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A Paradigm for Examining Toxicant Effects on Viability, Structure, and Axonal Transport of Neurons in Culture

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Contents

Abstract

NIE.115 murine neuroblastoma cells differentiating in serum-free medium were used to develop a paradigm for testing neurotoxicity in vitro. The paradigm was designed to test the effects of toxicants on four different aspects of cell function or structure:

1. Viability as shown by the retention of cellular radiolabel (^{51}Cr);
2. Growth and maintenance of neurites as reflected by the incidence and average length of these processes;
3. Gross structure of neurites; and
4. Velocity and flux of rapid anterograde and retrograde axonal transport as judged by video-enhanced differential interference contrast microscopy.

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To evaluate this paradigm, colchicine and vinblastine were used as neurotoxicants with a well-understood mechanism of action. These agents were only weakly cytotoxic according to the Cr-release assay, but were able to interfere with neurite outgrowth at nanomolar concentrations. Neurites that were elaborated in the presence of vinblastine and colchicine were often disfigured by numerous swellings packed with organelles. In established neurites, micromolar concentrations of vinblastine inhibited organellar motility with great rapidity, blocking all signs of transport within 20 min. The effect of colchicine was slower and less complete, but still impressive. We suggest that this four-part analysis represents a highly sensitive in vitro test for neurotoxicity, and a means of analyzing the relation between abnormalities of transport and structural damage of nerve cells.

Index Entries: Neurotoxicity; neurite outgrowth; axonal transport; video microscopy; colchicine; vinblastine.

Introduction

Peripheral and central neuropathies are, for the most part, disorders of major clinical significance whose pathogenesis is poorly understood. Our research is based on the premise that the phenomenon of axonal transport is highly relevant to these problems. Although there are many causes of neurological dysfunction, one reason for special vulnerability lies in the cellular anatomy that forces neurons to transport metabolic and structural elements over great distances. In humans, spontaneous "dying back" peripheral neuropathies are characterized by distal to central progression of symptoms (a stocking and glove distribution of initial signs), suggesting some disturbance of a distance-dependent function, like axonal transport (Cavanagh, 1964). Toxicant-induced peripheral neuropathies in animals may serve as experimental models of dying back diseases in humans. Like their human counterparts, these models share pathological features consistent with local interruptions of axonal transport. Typical examples are the intermittent distal axonal swellings and accumulations of tubulomembranous and filamentous structures induced by acrylamide (Prineas, 1969b), certain neurotoxic organophosphates (Prineas, 1969a), and *p*-bromophenylacetylurea (Cavanagh et al., 1968). Such observations have provoked many studies of the capability of toxicant-exposed nerves to support rapid and slow axonal transport (*see* Brimijoin and Ochs, 1992).

Classical methodologies for assessing axonal transport rely heavily on monitoring the displacement of radiolabeled proteins after radiolabeled metabolic precursors are supplied to nerve cell bodies (Ochs et al., 1969). Velocity of transport is read from the displacement of the wavefront. Nerve ligations applied at variable times can yield information on bidirectional transport fluxes (Bisby, 1976). Finally, transported proteins can be characterized by electrophoresis (Elam and Agranoff, 1971; Willard et al., 1974; Stone et al., 1978). Unfortunately, the classical approaches have limited spatial or temporal resolution. In order to establish pathogenic links in toxicant-induced neuropathies, one must measure subtle disturbances and determine whether they precede the neuropathology. To achieve the kinetic precision required, real-time analysis of organelle transport in cultured nerve cells may prove useful. Recent technical breakthroughs in video microscopy and the advent of neuronal culture systems now make it attractive to couple in vitro neurotoxicology with optical assessments of organellar motility.

In Vitro Optical Detection of Axonal Transport

Real-time, optical analysis of fast axonal transport hinges on detecting unstained organelles that are too small to resolve by light microscopy. To achieve adequate contrast, differential interference contrast optics can be combined with video detection and computerized enhancement routines. The physical basis for this method is nicely reviewed by Allen (1985).

Optical analysis of neurotoxicant-induced perturbation of axonal transport in vitro affords many advantages. Results from pure neuronal

cell cultures may be interpreted in the absence of confounding influences from glial and vascular tissues, and are free of artifacts induced by surgery and anesthesia. Control of dose and time of drug administration are much more precise in cell culture. Finally, optical methods have superior temporal resolution, allowing detection of drug effects *as they happen*.

The advantages of work in vitro are offset by certain limitations. Among these, the maximal duration of toxicant exposure may be most serious. Agents, such as acrylamide and 2,5-hexanedione, require weeks or months to induce pathology in intact animals (Le Quesne, 1980; Spencer and Schaumburg, 1984). Keeping healthy differentiated cells in culture for indefinite periods is difficult. The lack of enzymes that may be needed to bioactivate toxicants is also a potential problem with cultures. Such drugs as cyclophosphamide and acetaminophen must be converted to active cytotoxicants by hepatic metabolism, although adding liver microsomes to cultures is a possible remedy (Spielberg, 1980). A last point is the small sample mass in any cell culture, which often precludes biochemical analysis of presumptive cellular targets. Despite these limitations, in vitro neurotoxicology shows promise that is only beginning to be exploited.

