Regulation of Aberrant Neurofilament Phosphorylation in Neuronal Perikarya.
II. Correlation with Continued Axonal Elongation Following Axotomy

BRUCE G. GOLD, PH.D., DANIEL R. AUSTIN, M.S., AND JOHN W. GRIFFIN, M.D.

Abstract. Neurofilaments (NF) are normally poorly phosphorylated in neuronal perikarya and highly phosphorylated in axons. Aberrant NF phosphorylation in the neuronal perikaryon has been demonstrated in a number of human and experimental disorders. In this study, we have asked whether expression of these phosphorylated NF (pNF) epitopes is dependent upon continued axonal regeneration following nerve transection (axotomy). This hypothesis was tested using the neurotoxic chemical acrylamide (AC) which is known to inhibit axonal regeneration following systemic administration. First, we examined whether AC acts at the level of the neuronal perikaryon to inhibit axonal elongation. Systemic, high dose intraperitoneal (IP) AC administration totaling 150 mg/kg (75 mg/kg x 2) did not impair the axotomy-induced reordering of slow axonal transport in the neuronal perikaryon. Next, we studied the ability of AC to directly prevent nerve outgrowth at the growing tips of axons. Superficial injection of AC (0.1 M), which in preliminary studies was found not to produce nerve fiber damage, markedly reduced the extent of nerve outgrowth when injected proximal to a nerve crush; this was shown by a reduction in the extent of radiolabeling and number of axonal sprouts in the distal stump seven days following nerve crush. Using this protocol, a 67% decrease in the number of neuronal perikarya in the L4 and L5 dorsal root ganglia demonstrating immunoreactivity to antibody 07-05 (directed against pNF epitopes) was observed in AC-injected compared to contralateral saline-injected crushed nerves. Taken together, the results indicate that inhibition of axonal regeneration in the distal stump by AC reduces aberrant NF phosphorylation in the neuronal perikaryon following axotomy.

Key Words: Acrylamide; Axon reaction; Axotomy; Dorsal root ganglion; Neurofilament epitopes, phosphorylated; Regeneration; Slow axonal transport.

INTRODUCTION

The two high molecular weight polypeptides comprising the mammalian neurofilament (NF) triplet proteins (1) are extensively phosphorylated (2–6). Monoclonal antibodies which recognize phosphorylated NF (pNF) epitopes reveal a regional heterogeneity in the distribution of pNF in neurons (7, 8); pNF epitopes are present in very low levels in neuronal perikarya but are abundant in axons. Abnormal expression of pNF epitopes in neuronal perikarya has been observed in a variety of human and experimental conditions: motor neuron disease (9–11); Creutzfeldt–Jakob disease (12); progressive supranuclear palsy (13); Alzheimer’s disease, where

From the Center for Research on Occupational and Environmental Toxicology, Oregon Health Sciences University, Portland, Oregon (BGG, DRA) and the Neuromuscular Laboratories, Departments of Neurology and Neuroscience, The Johns Hopkins School of Medicine, Baltimore, Maryland (JWG).

Correspondence to: Bruce G. Gold, Ph.D., Center for Research on Occupational and Environmental Toxicology, L606, The Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, OR 97201.

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they are found in neurofibrillary tangles (14–20); the copper deficiency disorder in lambs, swayback (11); and intoxication by the neurotoxic chemicals acrylamide (AC) (21, 22), aluminum (23, 24), and organophosphates (25). The observation that aberrant NF phosphorylation is also produced by axotomy (26–28) suggests that, in at least some situations, this alteration may arise in neuronal perikarya secondary to axonal injury, i.e. abnormal expression of pNF epitopes in neuronal perikarya is a component of the axon reaction.

Little is known concerning the regulation of NF phosphorylation in the neuronal perikaryon. Previous studies (28) indicate that continued expression of pNF epitopes in neuronal perikarya following axotomy is dependent upon axonal regeneration; prevention of axonal elongation by nerve transection and ligation results in a reduction in the number of dorsal root ganglia (DRG) neurons expressing pNF epitopes compared to crushed nerves three weeks after axotomy. However, interpretation of these results was confounded by differences in the experimental protocols employed (i.e. nature of the lesion and its location along the sciatic nerve) in that study. Thus, in this study we examined whether the neurotoxic agent AC (29, 30), which is known to impair axonal regeneration (31, 32), could also inhibit the abnormal expression of pNF epitopes in neuronal perikarya.

