Brain Research, 440 (1988) 18–24
Elsevier

BRE 13230

Progressive deficits in retrograde axon transport precede degeneration of motor axons in acrylamide neuropathy

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(Accepted 14 July 1987)

Key words: Acrylamide; Retrograde transport; Axonal transport; Sciatic nerve; Neurotoxin; Neurotoxicology; Axonopathy; Neuropathy

Single injection of acrylamide (1.3 mmol/kg, i.p.) inhibited retrograde axon transport of [^{125}I]tetanus toxin in hen sensory and motor axons. Retrograde axon transport deficits appeared within hours of dosing with acrylamide. The inhibitory effect of acrylamide on retrograde axon transport was transient since transport deficits were not detectable 35 h after dosing. Acrylamide impaired the retrograde movement but not the uptake of [^{125}I]tetanus toxin in the axon. Multiple doses of acrylamide (0.42 mmol/kg, i.p.) induced progressive clinical signs of acrylamide neuropathy that correlated with increasing deficits in retrograde axon transport of [^{125}I]tetanus toxin to ventral spinal cord. Deficits were also observed in sensory neurons but were not statistically significant. Accumulated decrements in retrograde axon transport may be the underlying cause of degeneration of motor axons in acrylamide neuropathy in fowl.

INTRODUCTION

Acrylamide monomer is a neurotoxin that produces distal axonal degeneration in the central and peripheral nervous system of humans and animals alike[7]. Affected axons display abnormal accumulation of 10-nm neurofilaments[34,44] and smooth vesicular structures[9]. The pathophysiological mechanisms underlying axonal degeneration are not understood. The most widely accepted hypothesis is that repeated doses of acrylamide progressively impair some component of the axon essential for the maintenance of its normal functions, including axonal transport[42,50]. A major defect in slow anterograde axonal transport was initially implicated in acrylamide neuropathy[32], but subsequent experiments demonstrated normal rates of transport[3,46]. Recent studies have shown that a large single dose of acrylamide inhibits slow anterograde transport of proteins and causes accumulation of 10-nm neurofilaments in proximal axons[12].

Examination of fast axon transport in rat sciatic nerves has shown that multiple doses of acrylamide produce bidirectional deficits in distal axons[17,43,54]. Single doses of acrylamide impair retrograde transport in sensory and motor axons[5,25,37,42] and induce dose-dependent deficits in the accumulation of retrogradely transported [^{125}I]nerve growth factor and tetanus toxin in dorsal root ganglia[24,25]. Repeated daily dosing increases the degree of transport impairment until axon degeneration appears.

These studies suggest the possibility that deficits in fast axonal transport cause axonal pathology. This possibility has been examined in an avian species (hen) to determine whether single or repeated doses of acrylamide induce deficits in retrograde axonal transport prior to axonal degeneration. Distal-to-proximal movement of [^{125}I]tetanus toxin was used as a marker of retrograde axon transport[33,53], a method

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that does not require nerve-fiber ligation or crush, the trauma from which may obscure acrylamide-induced changes in retrograde axon transport. Moreover, measurement of retrograde transport with [^{125}I]tetanus toxin does not depend upon protein synthesis in perikarya or on anterograde transport[1,4,33]. A preliminary report of this work has appeared elsewhere[40].

MATERIALS AND METHODS

Acrylamide (99.9% purity) was obtained from BioRad Laboratories, Richmond, CA. All other chemicals used were of analytical grade. Purified tetanus toxin was kindly supplied by Dr. K. Field (Bronx, NY).

Animals

Randomly bred adult white leghorn hens (1.5–1.9 kg body weight) were obtained from a local supplier. The hens were maintained in an air-conditioned room (21–23 °C) on a 12-h light–dark cycle and allowed food and water ad libitum. Birds were randomly divided into groups and treated (i.p.) with single or multiple injections of saline or selected concentrations of acrylamide dissolved in sterile saline just prior to use.

Iodination of tetanus toxin

Tetanus toxin (100 μg) was iodinated by reaction with 5 mCi of Na^{125}I (Amersham, Arlington Heights, IL) and chloramine-T (15 μg) in phosphate buffer pH 7.3, as previously described[25]. Since iodinated tetanus toxin remained biologically active for no more than 2 days, fresh batches of [^{125}I]tetanus toxin were prepared for each experiment.