Model Systems in Neurotoxicology

Model in vitro systems for neurotoxicology are now plentiful (for review, *see* Goldberg, 1987), and both primary culture and continuous cell lines are used successfully. Among primary cultures favored for assessing effects on neuronal growth are explants of embryonic sympathetic and dorsal root ganglia from chick or rat (*see* Banker and Goslin, 1991). Ganglia from these sources extend neurites in the presence of nerve growth factor (NGF). Inhibition of neurite outgrowth from cultured ganglia has been used to study neuronal injury by clinical drugs, industrial compounds, and heavy metals. A critical disadvantage of explants is the difficulty of quantitating cell viability. Dissociated cortical, cerebellar, and hippocampal cell cultures have also been used for toxicological study (Goslin and Banker, 1991; Trenkner, 1991). Cellular viability can be evaluated by methods discussed below. These cultures usually have a significant nonneural component, however, that is often required for neural survival and may complicate the interpretation of toxic effects.

Another interesting primary culture is the chromatophore of teleost fish (McNiven and Porter, 1984). These cells translocate pigment granules centrally and peripherally at high speed in response to defined stimuli. Emerging information suggests that the streams of pigment granules are analogous to anterograde and retrograde axonal transport in vertebrate neurons (McNiven and Ward, 1988). Disruptions of pigment translocation do not necessarily represent transport-specific toxicity, because they can reflect the general metabolic status of the cell. Also, chromatophores may differ somewhat from neurons with respect to the mechanisms and regulation of motility. Nonetheless, quantitation of organellar motility in chromatophores is attractive, because the cells are inexpensive to prepare, and the transport phenomena are robust and readily visualized. Though no published studies of neurotoxicants have capitalized on this unique system, its potential should soon be recognized.

Continuous cell lines may be even more useful for neurotoxicology than primary cultures. Within a given passage, such lines as neuroblastoma (Prasad, 1975), PC12 cells (Greene and Tischler, 1976), and neuroblastoma X glioma hybrids (Nelson et al., 1976) reliably respond as homogeneous populations. Each line has at least three other favorable qualities:

1. Rapid division during log-phase growth;
2. Reproducible extension of neurites in response to appropriate stimuli; and
3. Growth on substrates compatible with high-contrast microscopy.

The last quality is very important. Many primary cultures require collagen or glial cells as substrates. Unfortunately, the 3-D topology of these substrates makes it difficult to keep a neurite in one focal plane, and their birefringence inter-

feres with imaging. By growing directly on glass or plastic, certain neuroblastoma and PC12 lines avoid this difficulty. Some neuroblastoma X glioma hybrids offer the additional advantage of forming functional synapses with cocultured rat myotubes, thereby modeling mature neural systems in vivo (Nelson et al., 1976). The inherent difficulties of cocultures, however, make it natural to choose simpler systems for most purposes. In the work described below, we used only the N1E.115 neuroblastoma line described by Amano et al. (1972).

Methods

Paradigm for In Vitro Studies

A reasonable paradigm for testing neurotoxicants in vitro would measure general cytotoxicity, outgrowth, and maintenance of neurites, neurite morphology, and also the capacity and velocity of rapid anterograde and retrograde axonal transport. The general approaches taken in this laboratory to obtaining and interpreting such data are described in the following sections.

Cytotoxicity

In vitro testing of a potential neurotoxicant must begin by characterizing the dose–response relations of general cytotoxicity. Most assays of cytotoxicity rely on the leakiness of the surface membrane in dying cells. One set of methods assesses cytotoxicity by counting the cells that take up a dye like Trypan Blue, which is excluded from healthy cells. With fluorescent dyes, such as ethidium bromide and propidium iodide, also excluded from live cells, a fluorescence-activated cell sorter can do this task automatically (St. John et al., 1986). All dye-uptake methods may miss cells that lose adherence or disintegrate. Assays that overcome this shortcoming are those measuring components *released* from leaky cells. One popular marker is the cytosolic enzyme, lactate dehydrogenase (Koh and Choi, 1987), which can be monitored during toxicant exposures that last for days or weeks. Another option is a radioactive marker. Healthy cells accumulate and retain ^{3}H-proline, ^{14}C-adenosine, or ^{51}Cr, so excess release is a reliable and easily monitored index of toxicity (Schlager and Adams, 1983; Brunner et al., 1976; Kachel et al., 1990). In our hands, the chromium release assay involves preincubation with ^{51}Cr in culture medium, removal of unincorporated label, and exposure to toxicant for 12 h in balanced salt solution. After centrifugation to separate cells from medium, radioactivity in both compartments is measured. A cytotoxic index (CI) is defined as:

$$CI = 100^{*}(R_{exp} - R_{con})/(1 - R_{con}) \quad (1)$$

In this formula, R is released radioactivity as a fraction of the total. The CI is zero when release is at control levels, and it is 100 when all ^{51}Cr is released (complete lysis).