Although AC has been shown to prevent axonal regeneration (31, 32), the mechanism by which this neurotoxic agent inhibits nerve outgrowth is unknown. Based upon previous studies (33–38), at least two possibilities exist. Acrylamide could prevent the production of the regenerative response in the neuronal perikaryon (33–38), perhaps by impairing the reordering of protein synthesis produced following nerve transection (axotomy) (39–43). Alternatively, AC could act directly at the level of the axonal sprouts to inhibit axonal elongation; one possibility, suggested by the presence of giant axonal sprouts filled with membranous materials following AC intoxication (32), is that AC prevents the incorporation of materials into the growing tips of axons. Some evidence (33–36) appears to support a direct toxic effect of AC on the cell body. However, more recent studies (22, 37, 38, 44, 45) demonstrating that chronic AC administration recapitulates many of the alterations observed in the neuronal perikaryon and proximal axon following axotomy (i.e. reordering of slow axonal transport, reduction in proximal axonal caliber, abnormal expression of pNF epitopes in neuronal perikarya, and increased synthesis of the growth-associated protein GAP-43) indicate that the neuronal perikaryon is capable of producing a regenerative-like response in AC-intoxicated animals. Thus, an inability of the cell body to reorder protein synthesis does not appear to explain the marked deficit in nerve outgrowth observed in AC-intoxicated animals. Taken together, these observations suggest that AC may act locally (i.e. at the level of the axonal sprouts) to prevent axonal elongation.

In this study, we have further tested between these two hypotheses. First, we examined the ability of systemic, high doses of AC to directly impair the reordering of slowly transported proteins induced by direct mechanical (crush) injury. Second, we used subperineurial AC injections proximal to a crush injury in the sciatic nerve to study the ability of AC to directly impair nerve outgrowth. Two approaches were used to assess regeneration following subperineurial injection of a dose of AC which was found, in preliminary studies, not to produce any structural damage in the sciatic nerve for up to seven days: 1) radiolabeling of injured motor axons to determine the extent of axonal elongation past the crush site, and 2) electron microscopy of axonal sprouts in the distal stump. Using this model, it was possible to ask whether abnormal expression of pNF epitopes in DRG neuronal perikarya is dependent upon
continued nerve outgrowth following axotomy. Portions of this work have appeared in abstract form (46).

MATERIALS AND METHODS

Slow Axonal Transport Studies

Four three week (wk)-old male Sprague-Dawley rats were given an intraperitoneal (IP) injection of AC (75 mg/kg); age-matched control animals (n = 3) received saline. Twenty-four hours (h) later, the animals were anesthetized with chloral hydrate (400 mg/kg, IP), and the sciatic nerve was crushed on one side; the contralateral side served as non-crushed AC- or saline-treated controls. One wk following the first injection (i.e. four-wk-old), rats were given a second injection of AC (75 mg/kg, IP) or saline. Twenty-four h later, the L4 and L5 ventral horns were microinjected with 3S-methionine (specific activity, 1,200 Ci/mmol) (Amersham Corp., Arlington Heights, IL), as previously described (32, 44). Briefly, the animals were killed six days following radiolabeling (i.e. six days of transport), sciatic nerves were dissected from their spinal roots to their distal branches and cut into 3 mm segments. Each segment was homogenized in sodium dodecyl sulfate/urea/2-mercaptoethanol and applied in sequence to single tracks on a polyacrylamide slab gel (gradient 5 to 17.5%). Following electrophoresis, the gels were fixed, stained with Coomassie blue, dried, and the radioactivity in the bands visualized by gel fluorography.

To determine the distribution of tubulin in AC- and saline-treated nerves (crushed and non-crushed), tubulin bands were cut out of each track, dissolved, and the radioactivity counted by liquid scintillation spectroscopy. Cumulative plots were made of the amount of radioactivity per segment for the first 13 segments (39 mm) since the radioactivity in the tubulin band approached background in more distal nerve segments (see Fig. 1).

Subperineurial Injection and Topical Application of Acrylamide

In preliminary studies, 22 male Sprague-Dawley rats (300–400 g) were anesthetized with chloral hydrate, the sciatic nerve exposed at mid-thigh, and either 10 μl of a solution containing AC (1–8 M) microinjected via a glass micropipette mounted on a micromanipulator connected to an infusion pump (Sage Inst.) or the nerve treated topically (45 minutes) with cotton pledges soaked in the AC solution; control animals (n = 4) had their sciatic nerves treated with saline. Sciatic nerves were fixed in situ with 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) at 1–12 h following AC exposure and processed for electron microscopy (see below).

