Neurofilament Antigens in Acrylamide Neuropathy

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Abstract. After repeated exposure, acrylamide (AC) produces degeneration of distal axons. Because neurons whose axons have been injured (e.g. by axotomy) show alterations in their structural and chemical properties, the present study was designed to differentiate the direct effects of AC intoxication from neuronal responses secondary to axonal injury caused by AC. Rats were given AC as either a single high dose (75 mg/kg), or as daily intraperitoneal injections (30 mg/kg, six days per week for four weeks). Dorsal root ganglia of the fifth lumbar level, L5, were examined using a variety of monoclonal antibodies directed against nonphosphorylated (2-135) and phosphorylated (03-44, 06-17, 07-05) epitopes of 145 and 200 kilodalton neurofilament proteins. In control rats, antibody 2-135 stained axons and neuronal cell bodies; antibodies against phosphorylated epitopes of neurofilaments stained only axons distal to the glomerulus. Following chronic AC intoxication, all three antibodies directed against phosphorylated epitopes of neurofilaments (particularly 07-05) demonstrated intense immunoreactivity in 20–30% of neuronal cell bodies. In addition, the glomerular region of these axons was stained. Electron microscopy revealed many chromatolytic cells containing few neurofilaments. In contrast, a single high dose of AC produced no abnormal staining of neuronal cell bodies at a time when slow axonal transport was impaired. Our findings are compared to those observed following axotomy and to those occurring in aluminum-intoxicated rabbits, two experimental disorders in which altered distributions of phosphorylated filaments have been documented.

Key Words: Acrylamide; Dorsal root ganglia; Neurofilament epitopes; Neurofilament, nonphosphorylated; Neurofilament, phosphorylated.

INTRODUCTION

Chronic exposure to acrylamide (AC) produces a distal axonopathy in which affected fibers undergo Wallerian-like degeneration (1). Acrylamide intoxication is associated with impairment in both fast (2–5) and slow (6) axonal transport. Of particular interest in the present report are changes in slow transport occurring with different schedules of AC administration: single high doses of AC appear to directly impair slow axonal transport, leading to the subsequent development of neurofilamentous swellings in proximal axons; with lower doses of AC during repeated intervals, changes in slow transport are similar to those induced by axotomy and include a reduction in the amount of transported neurofilament proteins with sec-
ondary reductions in axonal caliber (axonal atrophy) (6, 7). Thus, comparison of abnormalities occurring with these two schedules (i.e. single dose, acute intoxication, and chronic exposure) should allow differentiation of the direct neurotoxic effects of AC from responses secondary to axonal degeneration.

The present investigation was designed to examine the staining patterns of different epitopes of neurofilament proteins in these two types of experimental manipulations. Immunocytochemical methods were used to visualize the distribution of phosphorylated and nonphosphorylated epitopes of neurofilament proteins in neurons of the dorsal root ganglia (DRG). Previous studies using well-characterized antibodies (8–10) have disclosed that phosphorylated epitopes are particularly enriched in axons (but not expressed in perikarya), whereas nonphosphorylated epitopes are present in perikarya, dendrites, and axons. In this study, we have demonstrated a maldistribution of phosphorylated epitopes of neurofilament proteins in rats following chronic, but not single high dose, AC intoxication. This result is consistent with the concept that changes in the distribution of phosphorylated neurofilaments in AC intoxication reflect a secondary response of neurons to axonal injury.

MATERIALS AND METHODS

Subjects and Schedule of Intoxication

Fifteen three-week old Sprague-Dawley male rats were used for immunocytochemistry and were divided into two groups: single high-dose animals (n = 9); and chronically intoxicated rats (n = 6). The former group received a single intraperitoneal (IP) injection of AC (75 mg per kg); the latter group was given daily intraperitoneal injections (30 mg per kg, six days/week (wk) for four wk) of AC. Three four-wk-old and three seven-wk-old rats served as age-matched controls for immunocytochemical studies. Three additional chronically intoxicated rats and three age-matched controls from a previous study (6) were used for electron microscopy.