Neurite Outgrowth

Neurite extension, a fundamental property of neuronal cells in vitro, depends at least in part on axonal transport. Furthermore, outgrowth is relatively easy to quantitate. Therefore, the ability of environmental and industrial compounds at sublethal concentrations to interfere with outgrowth is often used to screen new compounds and to assess potencies of known toxicants (Dow and Riopelle, 1985; Windebank and Blexrud, 1986,1989). Observations of neurite outgrowth are a logical second step in tests of neurotoxicity in vitro.

In our work, N1E.115 cells are induced to extend neurites by elimination of serum from the medium. Up to 35% of the cells put forth neurites within 48 h in serum-free medium (average length ~140 μm). To test effects on outgrowth, a toxicant is added at the onset. After a standard period (usually 48 or 72 h), we determine (1) the fraction of cells bearing neurites at least one cell diameter in length and (2) the mean length of the neurites. Effects on *established* neurites are tested by allowing cells to differentiate in serum-free medium for 48 h. The medium is then replaced with toxicant solutions in fresh, serum-free medium or in buffered balanced salt solution (to eliminate

confounding effects of continued neurite outgrowth). Neurite incidence and average length are monitored for up to 72 h.

Neurite Morphology

Neurons cultured with toxicants often develop structurally abnormal neurites. A common phenomenon is "beading," the formation of gross, organelle-filled dilatations, as many as 10 or 15, spaced at intervals along the neurite. Beading occurs in cells exposed to colchicine (Takenaka, 1986), acrylamide (Walum et al., 1987), or ethylene oxide (Garnaas et al., 1991). Parallels with axonal swellings in experimental neuropathies induced by related agents are worth noting, particularly in view of the suspected role of abnormalities in axonal transport (Griffin et al., 1977; Sahenk and Mendell, 1981). We routinely examine N1E.115 neurites for beading during toxicant exposure.

Organelle Transport

The technically most difficult, but possibly most significant measures of neurotoxicity in vitro are those defining organellar motility. For optical studies of motility, cultured neurons must establish neurites suitable for VEC-DIC microscopy. Big neurites with no underlying fibrous substrate give the best images. Cells of the N1E.115 neuroblastoma line (Amano et al., 1972) extend neurites 2–5 μm in diameter and up to 400 μm in length on uncoated glass in serum-free medium. Easy detection of transported organelles was a major factor driving our choice of N1E.115 cells for studies on organellar motility.

Measurements of organellar motility must encompass both speed and flux (i.e., total flow of organelles). Speed can be assessed with precision, but organellar flux shows a high coefficient of variation (approx 50%), possibly owing to heterogeneity in the growth states of individual differentiating cells. In the future, it might be desirable to stabilize the growth state or arrest growth by supplying deficient media (balanced salt solution). Alternatively, one might turn to the NG108-15 neuroblastoma X glioma hybrids, which stop growing after forming stable synapses in vitro. For the present, we continue to utilize differentiating N1E.115 cells.

Differentiating N1E.115 cells growing on glass coverslips are placed in a Dvorak-Stotler cell culture chamber, containing two coverslips separated by a 1-mm ring with perfusion ports. The chamber, superfused during the experiment with preoxygenated medium, is mounted on the thermostatically controlled stage of a Zeiss Axiomat with DIC optics, planapo 100X/1.3 NA objective, 1.4 NA condenser, 1.6× zoom, 4× extension lens, and Newvicon 68 video camera (DAGE-MTI, Michigan City, IN). A fiber optic scrambler on the 100 W Hg lamp (Technical Video, Woods Hole, MA) uniformly illuminates the rear focal plane of the objective. Video signals, passed to a Quantex 9200 image processor (Quantex Corp., Sunnyvale, CA) for real-time digitization, background subtraction, and contrast enhancement, are viewed on a high resolution monitor and recorded on S-VHS tape for later analysis.

Analysis of motility begins with speeds. Organelles are tracked at 0.33-s intervals (10 video frames) for at least 3 s. Sequential positions are plotted on a transparency affixed to the video screen. The transparency is then transferred to a calibrated Macintosh monitor to measure X and Y coordinates, capture the numerical data, and compute mean speed in μm/s over an entire trajectory for each of up to 10 organelles/neurite.

Perhaps the best indicator of transport integrity is organellar flux, or total delivery of organelles per unit time. Flux can be estimated from the number of organelles traversing a line drawn perpendicular to the long axis of the neurite. A parameter that is comparable across neurites is the number of crossings/min/μm of neurite diameter. This parameter is directly proportional to the number of organelles undergoing rapid transport ("traffic density") and to the mean speed of those organelles. Consequently, measurements of flux sense the behavior of the entire population of transported organelles in a given neurite.

