Assessment of Potential Neurotoxic Actions of Organoarsenic Compounds Using Human Neuroblastoma NB-1 Cells and Rat Cerebellar Neurons in Primary Culture

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The potential neurotoxic effects of diphenylarsinic acid (DPAA) was investigated using two *in vitro* assay systems, since DPAA could be formed from phenylarsenic compounds as chemical warfare agents in the environment and pose potential health risks to the population using ground water contaminated with DPAA as a source for drinking water. DPAA showed inhibitory effects on the neurite extension in human neuroblastoma NB-1 cells similar to that of methylmercury, although it required a several hundred times higher dose than that of methylmercury. In addition, DPAA affected the expression and localization of 440-kDa ankyrin$_B$, a neuron-specific marker protein, in rat cerebellar neurons in primary culture. These results suggest that DPAA may have weak neurotoxic actions similar to those of methylmercury, even though it requires a 1000 times higher dose than methylmercury.

Key words —— diphenylarsinic acid, neurotoxicity, human neuroblastoma NB-1 cells, neurite extension, rat cerebellar neuron, 440-kD ankyrin$_B$

INTRODUCTION

Phenylarsenic compounds as chemical warfare agents were produced in large amounts during World Wars I and II. After World War II, the production sites and filling plants were destroyed and part of the chemical warfare agents were deposited in the production sites and filling plants.[1] Residues of these chemical warfare agents are still present and contaminate soil and ground water. In ground water, soils, and sediments the phenylarsenic compounds can be metabolized *via* hydrolysis and oxidation, resulting in the formation of diphenylarsinic acid (DPAA) and other phenylarsenic compounds.[2,3] These chemicals are likely to pose potential health risks to the population using such ground water as a source for drinking water. It is therefore important to assess their potential adverse effects on human health, especially on the developing nervous system, as suggested by congenital Minamata disease cases.[4,5]

Recently, we developed a new testing method for the assessment of hazardous environmental chemicals using human neuroblastoma NB-1 cells. This method is based on the image analysis of the neurite extension in NB-1 cells: the length of the extended neurites was determined using image analysis software. By screening 255 chemicals including methylmercury, an extremely hazardous environmental pollutant known to be a cause of Minamata disease, and endocrine-disrupting chemicals with this system, it was found that methylmercury and several chemicals inhibited neurite extension, while cadmium chloride, phthalates, and many other chemicals promoted it.[6–8]

Furthermore, we reported that the expression and localization of 440-kDa ankyrin$_B$, a neuron-specific protein confined to the unmyelinated axon,[9,10] in rat cerebellar neurons in primary culture could be one of the useful indicators for the early stage of neuronal degeneration caused by methylmercury.[11,12]

In the present study, we investigated the potential neurotoxic effects of DPAA based on the neurite extension in NB-1 cells and the expression of

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440-kDa ankyrin$_B$ in rat cerebellar neurons in primary culture. The results obtained indicate that DPAA may have weak neurotoxic actions similar to those of methylmercury, even though it a requires 1000 times higher dose than methylmercury.

MATERIALS AND METHODS

Chemicals —— DPAA was synthesized at the National Institute for Environmental Studies. Dimethylarsinic acid (DMAA) and methylmercury chloride were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

Cell Culture —— Human neuroblastoma NB-1 cells obtained from the Japanese Cancer Research Resources Bank were grown and stored as described previously.[6–8] All tissue culture media and supplements were from GIBCO BRL/Invitrogen (Carlsbad, U.S.A.). For the exposure of NB-1 cells to chemicals, the cells from frozen stock were directly plated in 96-well plates at a density of 5×10^3 cells/well, precultured for 2 days, and further cultured in the presence of various concentrations of chemicals for 2 days.

Cerebellar cells were prepared from neonatal Jcl-Wistar rats within 24 hr after birth, plated onto poly-lysine-coated dishes or glass coverslips, and maintained in serum-free medium as described previously.[13]

Assays for Cell Viability and Neurite Extension of NB-1 Cells —— The viable cell number of the NB-1 cells in culture was estimated by crystal violet staining as described previously.[13] Fixed, stained, and dried cells in 96-well plates were photographed under a microscope equipped with a digital camera (Polaroid PDMC II). The digital images obtained were then analyzed using image analysis software (NeuroZoom, Scripps Institute and Mount Sinai School of Medicine) by counting the cell number and total neurite length in the image field. The degree of neurite extension is expressed as the total length of neurites in micrometers/cell in randomly chosen phase-contrast microscope fields.[8]