Tissue Preparation, Histological Processing, and Electron Microscopic Preparation

Three rats from the single high-dose group were perfused seven days following administration of AC together with four-wk-old control rats; additional rats (two per group) were perfused at 1, 3, and 14 days. Chronically intoxicated rats were perfused at seven wk of age (four wk of intoxication) together with age-matched controls. Animals were anesthetized with chloral hydrate (400 mg/kg, IP) and perfused through the ascending aorta with either 4% formaldehyde (for immunocytochemistry) or 5% glutaraldehyde (for ultrastructural study) in 0.1 M sodium phosphate buffer (pH 7.4). The fifth lumbar (L5) DRG were dissected, and formaldehyde-fixed tissues were postfixed in 10% formalin overnight. Formaldehyde-fixed DRG were embedded in paraffin and sectioned (10 μm). Glutaraldehyde-fixed DRG were postfixed in osmium tetroxide, embedded in Epon/Araldite, and sectioned (1 μm). Paraffin sections were used for immunocytochemistry (see below). The 1-μm sections were stained with toluidine blue and examined by light microscopy; thin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H-600 electron microscope.

Antineurofilament Antibodies and Immunocytochemistry

Antibody 2-135 is directed against a nonphosphorylated epitope of the 200 kilodalton (kD) neurofilament polypeptide, 03-44 recognizes a phosphorylated epitope in the 200 kD polypeptide, and antibodies 06-17 and 07-05 delineate phosphorylated epitopes shared by the 200 and 145 kD polypeptides (8–10). For immunocytochemistry, tissue sections were deparaffinized, incubated with 3% goat serum, and incubated overnight with one of the primary antibodies (1:1,000 dilution in 1% normal goat serum). Sections were washed and incubated for one hour (h) with the secondary antibody (goat antirabbit) diluted 1:40; after washing, sections
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were incubated for one h in mouse peroxidase-antiperoxidase diluted 1:200. Immunoreactivity was visualized with 0.05% diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxidase (eight minutes). All washes were carried out in 0.05 M Tris buffer, 1.5% saline; all incubations were performed at room temperature except for the incubation with the primary antibody, which was done at 4°C. Some sections were lightly counterstained with cresyl violet. Sections were coverslipped and examined by light microscopy. Immunostaining was assessed in three ganglia from three chronically intoxicated AC rats (selected for their representative degree of staining) by counting the total numbers of neuronal cell bodies stained with cresyl violet and the total number of immunostained neuronal cells.

RESULTS

Control DRG Neurons

The morphology of the DRG has been well described (11–18). On the basis of their size and the abundance and distribution of ribosomes, neurons have been divided into two types: large, light nerve cells; and small, dark neurons (11). Axons, particularly of larger neurons, coil to form a glomerulus; myelin ensheaths the distal part of the axon just proximal to its bifurcation into central and distal processes. In the DRG of control rats, both neuronal perikarya and axons exhibit immunoreactivity to nonphosphorylated neurofilaments that is best visualized with antibody 2-135 (Fig. 1A); this immunoreactivity was more intense in perikarya than in glomerular and myelinated segments of axons. Axons in dorsal and ventral roots were well stained with antibodies against phosphorylated epitopes (i.e. 03-44, 06-17, and 07-05), but glomerular regions were unstained. An occasional (<1%) large neuronal perikaryon showed slight immunoreactivity when stained with antibodies directed against phosphorylated epitopes; of these antibodies, 07-05 tended to show more perikaryal immunoreactivity than the other antibodies (Fig. 1B).

Chronic AC Intoxication

At the light microscopic level, chromatolysis was observed in some neuronal perikarya, particularly in large, light nerve cells (Fig. 2A). Chromatolytic cells were recognized by eccentrically placed nuclei, infolding and capping, and displacement of Nissl material to the periphery. Ultrastructural analysis of chromatolytic parts of these cells disclosed alterations in the normal arrangement of the rough endoplasmic reticulum (Fig. 2B), as has been described in other types of neurons following axotomy (19–24). Perikarya of these chromatolytic neurons appeared to have reduced neurofilament content (Fig. 2B), compared to normal perikarya from control rats (Fig. 2C), a finding in agreement with results of other investigators (25, 26). The most striking alteration following chronic AC intoxication was observed with antibodies against phosphorylated neurofilament epitopes (Fig. 3) in all six animals studied. With all three antibodies, particularly 07-05 (Fig. 3B), we demonstrated homogeneous, often intense, immunoreactivity in approximately 15–30% of DRG perikarya as well as immunoreactivity in the glomerular portion of axons; the wide range in the number of stained cells was due to interanimal variability. This staining appeared to be most conspicuous in neurons with eccentric nuclei, but this was not always the case and not all cell bodies with eccentric nuclei were stained. The distribution of nonphosphorylated epitopes (2-135) (Fig. 3A) did not differ from controls.