Measurement of retrograde axon transport in sensory and motor nerve fibers

[^{125}I]Tetanus toxin was unilaterally injected (10 μCi, 5 μl in 0.1 M potassium phosphate pH 7.3) in hen gastrocnemius muscle with the aid of a 10-μl Hamilton syringe and a 30-gauge disposable needle. Retrograde axon transport was then assessed as: (a) accumulation of [^{125}I]tetanus toxin in dorsal root ganglia and ventral spinal cord; and (b) the retrograde movement of [^{125}I]tetanus toxin in sciatic nerve as described by Moretto et al.[28]. Briefly, ipsilateral and contralateral dorsal root ganglia of the 2nd, 3rd and 4th roots of the plexus ischiaticum and corresponding sections of ventral spinal cord were excised 36 h after injection of [^{125}I]tetanus toxin and the ^{125}I-content determined with a Packard Multiprias gamma counter (80% approximate efficiency). Protein content in each tissue sample was determined by the method of Lowry et al.[22]. Retrograde axonal transport of [^{125}I]tetanus toxin was estimated to be the quantity of ^{125}I per mg protein in ipsilateral tissue minus the amount in contralateral tissue.

$$\text{Retrograde transport} = (\text{cpm/mg protein})_{\text{ipsi}} - (\text{cpm/mg protein})_{\text{contra}}$$

Retrograde movement of [^{125}I]tetanus toxin in ipsilateral sciatic nerve was determined by counting ^{125}I content in 5-mm sciatic nerve segments. The leading edge of transported label was considered to be the most proximal segment containing greater than 1% of total radioactivity of the whole nerve, calculated as follows:

$$\text{Total nerve radioactivity} = \Sigma_1^n (\text{cpm}_{\text{ipsi}} - \text{cpm}_{\text{contra}})$$

This value was taken as an estimate of nerve fiber-uptake of [^{125}I]tetanus toxin. The distance of the leading edge from the site of injection was plotted against the time between [^{125}I]tetanus toxin injection and sacrifice. The slope resulting from linear regression analysis of the data was considered the rate of retrograde transport of [^{125}I]tetanus toxin[28].

Effect of acrylamide on retrograde axon transport

The effect of a single injection of acrylamide on the accumulation of [^{125}I]tetanus toxin in dorsal root ganglia and ventral spinal cord was investigated as follows: 14 animals were injected with [^{125}I]tetanus toxin as above. One hour after tetanus toxin injection, 7 hens received acrylamide (1.3 mmol/kg intraperitoneally, i.p.), and the remaining 7 animals received vehicle (saline) by the same route. Animals were sacrificed 36 h after [^{125}I]tetanus-toxin injection whereupon accumulation of ^{125}I in ipsilateral and contralateral dorsal root ganglia and ventral spinal cord was estimated[28].

The effect of a single injection of acrylamide on the rate of retrograde transport was determined as fol-

lows: hens were uniformly injected with [^{125}I]tetanus toxin. One hour later, the animals were injected with acrylamide (1.3 mmol/kg) or with saline and sacrificed 3 or 6.5 h thereafter. The rate of retrograde transport was determined by subtracting the distance of the position of the leading edge of radioactivity at 4 h from that at 7.5 h after [^{125}I]tetanus toxin injection. In another experiment, hens singly dosed with acrylamide (1.3 mmol/kg i.p.) or saline, were injected with [^{125}I]tetanus toxin 48 h later and after 6 h, the position of the leading edge of retrogradely transported [^{125}I]tetanus toxin was determined.

The effect of multiple doses of acrylamide on retrograde transport was determined as follows: hens were daily administered with acrylamide 0.42 mmol/kg or saline. At days 5, 8, and 11, 7 hens per group were unilaterally injected with [^{125}I]tetanus toxin in gastrocnemius muscle, and accumulation of ^{125}I was measured after 36 h in dorsal root ganglia (DRG) and ventral spinal cord (VSC). The 36 h interval was chosen because previous studies had shown accumulation of ^{125}I in VSC and DRG was not yet maximal[28], and both inhibition or activation of retrograde transport by acrylamide could be detected.

Clinical evaluation

Each bird was monitored daily (from day 1 until sacrifice: day 5–11) for appearance of neurological signs. Walking performance was evaluated according to the 0–4-point scale of Johnson and Barnes[19] (0 = no defect; 1 = slight abnormal gait; 2 = severely abnormal gait; 3 = animal can stand but frequently collapses; 4 = animal unable to stand). The 'leg-reaction' reflex — namely, the ability of the bird to retract its legs from the dangling position when lifted under the breast — was evaluated as described by Lotti et al.[21]. (The legs characteristically remain extended and flaccid in animals with peripheral nerve lesions[21].)

