity, and is not a consequence of general toxicity of mercury compounds to PC12 cells. The current findings of mercury-induced inhibition of neurite outgrowth is consistent with previous findings in other test systems (Abdulla et al., 1995; Nakada et al., 1981; Pendergass et al., 1997; Söderström and Ebendal, 1995; Windebank, 1986). However, these investigations did not attempt to address the question of whether mercury preferentially affects differentiation versus overt cytotoxicity. Using other models to examine neurite outgrowth, significant differences in the potency of the 2 mercury compounds were observed. In chick embryonic sensory ganglia, the inhibitory effect of CH$_3$Hg (EC$_{50} = 2$ μM) on neurite outgrowth was about 25 times more potent than HgCl$_2$ (EC$_{50} = 50$ μM; Nakada et al., 1981). In contrast, there was no significant difference between the EC$_{50}$ of CH$_3$Hg and HgCl$_2$ on total neurite outgrowth per cell in our primed cell system. However, differences were noted between the effects of the 2 forms of mercury on the components of neurite outgrowth (i.e., number of branch points per cell and fragment length per cell). CH$_3$Hg caused significant decreases in the number of branch points per cell at 0.3 μM while HgCl$_2$ did not have any significant effects. Also, CH$_3$Hg significantly decreased fragment length per cell at a concentration of 0.01 μM; HgCl$_2$ decreased fragment length per cell at 0.03 μM. Analogous effects of CH$_3$Hg on differentiation have been observed *in vivo*. Early postnatal exposure of mice to CH$_3$Hg has been shown to diminish dendritic elaboration in Purkinje cells (Choi et al., 1981) as well as decreased laminar widths of the neocortex in rats prenatally exposed to CH$_3$Hg (Barone et al., 1998). From these studies, it can be hypothesized that CH$_3$Hg may interfere with trophic factor-induced elaboration of neuronal dendrites *in vivo*.

Although not examined in the present study, there are several possible mechanisms by which mercury compounds could alter NGF-induced neurite outgrowth. One possible mechanism is that the mercury compounds are disrupting neurotro-
phin signaling by directly affecting proteins of the neurotrophin signal-transduction cascade. Inhibition of neurotrophin signaling has been shown to block differentiation and neurite outgrowth (Altin et al., 1992; Coleman and Wooten, 1994; Cowley et al., 1994; Obermeier et al., 1994; Pang et al., 1995; Tsukada et al., 1994; Stephens et al., 1994). Since many of the proteins in the neurotrophin-signaling cascade contain significant amounts of sulphydryl groups, they may be targets for these mercury compounds. This alteration in neurotrophin signaling may then lead to a disruption in differentiation. Previously, we have shown that gestational exposure to methylmercury affects a number of steps in the neurotrophin signal-transduction cascade, including a decrease in the neurotrophin receptor, trk, (Barone et al., 1998), alterations in PKC isoform expression and activity (Haykal-Coates et al., 1998) and neurotrophin- and carbachol-stimulated phosphatidylinositol hydrolysis (Mundy et al., 2000) in a regionally- and temporally-specific manner.

Cell Viability

While differences in the effects of exposure to species of mercury were evident when examining neurite outgrowth in unprimed cells, the differential effect of the mercury species was even more evident when we examined cell viability. In unprimed cells, CH₃Hg was approximately 6 times more toxic than HgCl₂ in the presence of NGF, and 40 times more toxic than HgCl₂ in the absence of NGF. Similar results were obtained in the primed cells, with CH₃Hg being more toxic than HgCl₂ in both the presence and absence of NGF. CH₃Hg is probably a more potent toxicant than HgCl₂, because HgCl₂ is less efficient at penetrating the plasma membrane than CH₃Hg (Lakowicz and Anderson, 1980; Nakada et al., 1982).