Results and Discussion

In Vitro Study of Antimicrotubule Agents

The potential for analyzing neurotoxicants in vitro is exemplified by some new experiments on colchicine and vinblastine. These two compounds are well known for their ability to bind to tubulin (Owellen et al., 1972) and to disaggregate neuronal microtubules (Wisniewski et al., 1968). They also inhibit fast axonal transport in vivo (Dahlstrom, 1968; Dustin, 1978; Kreutzberg, 1969; Sjostrand et al., 1970; Paulson and McClure, 1975). We applied colchicine and vinblastine as model toxicants to cultured N1E.115 neuroblastoma cells to evaluate the paradigm for in vitro testing of

1. Neuronal cytotoxicity;
2. Impaired neurite outgrowth;
3. Altered neurite morphology; and
4. Inhibition of organellar motility.

Cytotoxicity determined by ^{51}Cr release showed that colchicine did not impair viability during 12-h exposures at concentrations up to 1 m*M* (Fig. 1). Vinblastine did cause nearly complete Cr release at 0.1 m*M*, with half-maximal effects at 26 ± 5 μ*M*. However, these levels were much higher than required to inhibit neurite outgrowth or cause neurite degeneration.

Forty-eight hours after serum reduction, 25% of control cells had grown out neurites at least one cell body diameter in length (Fig. 2). Both vinblastine and colchicine inhibited outgrowth at concentrations below 1 μ*M*. For an unbiased estimate of the potency of these drugs, EC_{50} values were derived by computer fitting to a nonlinear regression model (Brat et al., 1992). Half-maximal inhibition required roughly one-tenth as much vinblastine (EC_{50} = 33 ± 11 n*M*) as colchicine (EC_{50} = 210 ± 110 n*M*).

To examine toxicity toward *established* neurites, cells were induced to differentiate as usual for 48 h in serum-free medium. The medium was then replaced with balanced salt solution containing colchicine or vinblastine, and neurites were examined 20 h later (Fig. 3). Vinblastine at a concentration of 1.1 ± 0.6 n*M* caused a half-maximal loss of neurites; concentrations above 1 μm caused nearly complete degeneration. Colchicine was markedly less potent (EC_{50} = 360 ± 100 n*M*).

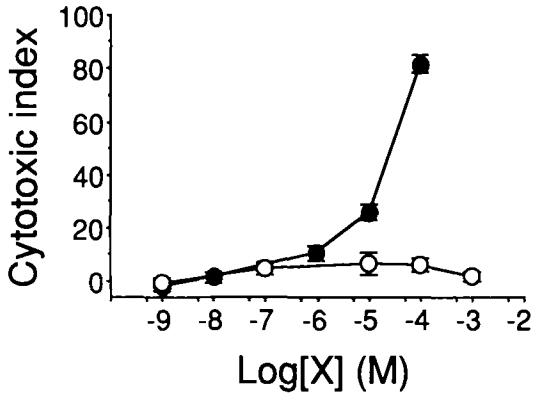

Fig. 1. Cytotoxicity of colchicine (○) and vinblastine (●). Data reflect release of preloaded ^{51}Cr from N1E.115 cells exposed to colchicine and vinblastine in a buffered balanced salt solution for 12 h. Cytotoxic index is fractional release of ^{51}Cr as a percentage of control release. Data are means ± SEM (n = 3 at each point). Smooth curve was fitted by computerized nonlinear regression. EC_{50} for vinblastine was 26 ± 5 μ*M*.

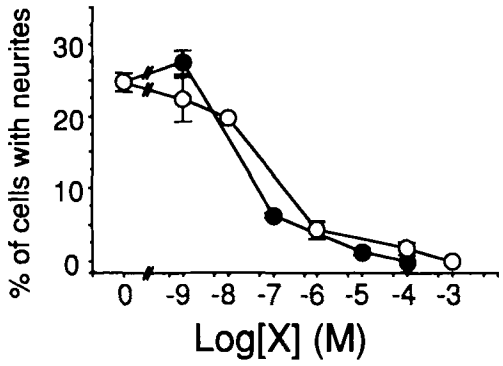

Fig. 2. Dose–response analysis of drug-induced inhibition of neurite outgrowth. N1E.115 cells were induced to differentiate for 48 h by serum reduction. Each point represents a mean (±SEM) of data from three identical samples. EC_{50} values derived by nonlinear regression analysis were 33 ± 11 n*M* for vinblastine (●), 210 ± 110 n*M* for colchicine (○).

