Novel Bioassay for the Assessment of Neurotoxicity of Chemicals Based on the Neurite Extension in Human Neuroblastoma NB-1 Cells

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A new testing method for the assessment of hazardous environmental chemicals using cultured human neuronal cells has been developed. This method is based on the image analysis of the neurite extension in human neuroblastoma NB-1 cells: the length of the extended neurites was determined using image analysis software. Using this system, 255 chemicals including methylmercury and endocrine disrupting chemicals were tested. Methylmercury and several chemicals inhibited neurite extension, while cadmium chloride, phthalates, and many other chemicals promoted it. These results suggest that the extended neurite length is a useful biological marker for the effects of neurotoxic environmental chemicals, especially on the developing nervous system.

Key words —— human neuroblastoma, neurite extension, image analysis, environmental chemicals

INTRODUCTION

While the possibility of heavy environmental pollution by certain hazardous chemicals is becoming less, our environment is still seriously suffering from the complex pollution by thousands of chemicals. Comprehensive bioassay systems have long been awaited for the risk assessment of environmental chemicals. Despite numerous efforts by biologists, toxicologists and engineers, no such bioassay system has yet been established. Therefore we have tried to develop bioassay systems for the assessment of neurotoxicity of chemicals including methylmercury and endocrine-disrupting chemicals (EDCs) using cultured neuronal cells or brain organotypic culture.[1–4)]

Methylmercury has been recognized as an extremely hazardous environmental pollutant.[5)] Various biochemical, physiologic, and morphologic investigations on the neurotoxic effects of methylmercury have demonstrated that the developing nervous system is vulnerable to the toxic effects of methylmercury[6,7)] and that cytoskeletal systems including microtubules are the possible target of methylmercury.[8)] Human neuroblastoma NB-1 cells extend neurites spontaneously in culture, and dibutyryl cyclic AMP (cAMP) further stimulates neurite outgrowth. When NB-1 cells were exposed to methylmercury, cell viability was not affected by exposure to up to 3 μM. However, the number and length of neurites of NB-1 cells were decreased dramatically by treatment with methylmercury 1 μM.[2)] In association with the retraction (and/or degeneration) of neurites induced by methylmercury, 440-kD ankyrinB, a neuronal growth-associated protein like growth-associated protein-43 (GAP-43),[9)] is down-regulated concomitantly.[2)] Since neurite extension is an integrated biological process intrinsic to neurons, it can be a promising biological index for the assessment of neurotoxic chemicals.

In this study, we developed a new testing method for the assessment of hazardous environmental chemicals using NB-1 cells. This method is based on the image analysis and biochemical analysis of the neurite extension in human neuroblastoma NB-1 cells: the length of the extended neurites was de-

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termined using image analysis software. Using this system, 255 chemicals including methylmercury and EDCs were screened.

MATERIALS AND METHODS

Cell Culture —— Human neuroblastoma NB-1 cells obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) were grown in 45% RPMI-1640 and 45% Eagle's minimum essential medium containing 10% fetal calf serum, penicillin G 50 units/ml, and streptomycin sulfate 50 mg/ml and subcultured once a week at a split ratio of 1 : 6.[1,9] All tissue culture media and supplements were from GIBCO/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 (Costar 3595) at a density of 5×10^3 cells per well, precultured for 2 days, and further cultured in the presence of various concentrations of chemicals for 48 hr.

Chemicals —— The 255 chemicals tested were selected with high priority for possible pollution of the environment, production scale, usage frequency, possible biological effects, and exposure level in the environment.[10,11] These 255 chemicals include 68 pesticides, 17 metal compounds, 41 polycyclic aromatics, 201 mutagens, 160 carcinogens, and 39 EDCs.

Assays for Cell Viablity 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.[1] Fixed and dried cells in 96-well plates were rehydrated with distilled water and photographed under a phase-contrast microscope (Leica DMIRB) 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, La Jolla, U.S.A.) 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 micrometer per cell in randomly chosen phase-contrast microscope fields.

RESULTS AND DISCUSSION

Human neuroblastoma NB-1 cells extend neurites spontaneously in culture.[9] While dibutyryl cAMP, a cAMP analogue, stimulates neurite extension, methylmercury inhibited it at concentrations at which the viability of NB-1 cells was not affected.[2] It therefore appeared promising that potentially neuroxic chemicals similar to methylmercury could be screened with this culture system. To establish a simpler screening system for those chemicals, a couple of unusual procedures were incorporated. First, cells from frozen stock stored in liquid nitrogen were directly plated and exposed to chemicals: NB-1 cells from the same batch of frozen stock can be used for the screening of hundreds of chemicals, which enables reproducible and labor-saving assay. With this procedure, neurite extension of control NB-1 cells in each assay showed a reproducible pattern (data not shown), even though the cells might not have been under ideal conditions. Second, microscopic images of the cells were recorded after fixation with glutaraldehyde, which allows almost indefinite storage of the cells on the plates and repetitive analysis of them later. Typical images analyzed with NeuroZoom image analysis software are shown in Fig. 1.