The effects of CH₃Hg were also dependent on the condition of the cells. In the absence of NGF, CH₃Hg was more cytotoxic in unprimed PC12 cells compared to primed PC12 cells (0.21 and 0.73 μM, respectively). This pattern was similar to that observed by Kunimoto et al. (1992), except that EC₅₀ values of 1.3 and 4.8 μM were obtained in unprimed and primed PC12 cells, respectively. In that study, the EC₅₀’s were approximately 6 times higher than in the present study. The increased potency of CH₃Hg in this study as compared to previous work could be due to differences in cell culturing conditions. Kunimoto et al. (1992) cultured their PC12 cells in medium that contained 5% horse serum and 5% newborn calf serum, which is in contrast to serum-free culture medium used in this study. This differential sensitivity of primed versus unprimed cells might result from state-dependent differences that relate to the degree of differentiation and the history of exposure to NGF. For example, certain enzyme systems are induced in PC12 cells by NGF, including catalase and glutathione peroxidase (Sampath et al., 1994), which are responsible for decreased sensitivity of primed PC12 cells. NGF has also been shown to protect against apoptosis following exposure to various compounds (Kamata et al., 1996; Nakajima et al., 1994; Satoh et al., 1996; Spear et al., 1997). Thus, NGF can provide protection against cytotoxicity in PC12 cells.

Acute exposure to NGF also affected the sensitivity of PC12 cells to CH₃Hg-induced cytotoxicity, since PC12 cells that did not have any NGF during the 24-h exposure period were more sensitive than PC12 cells exposed to NGF. Again, this may be attributed to NGF’s ability to promote survival as well as regulate and modulate differentiation in the PC12 cells (Jackson et al., 1990) and some investigators have suggested that NGF acts to help stabilize the reorganization of cytoskeletal elements of cells (Heidemann et al., 1985).

At concentrations which produced cytotoxicity (0.3 to 3 μM), CH₃Hg exposure resulted in a decrease in cell-body area, a morphological characteristic that would suggest apoptosis. Apoptosis has also been observed in vitro in organotypic cultures of cerebellar slices (Kunimoto and Suzuki, 1997), cerebellar granule cell cultures (Bulleit et al., 1998) and in vivo (Nagashima et al., 1996) following exposure to high concentrations of CH₃Hg (≥3 μM). Future experiments could determine the commitment pathways by which PC12 cells undergo CH₃Hg-induced cell death. Interestingly, the effects of HgCl₂ on cell viability in the presence or absence of NGF were not significantly different, suggesting that NGF exposure and signaling may not play a significant role in the commitment to cell death produced by HgCl₂.

Summary

Data from the present study indicated that significant morphological changes occurred in PC12 cells following acute exposures at relatively low concentrations to either CH₃Hg or HgCl₂. These changes in total neurite outgrowth occurred in a concentration range of 0.01–0.1 μM (0.002–0.02 ppm), which was significantly lower than the concentration range that resulted in overt cytotoxicity (1–10 μM; 0.25–2.5 ppm). Much of the description of neuropathological effects in humans following developmental CH₃Hg poisoning has come from high-level exposures (12–20 ppm brain concentrations) but morphological changes occurred at much lower brain concentrations (<3 ppm) in animal models (Burbacher et al., 1990; Khara et al., 1974; Nonaka, 1969). In both humans and animal models, the morphological changes observed included decreases in brain size, loss of neurons and myelin in the cerebellum and cortex, and neurobehavioral alterations (Burbacher et al., 1990). Because of the obvious differences that exist between in vivo and in vitro exposures, it is often difficult to interpret the potential in vivo significance of an in vitro result, particularly with mercury species that are highly reactive compounds. However the present results provide a characterization of the concentration response for differentiation and cytotoxicity that may provide some mechanistic insight to the effects observed previously following low-level exposures to mercury during critical windows of mammalian neural development. In addi-
tion, the results from this test system provide a foundation for future studies examining the role of mercury-induced alterations in cell-signaling pathways that regulate differentiation and apoptosis.

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