Aluminum-induced Neurofilamentous Changes in Cultured Rat Dorsal Root Ganglia Explants

Mark R. Gilbert,^{1,5} Beverly L. Harding,¹ Paul N. Hoffman,² John W. Griffin,^{1,3} Donald L. Price,^{1,4} and Juan C. Troncoso^{1,4}

Departments of ¹Neurology, ²Ophthalmology, ³Neuroscience, and ⁴Pathology, and the ⁵Oncology Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Intrathecal administration of aluminum (Al) salts to susceptible species causes prominent accumulations of neurofilaments (NFs) in neurons of the CNS. Involved nerve cells display abnormal phosphorylation of perikaryal NFs, impaired axonal transport of NFs, and reduced levels of mRNA for NF proteins. Further understanding of the pathogenesis of Al toxicity has been limited by difficulties inherent in the available *in vivo* systems. For this reason, we have developed a model to study the effects of Al on cultured sensory neurons.

Explant cultures of rat dorsal root ganglia (DRG) were exposed to 1 mM aluminum lactate for 1 d, 3 d, or 7 d and then examined morphologically. Accumulations of NFs were noted as early as 1 d after exposure, and prominent masses of NFs were seen at 3 and 7 d. Northern analysis of mRNA extracted from the cultured ganglia showed that high, medium, and low molecular weight NF protein mRNA levels were markedly reduced compared to control values by 1 d of exposure. Class II β -tubulin mRNA was also moderately decreased. Reversibility of toxicity was assessed by removing the aluminum lactate from the medium after a 3 d exposure and examining the cultures 1 week later. The perikaryal masses of NFs dispersed and the levels of mRNA coding for the NF proteins and class II β -tubulin increased.

The neurotoxic effects of Al on cultured DRG recapitulates the effects of intrathecal administration of Al on animals; this model produces similar changes in neuronal morphology with neurofilamentous masses and similar modifications of NF gene expression. The development of these NF abnormalities in neurons from rat DRG suggests that the apparent selective vulnerability of certain animal species and neurons to Al neurotoxicity is related to the access of the toxin to its target. This culture system will be useful for studying the effects of Al and possibly other toxins in a controlled setting.

The intrathecal administration of aluminum salts into young rabbits causes prominent accumulations of neurofilaments (NFs) (Terry and Pena, 1965; Embree et al., 1967; Wisniewski et al.,

1967; Exss and Summer, 1973; Selkoe et al., 1979; Troncoso et al., 1982; Bizzi et al., 1984; Cole et al., 1985; Bizzi and Gambetti, 1986; Muma et al., 1988) as well as abnormal phosphorylation (Bizzi and Gambetti, 1986; Troncoso et al., 1986) of NFs in the perikaryon of selected neuronal populations (Wisniewski et al., 1967; Troncoso et al., 1982), associated with a marked decrease in the axonal transport of NFs (Bizzi et al., 1984; Troncoso et al., 1985). In anterior horn cells, levels of mRNAs for all three NF proteins fall markedly, with a moderate reduction in levels of tubulin mRNA, as assessed both by *in situ* hybridization and Northern analysis (Muma et al., 1988).

Although the molecular pathogenesis of Al neurotoxicity is not well understood, recent studies have demonstrated that Al and other multivalent cations can cause aggregation of purified NFs *in vitro* (Troncoso et al., 1990). This observation suggests that free Al ions, acting as bridges among charged residues on neighboring filaments, may cause cross-linking and accumulation of NFs in the neuronal perikaryon. Further pathogenetic studies of Al toxicity, however, have been particularly difficult to pursue in animal models because the agent must be introduced intrathecally rather than systemically, only certain neuronal populations have been found susceptible, and only some species, notably rabbits, are affected by the agent. For these reasons, we used an *in vitro* model, which has inherent advantages in studies of pathogenetic mechanisms. Culture systems allow control over toxin exposure, permit frequent observation and sampling, and provide more uniform populations of cells for study. In this investigation, we asked whether explants of rat dorsal root ganglia (DRG) *in vitro* might be susceptible to the toxic effects of Al, even though the species and the neuronal population have previously been considered insusceptible to Al.