In subsequent experiments, eight ten wk-old male Sprague-Dawley rats were anesthetized with chloral hydrate, and the sciatic nerves were microinjected via a glass micropipette with 10 μl of 0.1 M (n = 4) or 1.0 M (n = 4) AC; contralateral nerves were injected with saline. Nerves were fixed in situ with phosphate buffered 3% glutaraldehyde at 24 h or seven days and processed for electron microscopy (see below).

For studies of nerve outgrowth following crush, six seven wk-old male Sprague-Dawley rats were anesthetized with chloral hydrate, and the sciatic nerves were microinjected with AC (0.1 M), as above, and crushed twice for 30 seconds using a jeweler's forceps immediately following injection; contralateral nerves were injected with saline prior to crush. Animals were used for radiolabeling (n = 3) or electron microscopic (n = 3) study (see below).

For immunocytochemical study (see below), three seven wk-old male Sprague-Dawley rats were anesthetized with chloral hydrate, and the sciatic nerves were microinjected with AC (0.1 M) and crushed, as above; the contralateral nerves were injected with saline prior to crush. Due to concerns over possible effects of AC in contralateral saline-injected nerves, both sciatic nerves were injected with saline and crushed in another seven wk-old rat. Since no difference was found between saline-injected crushed nerves contralateral to AC-injected nerves and those from the normal animal (see Statistical Analysis), the sciatic nerves were injected with AC and crushed bilaterally in two additional rats.

Animals used for measurement of nerve outgrowth and immunocytochemistry were all studied at eight weeks of age.
Tissue Preparation for Electron Microscopy

Sciatic nerve samples were postfixed in osmium tetroxide, dehydrated in a graded series of alcohols, and embedded in epon/alkaldite. Thick sections (1 μm) were stained with toluidine blue; thin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H-600 electron microscope.

Measurement of Nerve Outgrowth: Radiolabeling Studies

Six days following sciatic nerve injection, the animals (n = 3) were anesthetized with chloral hydrate and the L4 and L5 ventral horns were microinjected with 35S-methionine, as described above. Sciatic nerves were removed (from the spinal cord to its distal branches) 24 h after radiolabeling (i.e. seven days following nerve injection), frozen, cut into 3 mm segments, solubilized, and the radioactivity in each segment counted. Plots of disintegrations per minute (DPM) were made from these data.

Measurement of Nerve Outgrowth: Electron Microscopic Studies

Seven days following sciatic nerve injection, rats (n = 3) were anesthetized with chloral hydrate, heparinized and perfused through the ascending aorta with 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3). The sciatic nerves were sampled (2 mm segments) at 1–3 and 5–7 mm distal to the crush site. Following embedding (see above), the blocks were cut in half prior to sectioning. Sections from these tissue samples were therefore made at 2 and 6 mm distal to the crush site, respectively.

Immunocytochemistry

One wk following subperineurial AC injections, animals (n = 6) were heparinized, anesthetized with chloral hydrate and perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.6). The L4 and L5 DRG were dissected following overnight fixation in situ (4°C), dehydrated in a graded series of alcohols, and embedded in paraffin. Sections (10 μm) were cut and mounted on chrom-alum subbed slides, deparaffinized in xylene and ethyl alcohol, incubated in 3% normal goat serum (one h), and incubated overnight with one of the following primary antibodies (1:1,000 dilution in 1% normal goat serum): antibody 2-135 (directed against a nonphosphorylated epitope of the 200 kilodalton [kDa] NF polypeptide); or antibody 07-05 (directed against phosphorylated epitopes shared by the 200 and 145 kDa polypeptides). Sections were incubated for one h in goat-antimouse secondary antibody (1:20), washed, incubated for one h in mouse peroxidase-antiperoxidase (1:200), and the immunoreactivity visualized with 0.05% diamino benzidine tetrahydrochloride/0.01% hydrogen peroxidase (eight min).

Two additional rats from a previous study (45) were used for Po immunocytochemistry. These animals were given a single injection of AC (75 mg/kg, IP), perfused with 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) at 24 h, and samples of the sciatic and peroneal nerves were processed for light and electron microscopy, as described above. Sections (1 μm) demonstrating dense areas in the axoplasm by light microscopy, and confirmed as membranous materials by electron microscopy (see Results), were stained with Po antiserum (1:500 dilution) using the peroxidase-antiperoxidase method, as previously described (47).