With this screening system, 255 chemicals were tested. The results obtained indicate that the chemicals can be classified into three groups. Chemicals in the first group promoted the neurite outgrowth of NB-1 cells, the same as dibutyryl cAMP. As shown in Table 1, this group includes many unexpected chemicals such as cadmium chloride, di-2-ethylhexyl phthalate (DEHP), and 2-phenylenediamine. They stimulated the neurite extension at concentrations at which cell viability was not significantly affected (Figs. 2A–2C). Chemicals classified in the second group inhibited the neurite outgrowth of NB-1 cells, like methylmercury (Fig. 2D). Several chemicals had suppressive effects on the neurite extension, but the number of the chemicals in this group was much less than that in the first group (Table 1). The third group consists of the remainder of the tested chemicals, which induced disappearance of the neurites in parallel with a decrease in cell viability. Chemicals in this group are considered to be nonspecific toxicants, while those in the first and second groups are potentially neurotoxic.

There are numerous reports suggesting possible mechanisms for neurite outgrowth and factors controlling those processes. Among them, intracellular

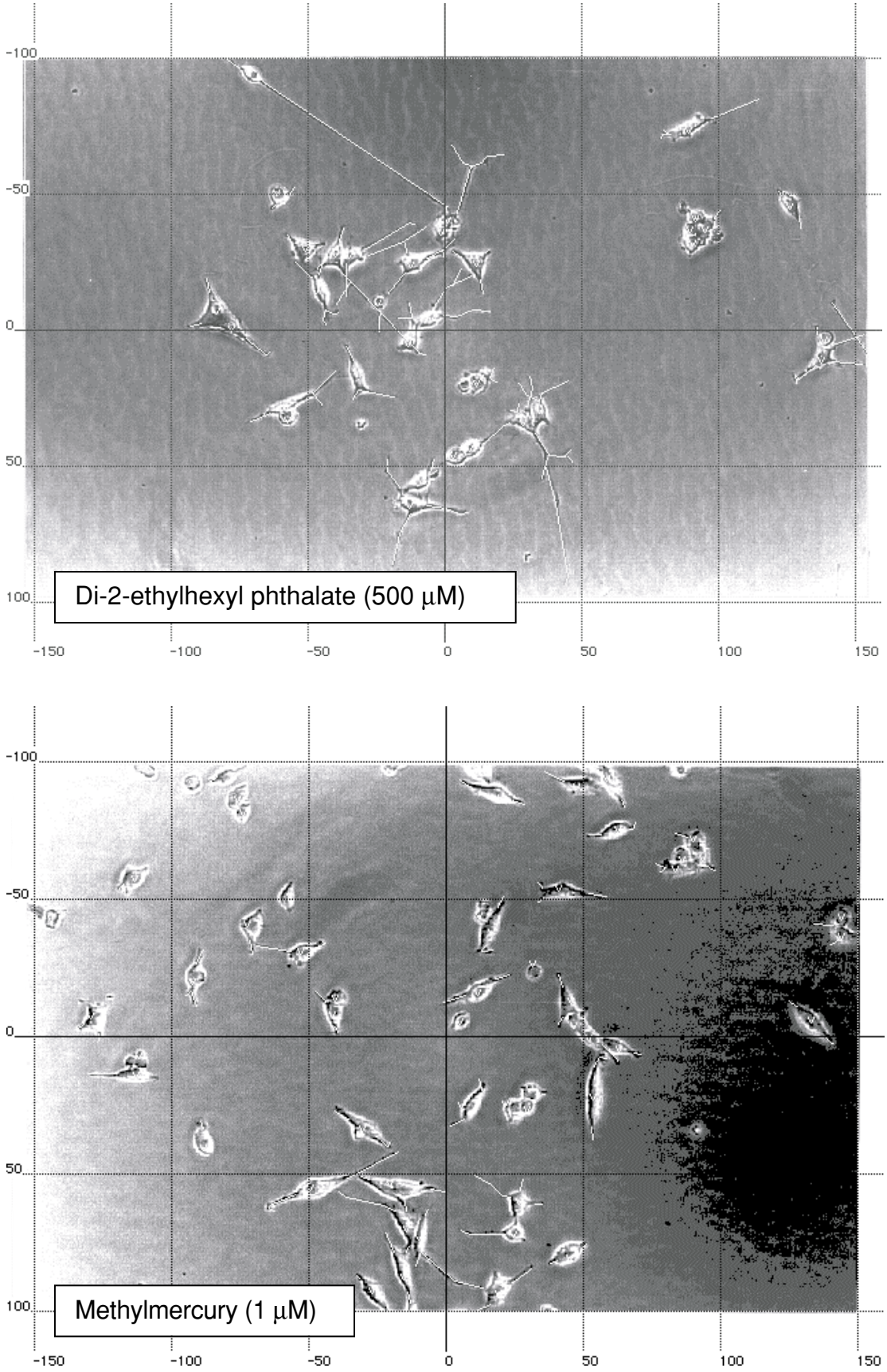

Fig. 1. Typical Images of NB-1 Cells Analyzed Using NeuroZoom

NB-1 cells treated with DEHP 500 μM or methylmercury 1 μM for 48 hr were digitally photographed and analyzed using NeuroZoom image analysis software.

Table 1. List of Chemicals Showing Stimulatory or Inhibitory Effects on Neurite Extension of NB-1 Cells

