The Pathogenesis of Primary Internodal Demyelination Produced by Acetyl Ethyl Tetramethyl Tetralin: Evidence for Preserved Schwann Cell Somal Function

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Abstract. The pathogenesis of primary internodal PNS demyelination produced by acetyl ethyl tetramethyl tetralin (AETT) has been studied using subchronically intoxicated rats with intact sciatic nerves (right side), and with focially traumatized nerves (left side) undergoing myelin breakdown and repair. Sixteen Sprague-Dawley rats were given approximately 50 mg/kg/d of AETT, dissolved in ethanol and placed in food. Six age-matched control animals received daily an equivalent amount of food treated with the same volume of alcohol. After six weeks and prior to the onset of demyelination, AETT treatment had increased the number of visible Schmidt-Lanterman incisures per internode of large-diameter fibers in tibial nerves. By ten weeks, the same group of fibers had begun to develop juxtannodal and internodal myelin bubbles. Subsequently, intramyellic phagocytes of hematogenous derivation removed entire internodes of edematous myelin. Schwann cell response to injury was studied in control and AETT-intoxicated animals which had undergone left hindlimb surgery 1 to 2 days after beginning toxin treatment: (a) a perineurial window was placed in the peroneal nerve to induce focal demyelination and remyelination, (b) the tibial nerve was transected between ligatures to study Wallarian degeneration of the distal stump, and (c) the sural nerve was focally crushed to induce axonal regeneration and remyelination. Qualitatively similar responses to nerve injury were seen 1 to 16 weeks later in AETT-treated and control animals. These results are compatible with the view that AETT damages myelin directly, that Schwann cell somal functions are not seriously affected by AETT, and that Schmidt-Lanterman incisures undergo changes prior to demyelination, which may represent a physiological response of the Schwann cell to toxic attack on its myelin sheath. Taken in concert, these observations challenge the long-held view that primary internodal demyelination is necessarily indicative of metabolic dysfunction of the Schwann cell soma.

INTRODUCTION

Central and peripheral myelinopathy and widespread neuronal ceroid-like pigmentation are the major pathological features of the neurological syndrome seen in rats intoxicated with the former fragrance compound acetyl ethyl tetramethyl tetralin (AETT) (17, 18). Demyelination is initiated by remarkable

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internodal myelin bubbling of the type seen in hexachlorophene or organotin intoxication (8, 19). Because pathological changes of myelinating cells and their sheaths develop slowly after many weeks of systemic intoxication, the AE TT model provides an opportunity to examine the evolution of myelin pathology.

The present study focuses on the peripheral nervous system and addresses the following aspects: (a) the spatial-temporal evolution of demyelination and remyelination, with special emphasis on the changes in the myelin sheath and Schwann cell which occur prior to overt myelin damage or loss, and (b) the response of the AE TT-exposed Schwann cell to traumatically induced demyelination and remyelination, and axonal degeneration and regeneration. These studies are used to examine the long-held concept, recently reiterated by Weller and Cervos-Navarro (21), that primary segmental demyelination results from a metabolic disturbance of the Schwann cell soma, and to consider the sites of vulnerability of the myelinating cell in toxic states.

MATERIALS AND METHODS

General

Sixteen young adult (approximately 200 g) Sprague-Dawley (CD-CRL:COBS®CD®-(SD)BR) rats received a diet containing 50 mg/kg/d of AE TT dissolved in 66% ethanol. Six age-matched control animals ate the same quantity of food treated with an equal volume of 66% ethanol. Animals were housed on smooth floors to minimize trauma to plantar nerves, weighed periodically, and observed for physical or functional deterioration. At the termination of intoxication, animals were anesthetized with sodium barbitone containing heparin, and perfused through the aortic arch with 4% paraformaldehyde for 30 sec followed by 5% glutaraldehyde for 10 min, each fixative in a 0.1 M phosphate buffer. Lumbar spinal roots and sciatic, peroneal, sural, and tibial nerves were removed and postfixed in 2% Dalton's chrome osmium solution, dehydrated stepwise, immersed in propylene oxide, and infiltrated with epoxy resin. The tibial nerve branches to the calf musculature were infiltrated with Spurr® resin and teased apart using mounted needles and a dissecting microscope. Nerve fibers were placed individually on glass slides, sealed in a coverslip, and examined by Nomarski differential interference contrast microscopy. Other tissues were embedded in Epon® and hardened into blocks. One-micrometer epoxy sections, cut with glass knives, were mounted on slides, stained with 1% toluidine blue, and examined by bright-field microscopy. Thin sections were prepared from selected blocks, stained with uranyl acetate followed by lead citrate, and examined by transmission electron microscopy.