Assessment of Immunostaining

Immunoreactivity was quantitated by counting all cells containing immunoreactivity above background; background staining was minimal in all sections. The proportion of cells demonstrating pNF epitopes (% phosphorylated) was determined for each DRG by counting the total number of neuronal perikarya stained with antibody 07-05 and dividing this value by the total number of neuronal perikarya in the DRG (determined by counting the total number of neuronal perikarya stained with antibody 2-135 and lightly counterstained with cresyl violet). For each treatment group (i.e. AC- and saline-injected), values from L4 and L5 DRG were averaged to give one mean data point for each sciatic nerve.
Statistical Analysis

The percentage of cells which demonstrated pNF epitopes was the same in saline-injected crushed nerves contralateral to AC-injected nerves and those from the normal animal. For purposes of further statistical analysis, AC-injected and saline-injected crushed nerves were pooled from the six rats used for immunocytochemistry. Student’s t-test was used to test for differences between the AC-injected (n = 7) and saline-injected (n = 5) groups.

All values are mean ± SEM.

RESULTS

Slow Axonal Transport Studies

Slow axonal transport in the rat sciatic nerve consists mostly of the cytoskeletal proteins comprising neurofilaments, microtubules and microfilaments (48, 49). Following axotomy, there is a decreased overall prominence of the neurofilament triplet proteins (relative to tubulin and actin) on gel fluorograms (39) (Fig. 1A, B). Previous studies (44) demonstrated a similar reordering of slowly transported proteins in rats given repeated AC exposures. In this study, we asked whether AC, given at a dose sufficient to impair both fast (50, 51) and slow (44) axonal transport, could directly prevent the ability of neuronal perikarya to undergo this regenerative-like response following a crush injury.

An increase in overall prominence of tubulin and actin was observed following axotomy in rats given systemic, high doses of AC (Fig. 1C, D). Quantitation of the radioactivity for tubulin showed a similar increase in the extent of outflow along the nerve in both normal and AC-intoxicated animals following nerve crush compared to the contralateral non-crushed sides (Fig. 2A, B). However, the distance of movement of the slowly transported proteins was reduced in AC-intoxicated rats, as previously described (44); a proportionate decrease in outflow was observed in both crushed and non-crushed nerves from AC-intoxicated rats (Fig. 2B). The data indicates that systemic, high dose AC administration does not prevent the ability of the neuronal perikaryon to reorder the synthesis and delivery of slowly transported proteins to the axon following axotomy (see Discussion). Thus, we next examined the possibility that AC impairs axonal regeneration by a direct axonal effect.

Subperineurial Injection into the Sciatic Nerve

Initial studies were performed to characterize the direct toxic effects of AC on the axon and determine a concentration which does not elicit structural abnormalities following subperineurial injection. In the preliminary studies, ultrastructural examination was performed on nerves either locally injected or soaked in situ (see Materials and Methods) with a range of AC concentrations (1–8 M). Few differences were noted using either methodologies, although non-specific axonal degeneration was less extensive in nerves soaked in 8 M AC than following subperineurial injection of 4 M AC. Local application allowed for delivery of higher doses which elicited specific alterations (see below) prior to the development of degenerative changes. Similar changes were observed between 1–12 h, although the extent of nerve fiber damage was greater at longer intervals following AC exposure; this was most apparent with higher AC concentrations (i.e. 4 and 8 M). Numerous fibers at all concentrations and time-points of study demonstrated accumulations of axonal neurofilaments, mitochondria and membranous materials indicative of axonal transport block. Figure 3A and B shows examples of nerves injected with 1.0 M AC at 24 h and seven days, respectively. These swellings, although characteristic of AC neuropathy (33,
Fig. 1. Fluorograms of representative polyacrylamide slab gels showing the slow component of axonal transport in sciatic motor nerve fibers from four wk-old rats six days after intraspinal injection of 35S-methionine. A: Control nerve in a saline-treated rat. B: Crushed nerve in a saline-treated rat. C: Control nerve in an AC-treated rat. D: Crushed nerve in an AC-treated rat. Each track represents a 3 mm nerve segment, extending distally to the right; the total amount of radiolabeled protein varies between gels, although a similar amount of protein was loaded into each track. A marked increase in the extent of radiolabeled tubulin along the nerve is apparent in the crushed nerves from control (B) and AC-treated (D) animals. All slow component proteins, including the neurofilament triplet proteins (200, 145, and 68 kDa), tubulin (55 kDa), actin (45 kDa), are mildly retarded in their extent of movement in both intact and crushed nerves from AC-intoxicated animals.