Materials and Methods

Establishing the cultures

Explant cultures of dorsal root ganglia were established as described previously (Wood, 1976). Timed pregnant Sprague–Dawley rats, at 16 d gestation, were deeply anesthetized with methoxyflurane (Metaflane, Pittman-Moore, Atlanta, GA), and the uteri were removed under sterile conditions in a laminar flow hood. Individual fetuses were removed from the uterus, and the spinal cord with attached DRG was then extracted using a dissecting microscope. The ganglia were removed at the dorsal roots by using jeweler's forces and placed into a 35 mm tissue culture dish that had been coated with rat tail collagen. Eight ganglia were placed in each dish containing 1 ml of N2 (defined medium) composed of Ham's F-12 (50%, GIBCO, New York, NY), Dulbecco's modified essential medium (50%, GIBCO), insulin, progesterone, selenium, putrescine, and glucose (Sigma, St. Louis, MO), and supplemented with 7S-NGF (nerve growth factor, Advanced Biotechnologies, Inc., Silver Spring, MD). The culture medium was replenished twice a

Received Jan. 10, 1991; revised Oct. 29, 1991; accepted Dec. 6, 1991.

We thank Frank Barksdale, Carol Rubright, and Adelaine Stocks for their assistance with the morphologic studies, Marcus Shagogue for the production of the photomicrographs, and Dr. Pamela Talalay for editorial assistance. This work was supported by NIH Grants NO1339 (M.R.G.), NS RO1 17484 (J.W.G.), and NS 25369 and NS 10580 (J.C.T.). A portion of this work was presented at the 1989 American Association of Neuropathology meeting in Dallas, Texas.

Correspondence should be addressed to Dr. Mark Gilbert, Department of Medicine, Montefiore University Hospital, 3459 Fifth Avenue, Pittsburgh, PA 15213. Copyright © 1992 Society for Neuroscience 0270-6474/92/121763-09\$05.00/0

Figure 1. Quantitation of argyrophilic perikaryal masses in DRG neurons. The percentage of neuronal nuclei with dark-staining masses was calculated in the aluminum-treated and untreated cultures. The error bars represent the SEM. *, $p < 0.01$ compared with control (untreated) cultures.

week. Cultures were maintained in a humidified incubator at 35°C containing 8% CO₂.

Experimental design

Cultures were maintained for 4 weeks in N2 defined medium. At this time, phase-contrast microscopic examination of the cultures showed dense radial outgrowth of neurites from the central ganglia. The average neurite length was 0.5–0.6 cm. The 4 week time point was chosen because previous experiments indicated that there is very little further elongation of neurites after this time on the collagen matrix. Additionally, previous investigations have shown that mRNA levels of low molecular weight NF protein (NF-L), class II β -tubulin, and GAP-43 changed little with increasing age after 4 weeks *in vitro* (Gilbert et al., 1989).

The explant cultures received aluminum lactate at a concentration of 1 mM in the nutrient medium for varied times (1 d, 3 d, 7 d); at physiologic pH the concentration of free ionic aluminum has been estimated to be 3×10^{-12} M (Macdonald and Martin, 1988). The start of the exposure was adjusted for each group so that the completion of the exposure time occurred exactly at 5 weeks *in vitro*. To assess recovery from exposure, cultures were incubated with 1 mM aluminum lactate in the medium for 3 d, washed, and then maintained in N2 defined medium for 1 week more. Control cultures continued to receive nutrient medium. At the end of the exposure period, 55–65 ganglia were removed from both experimental and control groups and stored at –80°C for mRNA analysis. The remaining cultures were fixed in either 10% for-

malin or 2% glutaraldehyde for subsequent histologic or electron microscopic examination.

Histologic techniques

Light microscopy. Ganglia explants fixed in 10% formaldehyde were dehydrated through alcohols, embedded in paraffin, and sectioned at 8 μ m. Sections were stained using the silver method of Bielchowsky.

Electron microscopy. Ganglia explants were fixed in 2% glutaraldehyde, postfixed with 0.1% osmium tetroxide, dehydrated, and infiltrated with an Epon/Araldite mixture in the tissue culture dishes. Following polymerization at 60°C for 1 d, the plastic layer containing the cells was separated from the dish, and explants were remounted and reembedded. Sections (1 μ m) were stained with toluidine blue for light microscopy. Ultrathin sections were cut at 80 nm, stained with uranyl acetate/lead citrate and examined in a transmission electron microscope at 80 kV.