Immunocytochemical Procedures —— Cells grown on coverslips coated with poly-lysine were fixed and stained with double-label immunofluorescence using anti-440 kDa ankyrin$_B$ antibody and anti-glial fibrillary acidic protein (GFAP) antibody as described previously.[12] Cellular nuclei were visualized by staining with Hoechst33258 as described.[14]

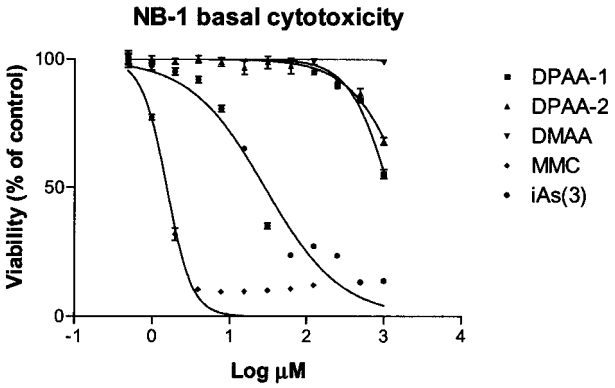

Fig. 1. Effects of Arsenic Compounds on the Viability of NB-1 Cells

NB-1 cells were exposed to DPAA, DMAA, sodium arsenite [iAs(3)], or methylmercury chloride (MMC) for 48 hr. Their viable cell numbers were estimated by crystal violet staining and expressed as percentages of the control value. Error bars indicate standard deviations of quadruplicate assays.

Stained samples were observed and photographed under a confocal laser fluorescent microscope system (LSM510, Carl Zeiss Co., Ltd., Jena, Germany).

RESULTS AND DISCUSSION

Effect of DPAA on the Viable Cell Number and Morphology of NB-1 Cells

When human neuroblastoma NB-1 cells were exposed to DPAA at 0–1000 μM for 2 days, the viable cell number of NB-1 cells estimated by crystal violet staining was decreased dose dependently with an IC_{50} of *ca.* 1.6 mM (Fig. 1). However, the cytotoxic effect was much weaker than those of sodium arsenite (IC_{50}: *ca.* 30 μM) or methylmercury chloride (IC_{50}: 1.5 μM). DMAA, another organoarsenic compound, showed no cytotoxic effect on NB-1 cells up to 1000 μM. These results indicate that DPAA is 1000 times less cytotoxic to NB-1 cells than methylmercury.

Since it was shown that methylmercury at sublethal concentration inhibits the neurite extension from NB-1 cells,[8,15] the effects of organoarsenic compounds on neurite extension were examined. The degree of neurite extension was measured using a computer image analysis system and expressed as percentage of the control. The length of extended neurites from NB-1 cells was decreased significantly in the presence of DPAA 250 μM, while viable cell number was not affected (Figs. 2 and 3). DMAA at the same concentration showed no effect on the neu-

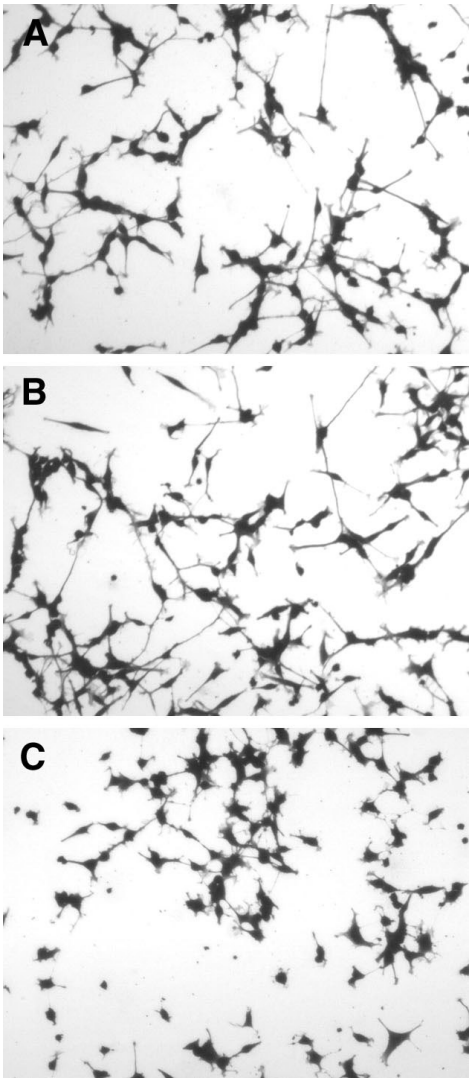

Fig. 2. Typical Images of NB-1 Cells Treated with Organoarsenic Compounds

NB-1 cells treated with dimethylarsinic acid (B) or diphenylarsinic acid (C) 250 μM for 2 days and control cells (A) were fixed and stained with crystal violet.