52), probably arise secondary to the extensive nerve degeneration observed in this model.

In contrast to previous local injection studies (53) using either 2,5-hexanedione or β,β'-iminodipropionitrile (IDPN), only a rare (0–2 per AC-injected nerve) neurofilamentous axonal swelling was observed following AC application (Fig. 4A); no swellings were observed following saline application. In addition to swellings, an occasional axon demonstrated whorls of disorganized neurofilaments and a paucity of microtubules following local application of 8 M AC (Fig. 4B).

Light microscopy revealed areas of dense staining in the axoplasm following direct application or injection of a single systemic dose of AC (Fig. 5A). Ultrastructural
Fig. 2. Comparison of cumulative plots of the distribution of tubulin in control (A) and AC-treated (B) five wk-old rats six days after labeling in intact (solid circles) and crushed (open triangles) nerves. There is an increase in the extent of radiolabeled tubulin in crushed compared to intact nerves from control and AC-treated animals. A mild retardation in the extent of tubulin transport is present in both intact and crushed nerves from AC-intoxicated animals compared to their corresponding controls. Similar results were obtained in nerves from three additional AC-treated and two additional saline-treated rats.
Fig. 3. Sciatic nerves from the site of subperineurial injection of 1.0 M (A, B) and 0.1 M (C, D) AC at 24 h (A, C) and seven days (B, D). A, B: Many swollen, vacuolated fibers (arrowheads) are present at 24 h (A) and most fibers demonstrate Wallerian-like degeneration at seven days (B) following injection of 1.0 M AC. C, D: Fibers appear normal at both time-points following injection of 0.1 M AC, except for the presence of a few degenerating fibers from placement of the micropipette. Note cellular infiltration at injection sites (arrows in A, C). Epon sections (1 μm) stained with toluidine blue. ×310.

examination did not reveal these to be the neurofilament-microtubule segregations observed following DPN and 2,5-hexanedione exposures (53, 54). Instead, these dense areas were found to correspond to structures composed of stacks of membranous materials (Figs. 4A, inset; 5B). These structures resembled myelin membranes
Fig. 4. Large myelinated nerve fibers packed with neurofilaments following topical application of AC to the sciatic nerve. A: Giant axonal swelling filled with neurofilament and membranous materials (arrows) in paranodal region of fiber (as demonstrated by numerous mitochondria in Schwann cell cytoplasm) 12 h following application of 8 M AC. Inset—Higher power view of neurofilaments and membranous accumulations in axoplasm (see Fig. 5). B: Whorls of disorganized neurofilaments in a fiber four h following application of 4 M AC. Inset—Higher power view showing numerous neurofilaments and paucity of microtubules in axoplasm. × 6,000; Insets: × 35,000.

and some appeared to form from the "pinching-off" of myelin into the axoplasm (Fig. 5B, inset). However, their periodicity (3.3–4.7 nm), as previously reported (45), was considerably less than that of myelin. Furthermore, Po antiserum demonstrated a lack of immunoreactivity of these structures (Fig. 5C).

Glycogen granules, often membrane bound, were observed in axons, particularly in nerves soaked in 8 M AC (Fig. 6). These granules were observed in axons as early as one h after AC application and were still present up to 12 h after treatment.

Local injection of 0.1 M AC produced no morphological alterations up to seven days following subperineurial injection into the sciatic nerve (Fig. 3C, D). This dose was therefore chosen to study the effect of local AC injection on nerve outgrowth and expression of pNF epitopes in neuronal perikarya following nerve crush.