Stimulatory	
Di-2-ethylhexyl phthalate	2,5-Dichloroaniline
Cucumechinoside D	1-Chloro-2,4-nitrobenzene
Aniline	2,4-Dinitroaniline
Antimony(III) chloride	Kelthane
Cadmium chloride	Phenylhydrazine
2-Phenylenediamine	Hydroquinone
Copper(II) sulfate	3′,4′-Dichloropropionanilide
EDTA 2Na	Bifenox
2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline	
Melamine	Inhibitory
2-Methylpyridine	β-Estradiol-17-acetate
Thallium(I) chloride	Methylmercury Chloride
N-Methylaniline	2,6-Di-t-butyl-4-methylphenol
4-Chloroaniline	Anthracene
2,4,6-Tribromophenol	Ethylene glycol
1,2,4-Trichlorobenzene	Chlorobenzene
2,4-Dinitrophenol	Adipic acid
2,2-Bis(3,5-dibromo-4-hydroxyphenyl)propane	Thiophanate-methyl
Dibutyl phthalate	1,3-Dichloropropene, mixture
2,4,6-Trichlorophenol	Microcystin RR
3-Nitrofluoranthene	*O*,*O*-Dimethyl *O*-4-methylthio-*m*-tolyl phosphorothioate
2,4-Diaminotoluene	

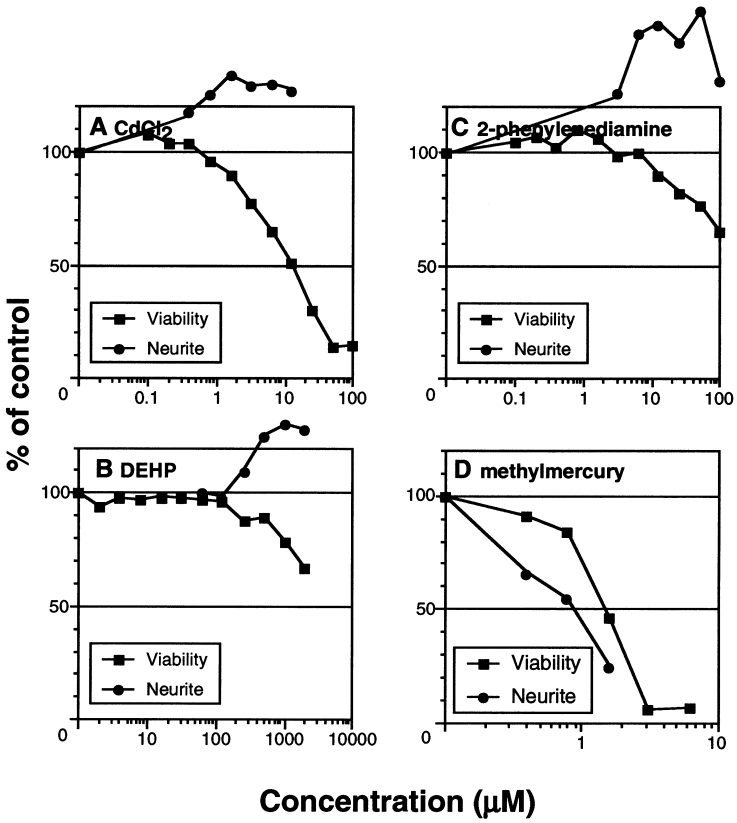

Fig. 2. Effects of Representative Chemicals on Neurite Extension and Viability of NB-1 Cells

NB-1 cells treated with $CdCl_2$ (A), DEHP (B), 2-phenylenediamine (C), or methylmercury (D) for 48 hr were subjected to cell viability assay and image analysis of extended neurites.

calcium level and protein kinase cascades are considered to be critical regulators.[12,13] Mechanistic studies on the effects of the chemicals listed above would be useful not only for their risk assessment but also for further understanding of the mechanisms of axogenesis.

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