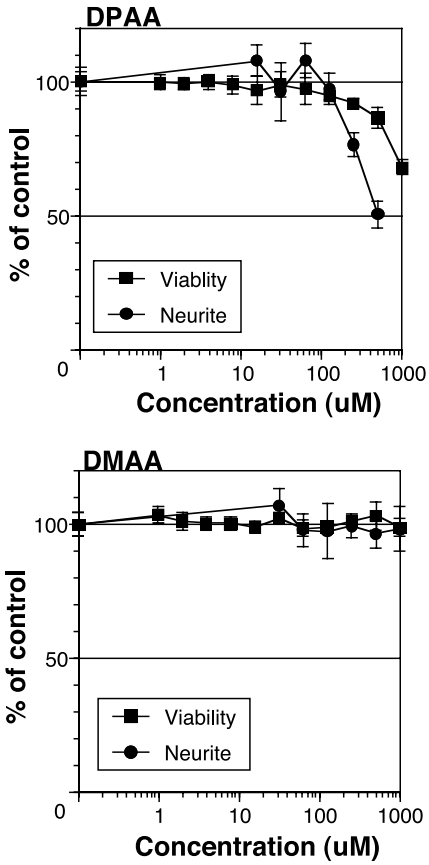

Fig. 3. Effects of Organoarsenic Compounds on Neurite Extension and Viability of NB-1 Cells

NB-1 cells treated with DPAA or DMAA for 2 days were subjected to cell viability assay and image analysis of extended neurites.

rite extension (Figs. 2 and 3). These results suggest that DPAA may have an inhibitory effect on neurite extension similar to that of methylmercury, although it requires a several hundred times higher dose than that of methylmercury.

Effects of DPAA on Rat Cerebellar Cells in Primary Culture

The cerebellum is one of the areas characteristically altered by methylmercury intoxication both in adults and during development. Rat cerebellar cells in primary culture comprised more than 90% neurons and small numbers of astrocytes.[9] After 5–7 days of culture *in vitro*, neurons extend extensive axons as revealed by staining with anti-440-kDa ankyrin$_B$ antibody (Fig. 4A), and astrocytes extend their processes as revealed by the staining with anti-GFAP antibody (Fig. 4B). When the cerebellar cells precultured for 2 days were exposed to DPAA at 0–100 μM for 7 days, viable cell number started to decrease from day 3 upon exposure to DPAA 100 μM (Fig. 5). Furthermore, even DPAA at 30 μM induced the death of cerebellar cells after 7-day exposure, while exposure to DPAA 10 μM for 7 days or 30 μM for 5 days did not induce significant death of cerebellar neurons (Figs. 5 and 6). Since it was shown that methylmercury 30 nM induces apoptotic death of cerebellar neurons,[12,14] DPAA is again 1000 times less toxic to cerebellar neurons than methylmercury.

We have shown that with exposure to methylmercury 30 nM for 2 days immunocytochemical staining of 440 kDa ankyrin$_B$ in cerebellar cells diminished drastically, while that of GFAP, a marker for astroglial cells coexisting in the culture, remained unchanged.[12] Upon exposure to DPAA 30 μM for