Neuritic Outgrowth Following Local Acrylamide Injection

Radiolabeling Studies: Radiolabeling of fast axonal transported proteins in motor nerves was used to determine the extent of neuritic outgrowth one wk following nerve crush (see Materials and Methods). In nerves injected with AC (0.1 M) from the animal, the extent of nerve outgrowth past the crush site was greatly reduced compared with contralateral saline-injected control nerves (Fig. 7). The majority of radioactivity in the AC-injected nerves was present in the segment proximal to the crush site, suggesting that nerve outgrowth was prevented in most fibers. However, the leading edge of transport was not different from the saline-injected nerves, suggesting that the maximal rate of regeneration was unimpaired by AC injection.
Fig. 5. Membranous materials in axoplasm of myelinated nerve fibers in the peroneal nerve 24 h following a single IP injection of AC (75 mg/kg). A: Light micrograph of peroneal nerve showing areas of dense staining in some fibers (arrowheads). Epon section (1 μm) stained with toluidine blue. × 820. B: Electron micrograph showing accumulations of membranous materials corresponding to the dense areas observed by light microscopy. Inset—Higher power view of a membranous structure which gives an appearance of being “pinched-off” from the myelin sheath. ×6,000; Inset: ×40,000. C: Light micrograph of the same peroneal nerve shown in A (not adjacent sections) stained with F(ab)2 antiserum showing lack of immunoreactivity in axoplasm; myelin sheaths demonstrate intense immunostaining. ×1,280.

Ultrastructural Studies: In accordance with the decreased extent of radiolabeling past the crush site, few axonal sprouts were observed in AC-injected nerves at 6 mm distal to the crush site at one wk (Fig. 8A, B). In contrast, numerous sprouts were present at this level from the contralateral saline-injected nerves (Fig. 8C, D). Closer to the site of crush (i.e. 2 mm distal to the crush site), giant axonal sprouts were observed in the AC-injected nerves (Fig. 9A–D).

Immunocytochemical Studies: Antibody 2-135 (directed against non-pNF epitopes) demonstrated similar intense staining in all neuronal perikarya and axons from normal (Fig. 10A), AC-injected (Fig. 10C), and saline-injected (Fig. 10E) nerves. In agreement with previous findings (7, 22, 28), antibody 07-05 (directed against pNF epitopes) did not stain neuronal perikarya in L5 DRG from normal animals (Fig. 10B) but demonstrated modest to intense staining of neuronal perikarya from saline-injected crushed nerves (Fig. 10D). In contrast, neuronal perikarya showed weak immunoreactivity from AC-injected crushed nerves (Fig. 10F); only a rare perikarya (0–2 per DRG) exhibited modest to intense staining (Fig. 10F, inset).
Fig. 6. Myelinated fiber filled with glycogen granules two h following application of 8 M AC to the sciatic nerve. Inset—Higher power view of glycogen granules and adjacent mitochondria. Granules on the left are membrane-bound (arrow), whereas those on the right appear to exist free in the axoplasm. ×15,600; Inset: ×30,000.

Quantitation of the percent of neuronal perikarya showing immunoreactivity (weak and intense) to pNF epitopes (see Materials and Methods) from AC-injected nerves revealed significantly (p < 0.001) fewer (67.1%) stained neurons compared to saline-injected nerves; immunoreactivity against pNF epitopes was present in 11 ± 1.8% (range 8–14%) and 34 ± 1.9% (range 31–37%) of neuronal perikarya in the L4 and L5 DRG from AC-injected and saline-injected crushed nerves, respectively (Fig. 11).

DISCUSSION

Impairment of Axonal Regeneration by Acrylamide

Previous studies (31, 32) have demonstrated that repeated, systemic AC administration impairs nerve outgrowth. The present study supports and extends these observations by showing that direct subperineural injection of AC inhibits nerve outgrowth following axotomy. Furthermore, our results demonstrate that a systemic dose of AC sufficient to impair both retrograde (50, 51, 55) and slow (44) axonal transport does not prevent the neuronal perikaryon from responding to a nerve crush injury, as shown by its ability to reorder slow transport in a fashion consistent with axonal regeneration (39); the modest reduction in the rate of slow transport is unlikely to account for the pronounced defect in axonal regeneration observed in AC-intoxicated animals. Reordering of slow transport following crush in animals given sys-
Fig. 8. Axonal sprouts 6 mm distal to the site of crush from saline-injected (A, B) and AC-injected (C, D) nerves. The saline-injected nerve (A) demonstrates numerous sprouts, whereas few sprouts are present in the AC-injected nerve (C). B, D: Higher power views of axonal sprouts from saline-injected (B) and AC-injected (D) nerves. A, C: ×6,000; B, D: ×20,400.