Axonal alterations in the L5 DRG in this model has been reported previously (6). Virtually all sensory fibers showed marked atrophy of axons at the level of the DRG, whereas motor fibers were not altered (Fig. 2A).
Fig. 1. L5 DRG from a seven-wk control rat. Peroxidase–antiperoxidase, ×55. Insets ×190. A. Antibody 2-135, against nonphosphorylated neurofilament epitopes, shows staining of perikarya and axons (including glomerular regions); staining is most intense in large, light cells. Regions devoid of staining correspond to nuclei of perikarya. Inset: Staining of cell bodies and glomeruli (arrow). B. Antibody 07-05, against phosphorylated neurofilament epitopes, shows axonal staining but a lack of immunoreactivity in perikarya and glomerular portions of axons. Inset: View of perikarya. Section was lightly counterstained with cresyl violet. Identical results were obtained in four-wk-old control rats.

Single High-Dose AC Intoxication

In confirmation of our previous findings (6), sensory axons were moderately enlarged and exhibited increased content and density of neurofilaments. Staining patterns obtained with neurofilament antibodies in single-dose AC-intoxicated rats were not different than controls at a time when slow transport was known to be altered
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(i.e. seven days) (6) or at earlier (i.e. greater than three days) or later (i.e. 14 days) time points. However, some suggestive qualitative differences were observed; the reaction product was very intense with 2-135, particularly in large cell bodies at seven days (Fig. 4A), and large axons of the dorsal root appeared more intensely stained with antibodies against phosphorylated epitopes, i.e. 03-44, 06-17, and 07-05, at this time (Fig. 4B).

At the level of the DRG, motor fibers appeared normal, but they were modestly enlarged in the region of the proximal ventral root (6).

DISCUSSION

Neurofilaments consist of three proteins (200, 145, and 68 kD) (27); the 68 kD subunit forms the core; the 145 kD proteins intertwine around the core; and the 200 kD subunit composes the sidearms (28, 29). The distribution of these proteins appears to have a regional topography within cells. Perikarya are enriched in the 68 and 145 kD proteins; and the 200 kD subunit appears to be more abundant in axons (30). Nonphosphorylated epitopes of the 145 and 200 kD proteins are present in perikarya, dendrites, and axons and phosphorylated epitopes, present at very low levels in the perikarya, are markedly enriched in axons (10). Sidearms, thought to play a role in linking neurofilaments to each other and to other organelles (31–33), are believed to be principally composed of the 200 kD subunit, which is rich in phosphorylation sites (34). The present investigation demonstrates that nonphosphorylated filaments are present in both perikarya and axons of normal DRG neurons but that the phosphorylated 200 kD protein is present at very low levels in perikarya and axonal glomerulus, but markedly enriched in axons distal to the glomerulus. This finding suggests that, in these neurons, phosphorylation of the 200 kD sidearms does not normally occur until after the initial segment, i.e. at the heminode proximal to the first myelinated internode.

Changes in Neurofilament Antigens in AC Intoxication

The major finding of this study is that phosphorylated epitopes of neurofilament proteins, which are not normally present in perikarya (10), appear in perikarya of some DRG neurons following chronic, but not single high-dose, intoxication with AC. In this investigation, we cannot be certain if antibodies recognize phosphorylation of the 145 kD protein or the 200 kD protein or both (see Materials and Methods). However, the finding that antibody 03-44, which normally recognizes only the 200 kD protein (8), produces similar results to those obtained with antibodies 06-17 and 07-05, which react with both the 145 and 200 kD protein (8, 10), suggests that at least some phosphorylation of perikarya and glomerular neurofilaments involve the 200 kD protein.