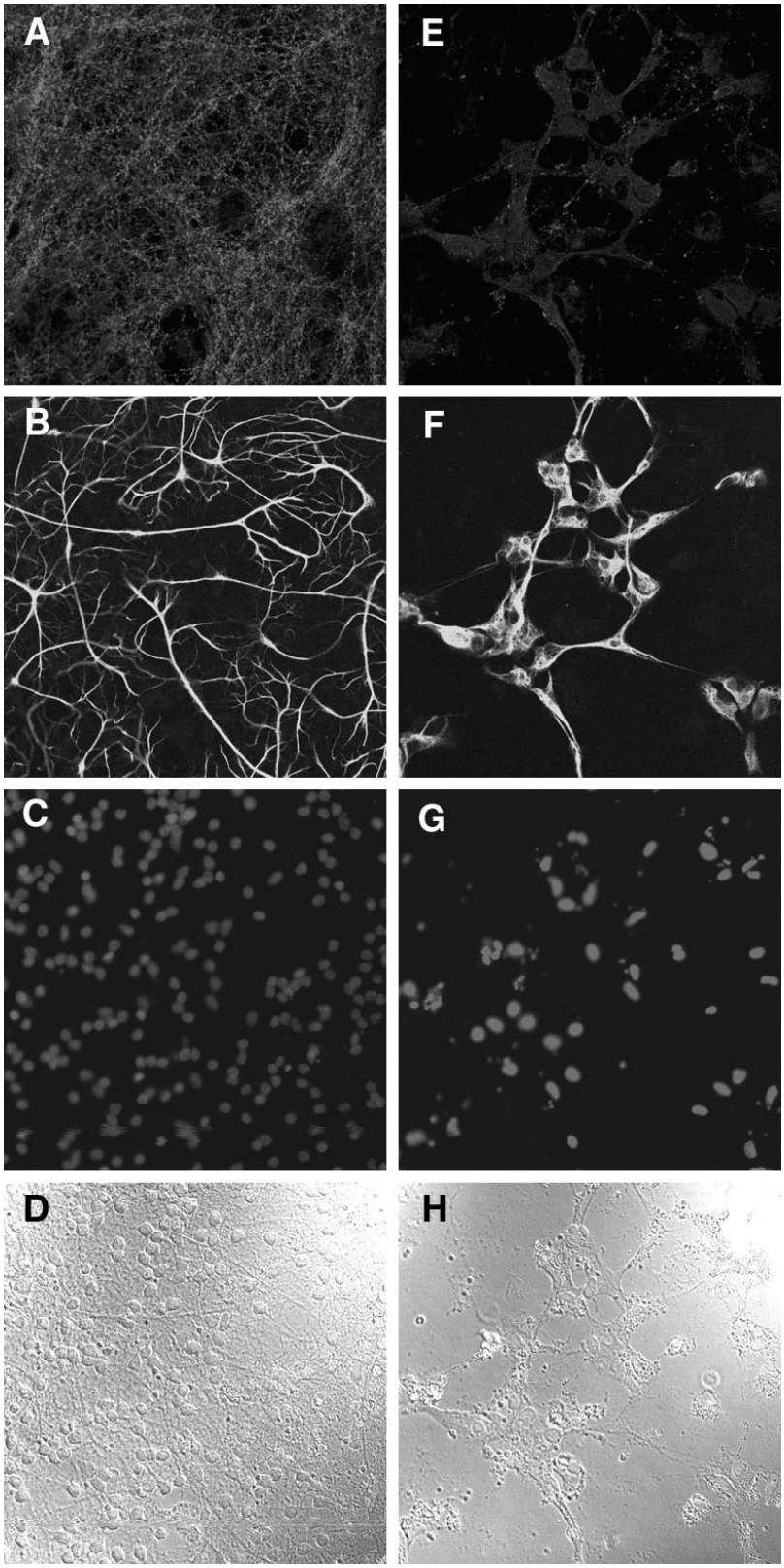

Fig. 4. Effects of Diphenylarsinic Acid on the Expression and Localization of 440-kDa ankyrin$_B$ and GFAP in Rat Cerebellar Cells in Primary Culture

Rat cerebellar cells treated with diphenylarsinic acid 30 μM for 7 days (E–H) and control cells (A–D) were stained with double-label immunofluorescence using anti-440-kDa ankyrin$_B$ antibody (A, E) and anti-GFAP antibody (B, F). Cellular nuclei were visualized by the staining with Hoechst33258 (C, G). Differential interference contrast images are also shown (D, H).

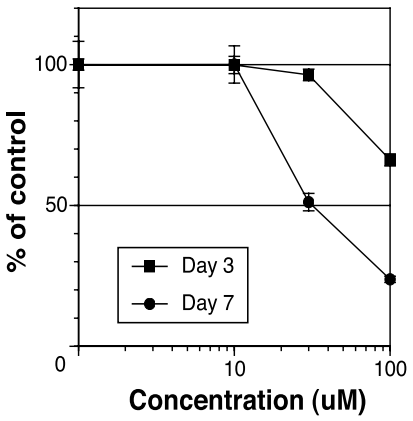

Fig. 5. Effects of Diphenylarsinic Acid on the Viability of Rat Cerebellar Cells in Primary Culture

Rat cerebellar cells in primary culture were exposed to diphenylarsinic acid 0–100 μM for 3 or 7 days. Their viable cell numbers were estimated by crystal violet staining and expressed as percentages of the control value. Error bars indicate standard deviations of quadruplicate assays.