RESULTS

The profile of radioactivity along the sciatic nerve at 4 and 7.5 h after unilateral injection of [^{125}I]tetanus toxin in gastrocnemius muscle is illustrated in Fig. 1. The rate of movement of [^{125}I]tetanus toxin is calculated to be 5.71 mm/h. This rate of retrograde movement of tetanus toxin is quite similar to that reported earlier in the hen[28] and mouse[25] sciatic nerves. Table I shows the accumulation of retrogradely transported ^{125}I in ipsilateral ventral spinal cord (VSC) and dorsal root ganglia (DRG) 36 h after unilateral injection of [^{125}I]tetanus toxin in gastrocnemius muscle of saline- and acrylamide-dosed hens. A single dose of acrylamide (1.3 mmol/kg) (which maximally inhibits retrograde axon transport of [^{125}I]tetanus toxin in mouse sciatic nerve[25]) had no effect on the accumulation of radiolabel in either VSC or DRG. These results indicate that either acrylamide has no inhibitory effect on retrograde transport in hen sciatic nerve, or a deficit in retrograde transport similar to that found in rodents[24,25] disappears a few hours after dosing

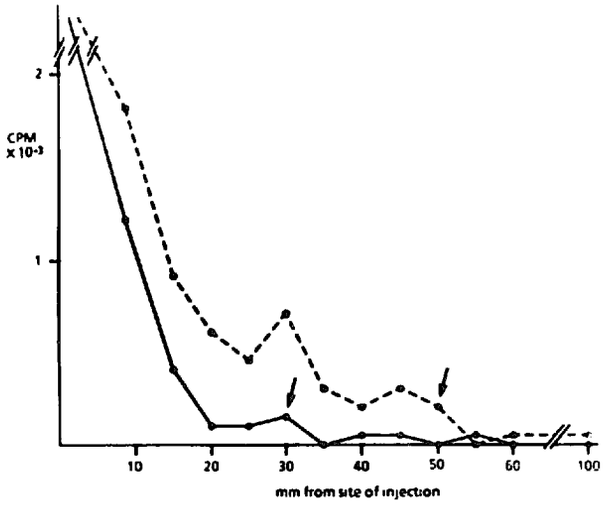

Fig. 1. Typical distribution of ^{125}I along the sciatic nerve of two control hens 4 (●——●) and 7.5 (●----●) h after unilateral injection with [^{125}I]tetanus toxin (5 μl, 10 μCi). Arrows indicate the positions of leading edges of radioactivity undergoing retrograde axonal transport.

TABLE I

Effect of acrylamide on accumulation of [^{125}I]tetanus toxin in hen ventral spinal cord (VSC) and dorsal root ganglia (DRG)

Accumulation of ^{125}I was measured 35 h after a single injection of saline or acrylamide (1.3 mmol/kg, i.p.). [^{125}I]Tetanus toxin was injected 1 h before dosing with acrylamide. Data in saline and acrylamide groups represent the mean ± S.E.M., n = 7, and are not statistically different from each other.

Treatment	*cpm/mg protein*	
	VSC	*DRG*
Saline	349 ± 19	127 ± 22
Acrylamide	297 ± 54	127 ± 23

TABLE II

Effect of acrylamide on the rate of retrograde axon transport of [^{125}I]tetanus toxin in hen sciatic nerve

[^{125}I]Tetanus toxin was injected 1 h before treatment with either acrylamide (1.3 mmol/kg, i.p.) or saline. Rate of retrograde transport (mm/h) was calculated from distance traveled by leading edge of radioactivity measured at 3 and 6.5 h after dosing. Data are the mean ± S.E.M.

Treatment	*Transport rate*
Saline (n = 8)	6.80 ± 1.90
Acrylamide (n = 8)	0.40 ± 0.08

TABLE IV

Position of leading edge of [^{125}I]tetanus toxin in hen sciatic nerve 48 h after dosing with acrylamide

[^{125}I]Tetanus toxin was injected 48 h after acrylamide (1.3 mmol/kg, i.p.) or saline administration and the leading edge of radioactivity was measured after 6 h. Data are the mean ± S.E.M., n = 7 per group.

Treatment	*Leading edge (mm/6 h)*
Saline	44.2 ± 6.4
Acrylamide	45.0 ± 2.2

with acrylamide. To investigate the latter possibility, retrograde transport of [^{125}I]tetanus toxin was examined 3, 6.5 and 48 h after dosing with acrylamide.