The alteration in the pattern of neurofilament phosphorylation does not appear to arise as a direct toxic effect of AC on perikarya of nerve cells. Based upon results using the present series of monoclonal antibodies (35) or other monoclonal antibodies that have either been shown to, or appear to, also recognize phosphorylated neurofilament epitopes (18, 36–38), the patterns of staining in chronically AC-intoxicated animals are clearly different from those present in acutely intoxicated rats and from those occurring in controls. In this study, control and acutely intoxicated animals showed small numbers (<1%) of DRG neurons with traces of immunoreactivity for phosphorylated epitopes of neurofilaments, but these images never approached the

intensity seen in chronically intoxicated animals or in rats in whom sciatic nerves had been crushed and allowed to regenerate (35). Dorsal root ganglia cells containing phosphorylated neurofilaments often demonstrated eccentric nuclei and appeared to correspond to large, light cells that exhibited ultrastructural features of chromatolysis. Similar staining patterns of DRG perikarya have also been observed following sciatic nerve crush, in which all nerve fibers are injured (35, 36, 38). Thus, it appears likely that patterns of immunoreactivity observed in chronically intoxicated animals represent an axotomy-like response of larger DRG cells and are not an indication of a direct toxic effect on these neurons (39, 40). Because the pattern of immunoreactivity in chronically intoxicated rats was not different than that observed in regenerating DRG cells (35, 36, 38), we believe that the present results support the hypothesis (6) that at least some alterations observed in chronically AC-intoxicated animals represent a secondary response of neurons to a toxin-induced axonal injury. This finding has important implications for understanding the pathogenesis of morphological alterations in DRG cell bodies in AC neuropathy.

Recent advances in understanding the pathogenesis of the neuropathy induced by AC indicate that this toxin probably acts at both the level of the axon and perikaryon (25, 26, 39–47). Unique alterations have been described in Purkinje cells of rats in early stages of AC administration (46). Sterman (25, 48) and Jones and Cavanagh (26) have described early ultrastructural alterations in cell bodies of DRG in AC neuropathy. These abnormalities, including increased numbers of mitochondria, eccentrically placed nuclei, peripherally placed rough endoplasmic reticulum, and regions devoid of neurofilaments, show some features in common with changes observed in chromatolytic neurons (19–22, 24). The concept that perikaryal changes may represent a direct toxic effect of AC on cell bodies (25, 26, 48) was based upon quantitative differences between AC neuropathy and axotomy (25, 48) as well as the observation that these changes preceded axonal degeneration (26). An alternative possibility, suggested by the present study, is that at least some of these changes might arise as a secondary response to axonal injury occurring before axonal degeneration (6), perhaps due to an impairment in retrograde transport (2–5). This hypothesis is supported by the observation that administration of botulinum toxin, an agent known to impair neuromuscular transmission (49–52), also induces atrophy of proximal axons (53). This finding suggests that changes in nerve–muscle interactions, possibly mediated by changes in trophic interactions, can initiate axotomy-like responses in the perikarya of neurons and that these changes can occur without evidence of axonal degeneration. Thus, although AC does appear to have a direct toxic effect upon perikarya (46), not all alterations in the perikarya represent direct toxic effects on neurons.

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**Fig. 2.** A. L5 DRG from chronically intoxicated AC rats. Many cell bodies, particularly large light cells, show typical chromatolytic changes including dispersed Nissl bodies and eccentric nuclei (*). Inset: higher power view of boxed region shows two chromatolytic neurons (*). Note axonal atrophy of sensory fibers. Epon sections (1 \( \mu \)m) stained with toluidine blue. \( \times 175 \). Inset \( \times 200 \). B and C. Large, light cell bodies from chronically intoxicated AC (B) and seven-wk-old control (C) rats. B) The cell shows typical chromatolytic changes, including peripherally displaced endoplasmic reticulum, nuclear eccentricity, capping, and infolding. Insets: Cytoplasm reveals paucity of neurofilaments in cell bodies of chronically intoxicated AC rats (B) compared to (C) the age-matched control rats. \( \times 2,220 \). Insets \( \times 25,000 \).