These two drugs caused abnormalities of neurite morphology, including the induction of focal dilatation or beading (not shown). A

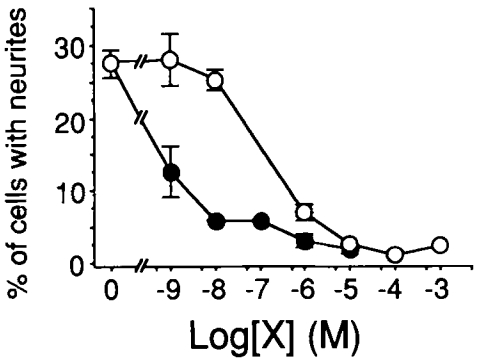

Fig. 3. Effect of colchicine (○) and vinblastine (●) on previously established neurites. N1E.115 cells were induced to extend neurites by serum reduction 48 h before addition of drug in a buffered balanced salt solution. The percentage of cells with neurites was determined 20 h after drug exposure. Data are means ± SEM (n = 3). EC_{50} values derived by nonlinear regression were 1.1 ± 0.6 n*M* for vinblastine, 360 ± 100 n*M* for colchicine.

similar phenomenon in chick dorsal root ganglia exposed to colchicine has been described by Takenaka (1986).

We also tested the effects of colchicine and vinblastine on axonal transport. To inhibit transport in intact nerves typically requires 12–48 h of treatment with high doses (up to 10 m*M* in the case of colchicine). With desheathed preparations of cat peroneal nerve in vitro, Ochs and colleags saw that 25 μ*M* vinblastine inhibited fast axonal downflow of radiolabeled protein after a few hours (Chan et al., 1980). In differentiating NlE.115 neuroblastoma, we found that even lower concentrations of colchicine or vinblastine blocked transport within minutes.

Fast axonal transport in normal N1E.115 cells was analyzed first in terms of organellar speeds. Organellar speeds in the anterograde and retrograde directions were distributed quasinormally and were in fair agreement with in vivo data from the literature. In a series of 16 control neurites, the maximal speed of anterograde transport 35°C was 4.5 μm/s (Fig. 4). This value nearly equaled the "textbook" 400 mm/d (4.6 μm/s) for radiolabeled protein in whole nerves at 37°C (Ochs, 1972). Corrected for temperature, the in vitro speed was actually higher. The same held for

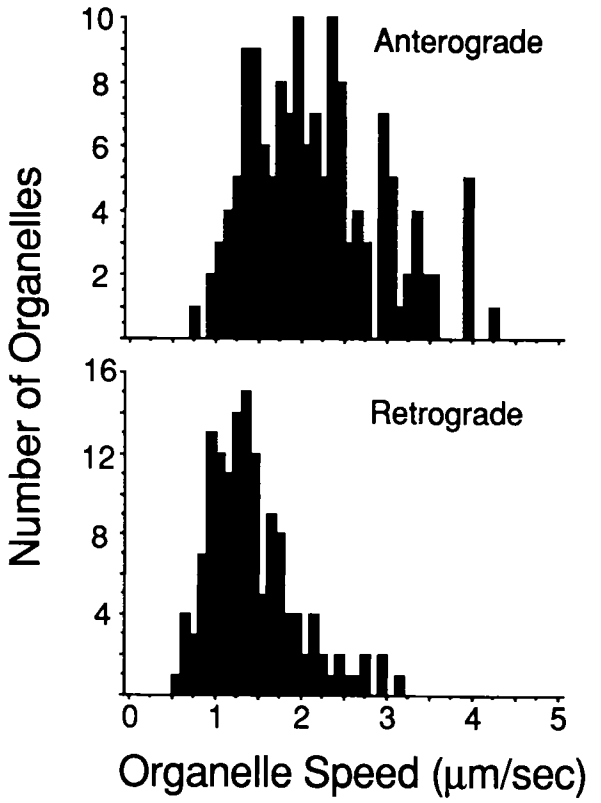

Fig. 4. Frequency histograms of anterograde and retrograde organelle speeds in control N1E.115 neurites at 35°C. Cells differentiated for 48 h in serum-free medium. Organelle motility was visualized in 16 neurites using VEC-DIC microscopy. In each neurite, speeds were determined for up to 10 organelles moving in the anterograde direction and an equivalent number moving in the retrograde direction (n = 144 for each histogram).

retrogradely moving organelles, which showed a top speed of 3.2 μm/s (Fig. 4) compared to the 200–220 mm/d (2.3–2.5 μm/s) typically determined in vivo (Ochs, 1982). Optical methods should yield relatively high peak values, since organelles do not maintain constant rates, may stop or momentarily reverse directions, and often move at rates well below the maximum (Smith, 1982).

Initial observations with vinblastine and colchicine suggested that these agents did not radically alter the speed of organellar transport in N1E.115 neurites, but did reduce the number of moving organelles. To define fully the transport effects, it would be necessary to examine the distribu-

tion of organellar speeds in detail. This relatively laborious process was carried out in our study of potentially toxic emulsifying agents (Brat et al., 1992). In the present case, we turned to analyzing the more impressive changes in organellar flux.