Measurement of mRNA levels

The ganglia were homogenized in 4 M guanidine isothiocyanate and purified by centrifugation through a cushion of 5.7 M cesium chloride at 41,000 rpm for 18 hr in a TLS-55 rotor. The RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water at 65°C, purified by phenol extraction, and concentrated by ethanol precipitation. RNA was redissolved in a final volume of 12 μ l of DEPC water, and its concentration was determined by measuring optical density at a wavelength of 260 nm. This procedure yielded 10–12 μ g of total RNA from the processed 55–65 ganglia. The RNA was analyzed on 1% agarose gels containing 6.6% formaldehyde; 5 μ g of RNA were used on each gel slot. After electrophoresis, the RNA was blotted onto nylon membranes (Gene Screen, Du Pont, Wilmington, DE) and hybridized with ³²P-cDNA probes for 2–3 d. Cloned DNA probe for high molecular weight NF protein (NF-H) was generously provided by Dr. Ivan Lieberberg (Athena Neurosciences, San Francisco, CA); the cDNA probe for medium molecular weight NF protein (NF-M) by Dr. David Nelson (Baylor College of Medicine); the cDNA probe for NF-L, by Dr. Nicholas Cowan (New York University); and the cDNA probe for class II β -tubulin, by Dr. Stephen Farmer (Boston University). The cDNA probes were labeled with ³²P by using Klenow agent in the presence of random primers and labeled dATP. Blots were washed with 0.1% SDS, 0.1 M saline-sodium citrate (3 $\times$ 20 min) at 50°C. The labeled hybrids were detected by autoradiography with x-ray film (Kodak, Rochester, NY). Integrated optical density from the autoradiographies was determined with a computerized image analysis system (Loats, Inc., Westminster, MD).

Results

Histology

As displayed in Figure 1, examination of histologic sections stained by the Bielchowsky method demonstrated argyrophilic perikaryal masses in 15% of neuronal cell bodies in untreated cultures. In contrast, in aluminum-treated cultures, up to 50% of neurons contained argyrophilic masses in the perikaryon. Furthermore, these masses stained more intensely in the aluminum-treated ganglia than in controls.

Electron microscopy

Electron microscopic examination confirmed a progressive accumulation of NFs in the perikaryon over the course of the Al

Figure 2. Electron micrograph of perikaryal region of cultured DRG neuron after 1 d of exposure to Al. *A*, Early coalescence of filamentous structures in the left perinuclear region. *B*, Higher magnification of the perinuclear region boxed in *A*, demonstrating disarray of filaments. *C*, Portion of the perinuclear region with NFs the predominant structures present (from boxed region in *B*). Scale bars: *A* and *B*, 1 μ m; *C*, 0.1 μ m.

Figure 3. Electron micrograph of perikaryal region of cultured DRG neuron after 3 d of exposure to Al. *A*, Marked coalescence of filaments in the perinuclear region. *B*, Higher magnification of the region of the perinuclear mass boxed in *A*, showing almost complete absence of other cytoplasmic elements. *C*, Section of the perinuclear mass showing almost exclusively NFs (from boxed region in *B*). Scale bars: *A* and *B*, 1 μ m; *C*, 0.1 μ m.

Figure 4. Electron micrograph of perikaryal region of cultured DRG neuron after 7 d of exposure to Al. *A*, Dense perinuclear mass with rare cytoplasmic elements. *B*, Disordered filaments in the perinuclear mass (from boxed region in *A*). *C*, Section of the perinuclear mass showing almost exclusively NFs (from boxed region in *B*). Scale bars: *A* and *B*, 1 μ m; *C*, 0.1 μ m.

Figure 5. Electron micrograph of perikaryal region of cultured DRG neuron 7 d after Al was removed from the medium. *A*, Perinuclear region without a distinct filamentous mass. *Inset*, Cytoplasmic elements and microtubules are noted with NFs (from boxed region in *A*). Scale bars: *A*, 1 μ m; *inset*, 0.5 μ m.

exposure. After 1 d, an increased number of NFs was detected in a portion of the perikaryon (Fig. 2*A,B*). NFs were scattered among normal organelles and other cytoplasmic elements (Fig. 2*C*). After 3 d, NFs accumulated in the perinuclear region (Fig. 3*A*) with almost complete exclusion of other elements (Fig. 3*B*). Higher magnification confirmed the nearly exclusive presence of NFs in the region (Fig. 3*C*). After 7 d, the neurofilamentous

mass was similar in appearance to the 3 d exposure (Fig. 4*A–C*). At both 3 and 7 d, filaments in the masses were densely packed and had relatively scarce side-arms similar to NFs in the perikaryon of untreated neurons.