7 days, the 440-kDa ankyrin$_B$ staining also diminished almost completely (Fig. 4E). On the other hand, GFAP staining survived the DPAA exposure, although the morphology of astrocytes was significantly altered by the exposure (Fig. 4F). These results indicate that neurons are more sensitive to the toxicity of DPAA than astrocytes as in the case of methylmercury. The morphology of the nuclei of cerebellar cells visualized by staining with Hoechst33258 was also affected significantly (Figs. 4C and 4G): condensed and fragmented nuclear staining with the dye was observed among the cells treated with DPAA, suggesting that the death of cerebellar neurons induced by DPAA is at least in part apoptotic. It is noteworthy that the significant decrease in the staining of 440-kDa ankyrin$_B$ was observed even in cells treated with DPAA 30 μM for 5 days, when the viability of the cells was not decreased at all (data not shown).

Based on the results obtained, it is concluded that DPAA may have weak neurotoxic actions similar to those of methylmercury, even though it requires

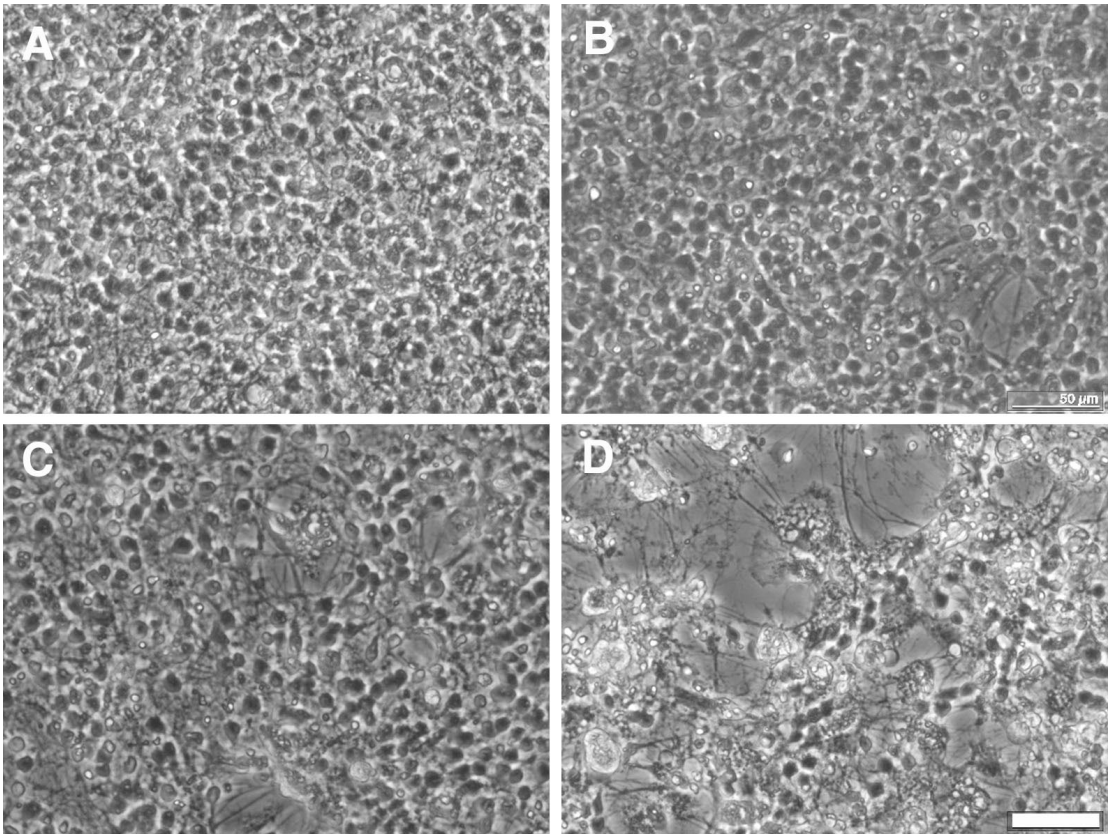

Fig. 6. Typical Images of Rat Cerebellar Cells Treated with Diphenylarsinic Acid

Rat cerebellar cells in primary culture were treated with diphenylarsinic acid at 0–100 μM for up to 7 days. Phase-contrast micrographs of the control cells (day 7) (A), cells treated with diphenylarsinic acid 10 μM for 7 days (B), 30 μM for 5 days (C), or 30 μM for 7 days (D) are shown. Scale bar, 50 μm.

a 1000 times higher dose than methylmercury. For the risk assessment of DPAA regarding the effects on the developing nervous system, information about its absorption, distribution, metabolism, and excretion is essential and must be awaited, in addition to its exposure levels.

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