Optically detected organellar flux was measured at various times after exposure to vinblastine or colchicine, 1 μ*M* in serum-free medium. Normative data collected in a sample of 13 neuroblastoma neurites incubated at 37°C gave control mean values (±SEM) of 4.9 ± 0.8 organelles/μm/min for flux toward the growth cone, and 3.0 ± 0.3 organelles/μm/min for flux toward the cell body. Vinblastine reduced bidirectional organelle flux by 75% within 20 min (Fig. 5), whereas longer exposures almost completely halted transport. Colchicine required 40 min to induce slightly smaller effects. This relative potency agrees well with results of earlier studies on antimicrotubule agents and axonal transport in intact nerves (Dahlstrom et al., 1975; Wooten, et al., 1975), although the effective doses were much smaller in culture.

Since vinblastine and colchicine blocked axonal transport at concentrations that did not increase the release of ^{51}Cr, the effects on transport were probably independent of general cytotoxicity. On the other hand, the micromolar concentrations that blocked transport were more than sufficient to inhibit neurite outgrowth and trigger the degeneration of established neurites. The interrelations among these three effects should now be explored in more detail.

Although neurotoxic effects of colchicine and vinblastine in vitro were expected, it was surprising how little drug was needed for neurite degeneration and how rapidly axonal transport was blocked. Evidently, neurite structure and organellar motility in cultured neurons are exquisitely sensitive detectors of neurotoxicity. The sensitivity, high temporal resolution, and controlled environment of such systems may be useful for testing new compounds. The paradigm described here has already helped us demonstrate that certain emulsifying agents in current clinical use have a potential for neurotoxicity (Brat et al., 1992). Similar approaches might also be taken to screen other potential toxicants and to deepen mechanistic understanding of known toxicants, such as acrylamide, BPAU, and cisplatin, whose pathogenic effects are likely to involve impairment of axonal transport.

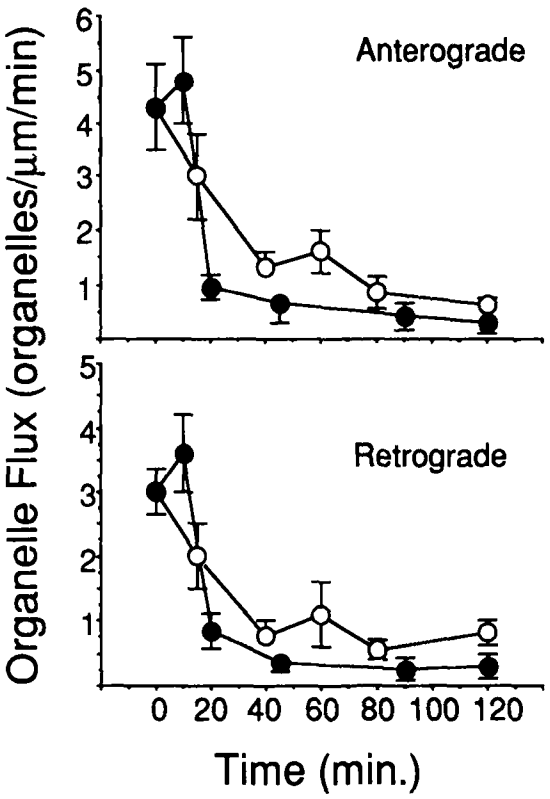

Fig. 5. Inhibition of bidirectional, rapid axonal transport by vinblastine and colchicine. N1E.115 cells were allowed to differentiate in serum-free medium for 72 h. The number of organelles traversing a line perpendicular to the neurite axis ("organelle-flux") was measured at various times after addition of fresh medium containing 1 μ*M* vinblastine (●) or colchicine (○). Data are means ± SEM ($n = 8$ for controls; $n = 5$ for experimental points).

Summary

1. Advantages of studying toxicant-related disturbances of axonal transport with in vitro optical methods include high temporal resolution and controlled drug delivery.

2. Many primary neuronal cultures and continuous cell lines are available for toxicological studies. N1E.115 neuroblastoma cells are attractive, because they extend large neurites on uncoated glass, facilitating high-contrast imaging.
3. Speed and capacity of axonal transport can both be measured optically, along with growth and maintenance of neurites in the presence of toxicants. Data on cell survival may suggest whether toxicity is specific to transport systems.
4. Vinblastine and colchicine affected neurite outgrowth and axonal transport at concentrations far below those causing cell death. EC_{50}s for suppression of neurite outgrowth were 33 n*M* for vinblastine and 210 n*M* for colchicine.
5. Micromolar concentrations of vinblastine and colchicine inhibited organelle motility by 75% or more. Vinblastine caused large effects within 20 min; colchicine took twice as long.
6. Optical analysis of neurite outgrowth and axonal transport in vitro may be useful for future studies of neurotoxicants

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References

Allen R. D. (1985) New observations on cell architecture and dynamics by video-enhanced contrast optical microscopy. *Annu. Rev. Biophys. Chem.* **14**, 265–290.

Amano T., Richelson E., and Nirenberg M. (1972) Neurotransmitter synthesis by neuroblastoma clones. *Proc. Natl. Acad. Sci. USA* **69**, 258–263.

Banker G. and Goslin K. (eds.) (1991) *Culturing Nerve Cells*, MIT Press, Cambridge, MA.