One week after removal of the aluminum from the medium, the cells showed evidence of recovery. There was dispersal of the neurofilamentous accumulations from the perinuclear region

Figure 6. Electron micrograph of perikaryal region of untreated cultured DRG neuron at the same day *in vitro* as treated cultures (*inset* is from boxed region in *A*). Scale bars: *A*, 1 μ m; *inset*, 0.5 μ m.

(Fig. 5*A*), and normal cellular organelles reappeared in this region (Fig. 5, *inset*). Control cultures showed a normal number and distribution of NFs in the perikaryon (Fig. 6).

mRNA analysis

mRNA levels for all three NF proteins were markedly decreased after only 1 d of exposure (Fig. 7) and remained very low for

the entire 7 d exposure. However, levels of class II β -tubulin mRNA showed only a moderate decrease over the course of the experiment. Control cultures showed levels of NF mRNAs that were intermediate between the low levels in DRG of newborn rats and the high levels found in adults. These observations are consistent with our previous evaluation of age-related changes in cultured ganglia (Gilbert, 1989). One week after removal of

Figure 7. Comparison of changes in NF (NF-H, NF-M, NF-L) and class II β -tubulin (TUB) mRNA levels with duration of exposure to Al. *NEWBORN*, mRNA from newborn rats; *ADULT*, mRNA from 10-week-old rats; *CONTROL*, untreated cultures of the same age as treated cultures.

the aluminum from the medium, levels of NF and tubulin mRNA levels were increased in comparison to cultures harvested without a recovery period (Fig. 8).

Discussion

This study demonstrates that exposure of neurons in DRG explant cultures to aluminum lactate causes perikaryal accumulations of NFs in the perikaryon and that this change is accompanied by a marked reduction in levels of NF mRNA. The changes are similar to those described following Al exposure of DRG explants from fetal rabbits (Seil et al., 1969), with *in vivo* exposure of rabbits (Wisniewski et al., 1967; Troncoso et al., 1982), and following Al exposure of a neuroblastoma cell line (Miller and Levine, 1974; Shea et al., 1989). This observation has important implications. It is now possible to use cultured rat DRG explants to explore the molecular pathogenesis of aluminum-induced accumulation of NFs in aluminum toxicity. In addition, the fact that cultured rat DRG neurons develop accumulations of NF after exposure to aluminum salts suggests that neither the differences in vulnerability among neuronal populations nor those among species are absolute.

There are several possible explanations for this apparent species related difference in susceptibility to aluminum exposure *in vivo*. The failure to induce NF changes with aluminum may be related to interspecies differences in clearance or access of the toxin to neurons. In contrast, permeability barriers are absent in the culture system. Cell viability depends on adequate diffusion of nutrient medium, thereby assuring that all cells are exposed to toxin. Furthermore, differences in the concentration or stability of the cellular pool of NFs during development may also be important in determining the susceptibility to aluminum. The neurite cytoskeleton in the cultured DRG is similar to that found in immature animals (Gilbert, 1989), such as the young rabbits used in aluminum studies.

Figure 8. Changes in neurofilament (NF-L) and class II β -tubulin (TUB) mRNA levels after removal of Al from the nutrient medium. *CONTROL*, Untreated cultures of the same age as treated cultures.

Measurement of levels of NF mRNA revealed a marked decrease for all three NF proteins even after only 24 hr of exposure to aluminum. This change in mRNA levels is associated with an increase in the percentage of neurons with perikaryal argyrophilic masses and segregation of NFs in the perinuclear region by ultrastructural evaluation. These morphologic and mRNA changes persisted for the entire 7 d exposure to aluminum. Levels of tubulin mRNA were moderately lowered, indicating that the consequences of Al exposure, although predominantly affecting the NFs, also cause a reversible decrease in class II β -tubulin gene expression. There is evidence that aluminum salts stabilize microtubules and may alter tubulin subunit equilibrium (Macdonald et al., 1987), which could account for the changes in tubulin mRNA levels. During recovery, manifested by a gradual dispersal of the neurofilamentous accumulation in the perikaryon, levels of NF mRNA were increased. The basis for this regulation of NF mRNA is not known, but our data suggest that the control of NF gene expression may be related to changes in the concentration of NFs in the perikaryon or to a direct effect of Al on mRNA production or stability.