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Fig. 3. Peroxidase–antiperoxidase staining of an L5 DRG from a chronically intoxicated AC rat. ×55. Insets ×185. A. Antibody 2-135, against nonphosphorylated neurofilament epitopes, shows a similar pattern of staining as in age-matched control animals (see Fig. 1A). Inset: Higher power view of axons. Note that axonal atrophy of fibers in the dorsal root (DR), in contrast to the normal caliber of ventral root (VR) fibers, can be visualized using this neurofilament stain (see Fig. 2A). B. Antibody 07-05, against phosphorylated neurofilament epitopes, demonstrates intense homogeneous immunoreactivity in many neuronal cell bodies. Some of these stained cells also contain eccentric nuclei (arrows). Inset: Higher power view of two of these stained cells, one shows staining of the glomerulus (arrow); this section was lightly counterstained with cresyl violet.
Fig. 4. Peroxidase-antiperoxidase staining of L5 DRG from a rat given a single high dose of AC (75 mg/kg, IP) one wk before fixation. ×100. Insets ×230. A. Antibody 2-135 against nonphosphorylated neurofilament epitopes. Inset: Higher power view showing staining of cell bodies and glomeruli. B. Antibody 07-05 against phosphorylated neurofilament epitopes. Inset: Higher power view showing lack of staining in cell bodies and glomeruli. Staining pattern with both antibodies was not different from control rats, although some suggestive increased staining was observed in the cell bodies and large fibers of the dorsal root with antibodies 2-135 (A) and 07-05 (B), respectively. This section was lightly counterstained with cresyl violet.

Relationship of AC Intoxication to Other Models of Disease and to Human Disorders

Previous studies have shown that, after axonal injury neurofilaments in axons are decreased, a change shown to be due to decreased expression of neurofilament genes (54) and that there is an increase in neurofilament phosphorylation within the cell body (35). This decrease in the synthesis of neurofilament proteins results in a reduction in the total number of neurofilaments within the perikarya and axon and eventually, a wave of reduced axonal caliber moves down the axon at the rate of slow axonal transport (55). The finding that phosphorylated neurofilaments are not observed in perikarya three wk following permanent axotomy (35), a model in which axonal elongation is prevented, suggests that a relationship may exist between the

occurrence of axonal elongation and the appearance of phosphorylated neurofilaments in the cell body. In this context, AC has been shown to impair axonal outgrowth following axonal injury (56, 57) and, in chronically intoxicated animals, only some DRG cells demonstrate neurofilament immunoreactivity.

Abnormal staining of DRG cell bodies has also been observed in aluminum poliomyelopathy (58), a disorder associated with neurofilamentous alterations in axons, perikarya, and dendrites (59–63) and with the impaired transport of neurofilaments (64, 65). However, patterns of neurofilament immunoreactivity in the aluminum model differ from those occurring in animals chronically intoxicated with AC. In aluminum poliomyelopathy, the most intense staining of cell bodies is observed with antibody 03-44, as opposed to 06-17 and 07-05 in the present study. This result does not appear to be a species difference, because staining is also different within cells. Compared to the homogeneous immunoreactivity observed in chronic AC-intoxicated rats, immunoreactivity in aluminum-intoxicated neurons is very heterogeneous, being most intense in regions where neurofilaments accumulate in the perikarya (58). The difference in patterns of immunoreactivity in these experimental models is not well understood.

Finally, abnormal staining by antibodies directed against phosphorylated neurofilament epitopes was not observed in neuronal perikarya up to two wk following a single high dose of AC. This dose has been previously shown to impair axonal transport and produce modest neurofilamentous axonal swellings in the proximal portions of large fibers (6). Thus, studies in AC neuropathy do not support a relationship between abnormal phosphorylation of neurofilaments in the perikarya and a retardation in neurofilament transport (66, 67) in at least some situations.

In conclusion, the present results, using single high-dose and chronic exposure models of AC neuropathy, clearly suggest that at least some morphological alterations in perikarya arising after repeated exposure may not be due to a direct toxic effect of AC upon DRG cell bodies. Therefore, it is important to differentiate direct toxic effects from secondary alterations that may arise in perikarya in situations with axonal degeneration. Thus, our findings may also be relevant to other degenerative disorders, e.g. Alzheimer’s disease, amyotrophic lateral sclerosis, and Parkinson’s disease, in which similar alterations can occur (68, 69), possibly as a secondary response to axonal degeneration.

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