Bisby M. A. (1976) Orthograde and retrograde axonal transport of labeled protein in motoneurons. *Exp. Neurol.* **50**, 628–640.

Brat D. J., Windebank A. J., and Brimijoin S. (1992) Emulsifier for intravenous cyclosporin inhibits neurite outgrowth, causes deficits in rapid axonal transport, and leads to structural abnormalities in differentiating N1E.115 neuroblastoma. *J. Pharmacol. Exp. Ther.* **261**, 803–810.

Brimijoin S. and Ochs S. (1992) Axonal transport, in *Peripheral Neuropathy*, 3rd ed. (Dyck P. J., Thomas P. K., Lambert E. H., and Bunge R. P., eds.) Saunders, Philadelphia, in press.

Brunner K. T., Engas H. D., and Cerottini J. C. (1976) The 51Cr release assay as used for the quantitative measurement of cell mediated cytolysis in vitro, in *In Vitro Methods in Cell-Mediated and Tumor Immunity* (Bloom R. and David J. R., eds.), Academic, New York, pp. 423–428.

Cavanagh J. B. (1964) The significance of the "dying-back" process in experimental and human neurological disease. *Int. Rev. Exp. Pathol.* **3**, 219–267.

Cavanagh J. B., Chen F. C. K. Kyu, M. H., and Ridley A. (1968) The experimental neuropathy in rats caused by *p*-bromophenylacetylurea. *J. Neurol. Neurosurg.* **31**, 471–478.

Chan S. Y., Worth R., and Ochs S. (1980) Block of axoplasmic transport in vitro by vinca alkaloids. *J. Neurobiol.* **11**, 251–264.

Dahlstrom A. (1968) Effect of colchicine on transport of amine storage granules in sympathetic nerves of rat. *Eur. J. Pharmacol.* **5**, 111–113.

Dahlstrom A., Heiwall P. O., Haggendal J., and Saunders N. R. (1975) Effects of antimitotic drugs on the intraaxonal transport of neurotransmitters in rat adrenergic and cholinergic nerves. *Ann. NY Acad. Sci.* **253**, 528–534.

Dow K. E., and Riopelle R. J. (1985) Ethanol neurotoxicity: Effects on neurite formation and neurotrophic factor production in vitro. *Science* **228**, 591–593.

Dustin P. (1978) *Microtubules*, Springer-Verlag, New York.

Elam J. S. and Agranoff B. W. (1971) Transport of proteins and sulfated mucopolysaccharides in the goldfish visual system. *J. Neurobiol.* **2**, 379–390.

Garnaas K. R., Windebank A. J., Blexrud M. D., and Kurtz S. B. (1991) Ultrastructural changes produced in dorsal root ganglia in vitro by exposure to ethylene oxide from hemodialyzers. *J. Neuropath. Exp. Neurol.* **50**, 256–262.

Goldberg A. M. (1987) Approaches to the development of in vitro neurotoxicological methods. *Prog. Clin. Biol. Res.* **253**, 1–11.

Goslin K., and Banker G. (1991) Rat hippocampal neurons in low-density culture, in *Culturing Nerve Cells* (Banker G. and Goslin K., eds.), MIT Press, Cambridge, MA, pp. 251–281.

Greene L. A. and Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424–2428.

Griffin J. W., Price D. L., Engel W. K., and Drachman D. B. (1977) The pathogenesis of reactive swelling: the role of axonal transport. *J. Neuropath. Exp. Neurol.* **36**, 214–226.

Kachel D. L., Moyer T. P., and Martin W. J. (1990). Amioderone-induced injury of human pulmonary artery endothelial cells: protection by alpha-tocopherol. *J. Pharmacol. Exp. Ther.* **254**, 1107–1112.

Koh J. Y. and Choi D. W. (1987) Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J. Neurosci. Meth.* **20**, 83–90.

Kreutzberg G. W. (1969) Neuronal dynamics and axonal flow: IV. Blockage of intra-axonal enzyme transport by colchicine. *Proc. Natl. Acad. Sci. USA* **62**, 722–728.

Le Quesne P. M. (1980) Acrylamide, in *Experimental and Clinical Neurotoxicology* (Spencer P. S. and Schaumburg H. H., eds.), Williams and Wilkins, Baltimore, pp. 309–325.

McNiven M. A. and Porter K. R. (1984) Chromatophores—models for studying cytomatrix translocations. *J. Cell Biol.* **99**, 152–158.

McNiven M. A., and Ward J. B. (1988) Calcium regulation of pigment transport in vitro *J. Cell Biol.* **106**, 111–125.

Nelson P., Christian C. and Nirenberg M. (1976) Synapse formation between clonal neuroblastoma x glioma hybrid cells and striated muscle cells. *Proc. Natl. Acad. Sci. USA* **73**, 123–127.

Ochs S. (1972) Rate of fast axoplasmic transport in mammalian nerve fibres. *J. Physiol. (London)* **227**, 627–645.