This tissue culture model provides a useful system to study the consequences of NF abnormalities because it allows careful control of the experimental conditions, including the concentration of the neurotoxic agent, as well as permitting ongoing observation of morphologic changes and toxicity. It also provides an isolated group of uniformly exposed neuronal cells that enable in-depth molecular investigation of the consequences of aluminum exposure and control of NF gene expression.

References

- Bizzi A, Gambetti P (1986) Phosphorylation of neurofilaments is altered by aluminum intoxication. *Acta Neuropathol (Berl)* 71:154–158.
- Bizzi A, Crane RC, Autilio-Gambetti L, Gambetti P (1984) Aluminum effect on slow axonal transport: a novel impairment of neurofilament transport. *J Neurosci* 4:722–731.
- Cole GM, Wu K, Timiras PS (1985) A culture model for age-related human neurofibrillary pathology. *Int J Dev Neurosci* 3:23–32.
- Embree LJ, Hamberger A, Sjostrand J (1967) Quantitative cytochemical studies and histochemistry in experimental neurofibrillary degeneration. *J Neuropathol Exp Neurol* 26:427–436.
- Exss RE, Summer GK (1973) Basic proteins in neurons containing fibrillary deposits. *Brain Res* 49:151–164.
- Gilbert MR, Harding BL, Hoffman PN, Griffin JW (1989) Cytoskeletal changes during growth and regeneration of cultured dorsal root ganglia. *Soc Neurosci Abstr* 15:350.8.
- Macdonald TL, Martin RB (1988) Aluminum ion in biological systems. *Trends Biochem Sci* 13:15–19.

- Macdonald TL, Humphreys WG, Martin RB (1987) Promotion of tubulin assembly by aluminum ion *in vitro*. *Science* 236:183-186.
- Miller CA, Levine EM (1974) Effects of aluminum salts on cultured neuroblastoma cells. *J Neurochem* 22:751-758.
- Muma NA, Troncoso JC, Hoffman PN, Koo EH, Price DL (1988) Aluminum neurotoxicity: altered expression of cytoskeletal genes. *Mol Brain Res* 3:115-122.
- Seil FJ, Lampert PW, Klatzo I (1969) Neurofibrillary spheroids induced by aluminum phosphate in dorsal root ganglia neurons. *J Neuropathol Exp Neurol* 28:74-85.
- Selkoe DJ, Liem RK, Yen S-H, Shelanski ML (1979) Biochemical and immunological characterization of neurofilaments in experimental neurofibrillary degeneration induced by aluminum. *Brain Res* 163:235-252.
- Shea TB, Clarke JF, Wheelock TR, Paskevich PA, Nixon RA (1989) Aluminum salts induce the accumulation of neurofilaments in perikarya of NB2a/d1 neuroblastoma. *Brain Res* 492:53-64.
- Terry RD, Pena C (1965) Experimental production of neurofibrillary degeneration. 2. Electron microscopy, phosphatase histochemistry and electron probe analysis. *J Neuropathol Exp Neurol* 24:200-210.
- Troncoso JC, Price DL, Griffin JW, Parhad IM (1982) Neurofibrillary axonal pathology in aluminum intoxication. *Ann Neurol* 12:278-283.
- Troncoso JC, Hoffman PN, Griffin JW, Hess-Kozlow KM, Price DL (1985) Aluminum intoxication: a disorder of neurofilament transport in motor neurons. *Brain Res* 342:172-175.
- Troncoso JC, Sternberger NH, Sternberger LA, Hoffman PN, Price DL (1986) Immunocytochemical studies of neurofilament antigens in the neurofibrillary pathology induced by aluminum. *Brain Res* 364:295-300.
- Troncoso JC, March JL, Häner M, Aebi U (1990) Effect of aluminum and other multivalent cations on neurofilaments *in vitro*: an electron microscopic study. *J Struct Biol* 103:2-12.
- Wisniewski HM, Narkiewicz O, Wisniewski K (1967) Topography and dynamics of neurofibrillary degeneration in aluminum encephalopathy. *Acta Neuropathol (Berl)* 9:127-133.
- Wood P (1976) Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. *Brain Res* 115:361-375.