Administration of a single dose of acrylamide (1.3 mmol/kg) completely blocked retrograde axon transport in hen sciatic nerve within 6.5 h of dosing (Table II). In saline-treated animals, the velocity of retrograde transport was 6.8 ± 1.9 mm/h; administration of acrylamide reduced the retrograde transport rate to a minimal 0.4 ± 0.08 mm/h. Total sciatic nerve-associated ^{125}I content was not significantly different in saline- and acrylamide-dosed animals (Table III). However, when retrograde axonal transport was measured 48 h after acrylamide dosing, the position of the leading edge of [^{125}I]tetanus toxin was identical in both saline- and acrylamide-treated animals (Table IV). Birds treated with multiple daily doses of acrylamide, displayed progressive abnormal gait and walking, and standing difficulty (Table V). Leg strength and reflexes of acrylamide-treated animals remained comparable to those of saline-dosed animals throughout the study period, suggesting the absence of clinically significant axonal degeneration in peripheral nerves. Edwards[11] found only scanty degeneration of sciatic-nerve fibers of severely ataxic hens exposed to a cumulative dose of acrylamide (6.3 mmol/kg) over a period of 3 weeks.

Multiple daily doses of acrylamide caused a progressive decrease in the accumulation of retrogradely transported [^{125}I]tetanus toxin in sensory and motor neurons (Fig. 2). Accumulation of ^{125}I in VSC was reduced by 30% and 54% of control values after eight and eleven days, respectively, of acrylamide treatment. By contrast, accumulation of the label in DRG was not significantly affected, although a non-significant decrease in ^{125}I was found 11 days after dosing with acrylamide.

DISCUSSION

This study demonstrates that a single dose of acrylamide inhibits retrograde axon transport of [^{125}I]teta-

TABLE III

Total nerve-fiber-associated ^{125}I after acrylamide administration

Gastrocnemius muscle of hen was unilaterally injected with [^{125}I]tetanus toxin. One hour later, acrylamide (1.3 mmol/kg) was administered i.p. Animals were sacrificed 7.5 h after [^{125}I]tetanus-toxin injection. Total nerve-fiber-associated ^{125}I was calculated by summing the radioactivity in ipsilateral sciatic nerve minus the radioactivity in contralateral nerve segments.

Treatment	*n*	*Total cpm ± S.E.M.*
Saline	5	12,028 ± 3,837
Acrylamide	4	16,290 ± 2,994

TABLE V

Neurological scoring in hens intoxicated daily with acrylamide

Hens were administered acrylamide (0.42 mmol/kg, i.p.) daily. Neurological scoring was done according to Johnson and Barnes (see Materials and Methods for clinical evaluation)[19] and is expressed as mean ± S.E.M.

Days	*n*	*Cumulated dose (mmol/kg)*	*Neurological score*
5	21	2.1	0.20 ± 0.1
8	14	3.4	1.10 ± 0.2
11	7	4.6	2.90 ± 0.5

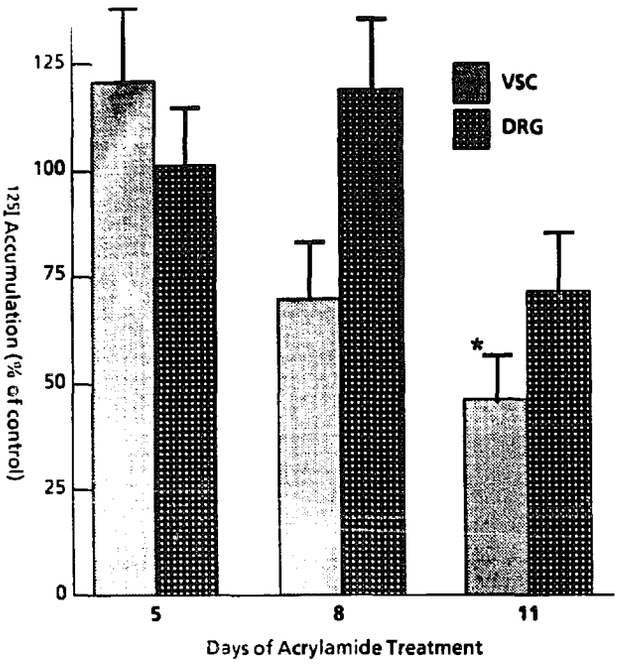

Fig. 2. Effect of multiple doses of acrylamide (0.42 mmol/kg, i.p.) on [^{125}I]tetanus toxin accumulation in hen ventral spinal cord (VSC) and dorsal root ganglia (DRG). Accumulation of ^{125}I was measured 36 h after [^{125}I]tetanus-toxin injection. Values are the mean ± S.E.M. of 6–7 hens and are expressed as percent ^{125}I accumulation in control (saline injected) hens. Accumulation in 7 control DRG was 160 ± 28 cpm/mg protein and 363 ± 50 cpm/mg protein in VSC. * $P < 0.05$ by analysis of variance and Scheff's test.

nus toxin in hen sciatic nerve. The inhibitory effect of acrylamide appears to be temporary since retrograde axon transport returns to a normal velocity within 48 h. The near total blockade of retrograde axon transport observed at 6.5 h, after a single dose of acrylamide, coupled with the normal rate of transport measured at 48 h, suggests the pathophysiological effect of single doses of acrylamide is reversible. Repeated daily dosing leads to a progressive blockade of retrograde transport. Impairment of retrograde axon transport precedes the onset of functional signs of peripheral neuropathy.