Fig. 7. Comparison of the distribution of radioactive proteins in AC-injected and contralateral saline-injected nerves seven days following crush (arrows). Data from three separate animals are presented. Little radioactivity has advanced past the crush site in AC-injected nerves compared to saline-injected nerves, although the maximal extent of nerve outgrowth appears similar in both groups.
temic, high dose AC injections is consistent with our previous finding (44) that repeated, systemic AC administration induces axotomy-like alterations in slow axonal transport; the ability of AC to block the retrograde transport (50, 51) of a "trophic" signal from the periphery (56, 57) most likely underlies the development of this axotomy-like response. Taken together with our previous findings (22, 44, 45), the present results suggest that the defect in axonal regeneration observed in AC-intoxicated animals does not arise from an inability of neuronal perikaryon to elicit an axotomy-like response but is due to an effect of AC at the level of the growing tips of axons (32).

The present findings, however, do not rule out the possibility that the neuronal perikaryal response to axonal degeneration is deficient in AC neuropathy. However, the relatively small reduction in the perikaryal response to axotomy which has been noted in AC-intoxicated animals (37, 38) makes this an unlikely explanation for the marked impairment in regeneration observed following systemic AC administration. Our observation that local (subperineurial) injection of AC reduces the extent of aberrant expression of pNF epitopes in neuronal perikaryon (see below) provides an alternative explanation to account for deficiency in the perikaryal response to axotomy in AC-intoxicated animals. We suggest that the failure of axotomy to elicit a robust response in neuronal perikaryon from AC-intoxicated animals (37, 38) arises as a consequence of an impairment in axonal elongation in the distal stump.

The mechanism by which AC inhibits axonal regeneration is unknown. Interference with either axonal or Schwann cell functions could lead to an impairment in nerve outgrowth. Based upon ultrastructural findings of giant axonal sprouts filled with membranous materials Griffin and co-workers (32) proposed that AC impairs incorporation of materials into the axolemma. An additional possibility, suggested from the present observation of an apparent reduction in microtubules in some axons, is that impaired outgrowth involves an alteration in microtubule and microfilament assembly in axonal sprouts (58–60). Furthermore, AC may affect the function of non-neuronal cells present in the distal stump. Schwann cells synthesize both soluble and cell surface constituents (61), such as nerve growth factor (NGF) (62) and its receptor (63), which are important for nerve outgrowth; the occurrence of numerous Schwann cell processes with few axonal sprouts observed in the present study argues against a mechanism involving an inhibition of Schwann cell mitosis (64, 65) by AC. Although each of these possibilities is entirely speculative, the present results clearly implicate a local effect of AC (i.e. in the region of the growth cone) in the development of impaired axonal regeneration.

Alterations in Axonally Transported Materials by Local Acrylamide Injection

The nature of the membranous materials observed in axoplasm from AC-injected nerves is unknown. These structures are occasionally present in normal axons. However, the present findings are remarkable in their great abundance in axoplasm. We have observed accumulations of similar profiles following systemic AC administra-

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**Fig. 9.** Axonal sprouts 2 mm distal to the site of crush from saline-injected (A, B) and AC-injected (C, D) nerves. A, B: Normal sprouts from saline-injected nerves. Axon in B shows early remyelination. Inset (B)—Higher power view of compacted and non-compacted myelin. C, D: AC-injected nerves demonstrate giant axonal sprouts filled with neurofilaments (C) and membranous materials (D). ×12,000; Insets: ×25,000.
Fig. 10. Peroxidase-antiperoxidase staining of L5 DRG from a saline-injected non-crushed nerve (A, B), a saline-injected crushed nerve (C, D), and an AC-injected crushed nerve (E, F). A, C, E: Antibody 2-135 (against nonphosphorylated neurofilament epitopes) demonstrates similar intense immunoreactivity in neuronal perikarya and axons from all groups. B, D, F: Antibody 07-05 (against phosphorylated neurofilament epitopes) shows no staining of many neuronal perikarya from the normal animal (B), modest to intense staining of many neuronal
PHOSPHORYLATED NEUROFILAMENT EPITOPES IN REGENERATION

Fig. 11. Percent of neuronal perikarya demonstrating immunoreactivity to phosphorylated neurofilament epitopes (antibody 07-05) in L4 and L5 DRG from saline-injected and AC-injected crushed nerves. AC-injected nerves demonstrate a 67.1% reduction in the number of neuronal perikarya expressing phosphorylated neurofilament epitopes. * = p < 0.001.

tion (45) and colchicine application to sciatic nerve (unpublished observation). Thus, it appears likely that these structures, regardless of their origin, accumulate in axons secondary to a blockade by AC in retrograde axonal transport (50, 51). Moreover, the presence of glycogen granules in some fibers, albeit at high concentrations, may support the involvement of a defect in energy metabolism (66, 67) in the development of the defect in fast axonal transport.