Ochs S. (1982) *Axoplasmic Transport and Its Relation to Other Nerve Functions*. Chapter 3. Wiley & Sons, New York, pp. 47–69.

Ochs S., Sabri M. I., and Johnson J. (1969) Fast transport system of materials in mammalian nerve fibers. *Science* **163**, 686,687.

Owellen R. J., Owens A. H., and Donigan D. W. (1972) The binding of vincristine, vinblastine and colchicine to tubulin. *Biochem. Biophys. Res. Comm.* **47**, 685–691.

Paulson J. C. and McClure W. O. (1975) Inhibition of axoplasmic transport by colchicine, podophyllotoxin and vinblastine: an effect on microtubules. *Ann. NY Acad. Sci.* **253**, 517–527.

Prasad K. D. (1975) Differentiation of neuroblastoma cells in culture. *Biol. Rev.* **50**, 129–165.

Prineas J. (1969a) The pathogenesis of dying-back neuropathies: I. An ultrastructural study of experimental tri-ortho-cresyl phosphate intoxication in the cat. *J. Neuropath. Exp. Neurol.* **28**, 571–597.

Prineas J. (1969b) The pathogenesis of dying-back neuropathies: II. An ultrastructural study of experimental acrylamide intoxication in the cat. *J. Neuropath. Exp. Neurol.* **28**, 598–621.

Sahenk Z. and Mendell J. R. (1981) Acrylamide and 2,5-hexanedione neuropathies: Abnormal bidirectional transport rate in distal axons. *Brain Res.* **219**, 397–405.

St. John P. A., Kell W. M., Mazzetta J. S., Lange G. D., and Barker J. L. (1986) Analysis and isolation of embryonic mammalian neurons by fluorescence-activated cell sorting. *J. Neurosci.* **6**, 1492–1512.

Schlager S. I. and Adams A. C. (1983) Use of dyes and radioisotopic markers in cytotoxicity tests, in *Methods in Enzymology*, Vol. 93 (Langone J. J. and Vunakis M. V., eds.), Academic, New York, pp. 233–245.

Sjostrand J., Frizell M., and Hasselgren P. O. (1970) Effects of colchicine on axonal transport in peripheral nerves. *J. Neurochem.* **17**, 1563–1570.

Smith R. S. (1982) Axonal transport of optically detectable organelles, in *Axoplasmic Transport* (Weiss D. G., ed.), Springer-Verlag, Berlin, pp. 181–192.

Spencer P. S. and Schaumburg H. H. (1984) Experimental models of primary axonal disease induced by toxic chemicals, in *Peripheral Neuropathy* (Dyck P. J., Thomas P. K., Lambert E. H., and Bunge R. P., eds.), Saunders, Philadelphia, pp. 636–649.

Spielberg S. P. (1980) Acetaminophen toxicity in human lymphocytes in vitro. *J. Pharmacol. Exp. Ther.* **213**, 395–398.

Stone G. C., Wilson D. L., and Hall M. E. (1978) Two-dimensional gel electrophoresis of proteins in rapid axoplasmic transport. *Brain Res.* **144**, 287–302.

Takenaka T. (1986) Particle movements in axonal transport, in *Axoplasmic Transport* (Iqbal Z., ed.), CRC, Boca Raton, FL, pp. 109–118.

Trenkner E. (1991) Cerebellar cells in culture, in *Culturing Nerve Cells* (Banker G. and Goslin K., eds.), MIT Press, Cambridge, MA, pp. 177–206.

Walum E., Ekblad-Sekund G., Nyberg E., and Gustafsson L. (1987) Cultured neuroblastoma cells as neurotoxicological models: acrylamide induced neurite disintegration, in *Model Systems in Neurotoxicology: Alternative Approaches to Animal Testing* (Shahar A., and Goldberg A. M., eds.), Alan R. Liss, New York, pp. 121–136.

Willard M., Cowan W. M., and Vagelos P. R. (1974) The polypeptide composition of intraaxonally transported proteins: evidence for four transport velocities. *Proc. Natl. Acad. Sci. USA* **71**, 2183–2187.

Windebank A. J. and Blexrud M. D. (1986) Specific inhibition of myelination by lead *in vitro*; comparison with arsenic, thallium and mercury. *Exper. Neurol.* **94**, 203–212.

Windebank A. J. and Blexrud M. D. (1989) Residual ethylene oxide in hollow-fiber hemodialysis units is neurotoxic in vitro. *Ann. Neurol.* **26**, 63–68.

Wisniewski H. M., Shelanski M., and Terry R. (1968) Effects of mitotic spindle inhibitors on neurotubules and neurofilaments in anterior horn cells. *J. Cell Biol.* **38**, 224–228.

Wooten G. F., Kopin I. J., and Axelrod J. (1975) Effects of colchicine and vinblastine on axonal transport and transmitter release in sympathetic nerves. *Ann. NY Acad. Sci.* **253**, 528–534.