The role of axon transport abnormalities in the pathogenesis of toxic neuropathies has been the subject of intense research[2,5,23,27,30,37,50]. Several investigators have observed an association between distal axonal degeneration and impairment of fast axonal transport within the distal axon. Although certain reports on toxin-induced deficits in axonal transport are complicated by the presence of axonal pathology[2,5,9,17,35,43,49], it seems certain from the present and previous data that acrylamide impairs fast retrograde transport long or shortly before the appearance of axonal pathology. Studies with another primary axonal toxin (an organophosphorus compound) revealed early (but irreversible) deficits in retrograde axon transport[28]. This suggests that altered retrograde axon transport may play a role in the pathogenesis of axonopathies induced by a variety of neurotoxic agents[5,18,42,51].

Acrylamide appears to be a more potent inhibitor of retrograde transport in motor fibers of the hen sciatic nerve than in associated sensory fibers. This is suggested by the greater reduction of ^{125}I-accumulation in VSC, than in DRG, although the data are not statistically significant. Acrylamide does not affect total nerve-fiber-associated [^{125}I]tetanus toxin, indicating that acrylamide fails to interfere detectably with the uptake of radiolabel from nerve terminals.

The molecular mechanisms underlying acrylamide-induced axonopathy are unknown. It has been proposed that chemically unrelated neurotoxic compounds[38,39], including acrylamide, deplete energy supplies[51] in the axon by inhibiting certain nerve fiber enzymes[14–16,36]. These studies led some investigators[10,14,36], but not others[6,52], to postulate that axonal degeneration in acrylamide-induced neuropathy is related to depletion of energy and consequent deficits in energy-dependent axonal transport in the distal axon. Recent work[44] has shown that acrylamide produces a dose-dependent inhibition of oxidative metabolism in motor neurons, thus supporting energy metabolism as the key site of acrylamide action. Impairment of glucose metabolism after single and repeated doses of 2,5-hexanedione[31] also indicates a defect in energy transformation and is thereby consistent with an earlier suggestion that disturbances in energy metabolism may underlie axonal degeneration in toxic neuropathies[50,51]. However, the matter is far from being resolved.

The specific role of decremental retrograde axon transport in the generation of acrylamide neuropathy is unknown. However, since it is thought that retrograde axon transport is an important intracellular communication system between axon and perikaryon, a delay in the delivery of retrogradely transported signals may compromise the perikaryal repair response to nerve injury. Others have reported changes in the perikaryal response to axotomy by

prior administration of colchicine[47] or vinblastine[20,48], agents that are known to disrupt the fast axonal transport system. It is possible that acrylamide blocks a retrogradely transported signal and thereby impairs the perikaryal response required to maintain (and regenerate) the axon. Support for this idea is drawn from previous studies showing that acrylamide significantly slows regeneration, inhibits axonal sprouting[13,29], produces 'dying-back' axonopathy proximal to ligation[8], and attenuates axotomy-induced increases of ornithine decarboxylase in dorsal root ganglia[26].

In summary, our studies demonstrate that a single dose of acrylamide rapidly impairs retrograde transport in hen sciatic nerve. Retrograde transport deficits occur within hours of dosing, reverse after single doses and, with repeated daily doses, accrue until axon degeneration commences. On the basis of this and previous studies, it can be hypothesized that primary axon degeneration in acrylamide neuropathy may result from a cascade of pathophysiological events[41]: (a) acrylamide initially impairs retrograde axon transport; (b) this is followed by an inhibition of a retrogradely-transported signal to the perikaryon (c) resulting in a perikaryal response insufficient to restore the integrity of the entire axon; (d) the resupply of required materials via anterograde fast transport reaches the proximal but not the distal axon, a situation that may be further aggravated by defective anterograde axon transport; and (e) the axon consequently undergoes distal-to-proximal degeneration.

ACKNOWLEDGEMENTS

The authors thank Monica Bischoff-Fenton, Linda Baboukis and Christine Esposito-Torres for their assistance in the preparation of this manuscript. These studies were supported by NIH Grants NS19611 and OH02065; and Italian Ministry of Education; Regione Veneto, CNR 85.00581.56, SP5.

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