Neurofilamentous axonal swellings are observed following chronic (52, 68-70) and acute, high dose (44) AC administrations. In contrast to previous local injection studies using 2,3-hexanedione and 1DFN (53), neurofilamentous swellings were rarely observed and may arise secondary to axonal degeneration in the present material. This suggests that, in contrast to the mechanisms which have been proposed for the hexacarbons (71-73), AC may not directly interact with neurofilaments to elicit axonal swellings.

Aberrant Neurofilament Phosphorylation in Neuronal Perikarya and Axonal Regeneration

Subperineurial injection of AC was employed to study the relationship between the abnormal expression of pNF epitopes in neuronal perikarya and axonal regeneration. The present findings suggest a correlation between these two events. It is unlikely that AC acts directly at the level of the DRG to suppress the development of this perikaryal response to injury since systemic AC administration induces the expression of pNF epitopes in neuronal perikarya (22). In fact, the present findings

perikarya from the saline-injected crushed nerve (D), and weak immunoreactivity in neuronal perikarya from the AC-injected crushed nerve (F). Insets (B, D, F) — Higher power views of neuronal perikarya. ×70; Insets: ×140.

suggest that the fewer number of DRG neurons demonstrating aberrant pNF epitopes in systemically intoxicated AC animals (22) compared to axotomized nerves (28) is due to an inhibition of axonal elongation by AC (31, 32).

The mechanism by which axonal elongation affects NF phosphorylation in neuronal perikarya is unknown, although it most likely involves a yet unidentified retrogradely transported "trophic" signal (59). It is possible that the putative "trophic" signal is produced as a consequence of the elongation process (58) and that AC acts to decrease the delivery of the signal to the perikaryon. For example, AC could prevent the "trophic" signal from reaching the neuronal perikarya by inhibiting retrograde axonal transport (50, 51), resulting in the diminution of this axotomy-like alteration in the cell body. Alternatively, AC may directly impair axonal regeneration (32) leading to decreased production of the "trophic" factor, thereby signaling the neuronal perikaryon that the elongation process has stopped. In the latter case, inhibition of nerve outgrowth by AC would mimic the situation produced in the nerve cell when the growing axon reaches its target. Regardless of the mechanism involved, the present results indicate that continued expression of pNF epitopes in neuronal perikarya following axotomy is dependent upon axonal elongation.

Initiation of Aberrant Neurofilament Phosphorylation in Neuronal Perikarya and Production of the Axon Reaction

Production of aberrant NF phosphorylation in neuronal perikarya may arise via a different mechanism. The finding that this alteration can be induced by axotomy (26–28) suggests that expression of at least some pNF epitopes represents a response of the neuronal perikarya to injury (i.e. axon reaction). This is supported by our recent finding that this alteration can also be induced in intact neurons following inhibition of fast axonal transport by colchicine (74). Previous studies (57) have provided direct support for the hypothesis (56) that loss of a retrogradely transported "trophic" signal initiates the axon reaction. Moreover, nerve growth factor (NGF) is the "trophic" substance whose loss following axotomy leads to induction of the axon reaction in NGF-responsive sensory neurons (57, 75). Future studies will examine whether loss of NGF plays a role in the induction of abnormal expression of pNF epitopes in sensory neurons.

Taken together with the present results demonstrating a relationship between aberrant NF phosphorylation and axonal regeneration, these studies indicate that at least two "trophic" signals are involved in the regulation of this component of the axon reaction. We suggest that there are separate signals which function in the initiation and maintenance of this perikaryal response to injury. Moreover, the ability of AC to both induce (22) and prevent the maintenance (present study) of this alteration may be explained by its capacity to prevent the retrograde transport (50, 51) of these putative "trophic" signals to the neuronal perikarya. This hypothesis is consistent with studies of fibers in the central nervous system (CNS) which indicate that although some CNS neurons initially produce axotomy-like changes, the response is not maintained (76) unless the axons are provided with a suitable environment (and, presumably, an appropriate "trophic" signal) capable of sustaining axonal regeneration (77, 78).

Finally, the present results suggest that neuronal perikarya inappropriately demonstrating aberrant pNF epitopes could be undergoing axonal regeneration. If neuritic plaques in Alzheimer's disease represent aberrant sprouts (79), this may indicate that the abnormal expression of pNF epitopes in this disorder (14–20) arises secondary to abortive axonal regeneration of surviving neurons.
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