Effects of AE TT on Schmidt-Lantermann Incisures (SLIs)

Morphometric analysis of teased fibers removed from tibial nerve branches was performed after 6, 10, and 14 weeks of AE TT intoxication. Internodal lengths (ILs) and numbers of SLIs per internode were determined at 400× and 1,000× magnification using an eyepiece micrometer. Internodal length, rather than fiber diameter, was chosen as an index of myelin sheath size, because the former could be determined with greater accuracy. At the six-week timepoint, ILs and number of SLIs per internode were determined for 49 control and 52 experimental internodes; at 10 weeks, 63 control and 64 experimental internodes and, at 14 weeks, 77 control and 52 experimental internodes were analyzed. A total of 8,770 SLIs was examined single-blind at the three timepoints (2,342, 3,251, and 3,177 SLIs, respectively). In addition, approximately seven thousand internodes of teased fibers were examined qualitatively.
Effects of AETT on Traumatically Induced Focal Nerve Injuries

One to two days after commencing intoxication, the left sciatic nerve complex of anesthetized experimental and control animals was surgically exposed under aseptic conditions. The following manipulations were then carried out with the aid of an operating microscope: (a) a perineurial window 1 to 2 mm long was placed in the peroneal nerve, (b) a distal nerve stump was produced by ligating the tibial nerve twice with 4/0 sutures in the mid-thigh region, and then transecting the nerve between the sutures, and (c) a focal sural nerve crush lesion was produced with the aid of watchmaker's forceps. Surgical wounds were closed and the animals were allowed to recover without interruption of intoxication. One or two intoxicated and control animals with surgical lesions were examined at 1- or 2-week intervals up to 16 weeks after surgery.

RESULTS

Effects of AETT Alone

Quantitative examination of teased nerve fibers: Teased fibers removed from control and AETT-intoxicated animals at the three experimental timepoints (6, 10, and 14 weeks of intoxication) revealed that the number of SLIs visible per internode increased in proportion to internodal length (p < 0.01). Figure 1 demonstrates these nerve fiber relationships at 6 weeks of intoxication. Comparison of the calculated regression lines for data obtained from control and AETT-intoxicated animals at 6 and 10 weeks showed a significant difference (p < 0.001) in the number of SLIs visible per internode. Comparable data obtained from animals at the 14-week timepoint were also significantly different (p < 0.01) from control values.

Data at each timepoint were also analyzed by placing ILs into 100-micrometer groups from 800 to 1200 micrometers (Fig. 2). At 6 weeks of intoxication (preceding demyelination), this analysis revealed that the mean number of SLIs visible per internode was significantly different only in the three larger fiber groups. The shorter internode group (i.e., 800 to 890 μm long) from treated animals showed a small increase over controls which did not reach statistical significance. At 10 weeks of intoxication, coinciding with the onset of demyelination, and at 14 weeks, when demyelination was advanced, the larger-sized internodes also showed a significant increase (p < 0.01) in the number of SLIs per internode as compared to controls.

Structural changes in the myelin sheath: Demyelination was first seen in the tibial nerve branches to the calf muscles at approximately ten weeks of AETT intoxication. At this time, myelin changes were restricted to individual internodes but, as intoxication continued, multiple internodes became affected. Demyelination began with splitting and bubbling of myelin lamellae at intraperiod lines, and was followed by the appearance of edematous intramyelinic vacuoles. Examination of the few myelinated internodes which exhibited single vacuoles indicated that the paranodal or juxtaparanodal region was often affected first (Fig. 3), although the initial myelin bubble sometimes appeared internodally. Cross sections through myelin bubbles demonstrated that splitting and vacuolation commonly manifest first deep within the myelin sheath (Figs. 4 and 5). Internodes with multiple myelin bubbles displayed a quilted appearance, the myelin sheath often remaining relatively intact close to SLIs. Although a range
Fig. 1. The relationship between internodal length and number of Schmidt-Lanterman incisures (SLIs) visible per internode in control rats (left) and in animals intoxicated with approximately 50 mg/kg/d of AETT for six weeks.

Fig. 2. Number of SLIs visible per internode, grouped according to four categories of internodal length (in micrometers), in animals intoxicated for 6, 10, and 14 weeks, and in age-matched controls. Tissue from same source as stated in Fig. 1 legend.
of nerve fibers was affected by the demyelination process, large-diameter fibers, particularly in roots (Fig. 4) and tibial nerve branches to the calf muscles, were the most vulnerable, both in terms of the time of onset and extent of involvement. When myelin splitting was evident, but demyelination had not yet commenced, phagocytes congregated in the walls of endoneurial blood vessels. Later, when myelin bubbling was advanced, intramyelinic phagocytes appeared and engulfed damaged myelin. Myelin stripping by phagocytes began in paranodal regions and appeared to advance in a pincer movement toward the somal region of the Schwann cell. Daughter Schwann cells occupied the denuded axon and commenced remyelination in the paranodal region concurrent with stripping and removal of damaged internodal myelin (Fig. 6). Eventually, the entire denuded internode underwent remyelination with the formation of short internodes of thin myelin.

There was an obvious relationship between the duration of intoxication and the number of myelin internodes affected on any single fiber. After 14 weeks of intoxication with AETT, many large-diameter fibers were composed almost entirely of short, thinly remyelinated internodes. A few fibers undergoing Wallerian-like degeneration, manifest by long chains of myelin ovoids, were also found at this time.

Effects of Trauma During AETT Intoxication

Perineurial window: A previous study (5) has shown that nerve fibers herniate through a surgical opening of the perineurium and subsequently undergo focal demyelination and remyelination. Myelinated fibers within the herniation became focally swollen and corrugated within a few days of surgery. Demyelination began at Day 3 and was complete by Day 7. At this time, nerve fibers from control and AETT-intoxicated rats showed Schwann cells associated with denuded regions of the axon. By two weeks after surgery, denuded fibers had commenced remyelination by forming short internodes of thin myelin (Fig. 7). No qualitative morphological differences were found at any timepoint between fibers in the perineurial windows of control and AETT-intoxicated rats.

Wallerian degeneration: Nerve transection caused all nerve fibers in the distal stump to undergo degeneration. This involved the dissolution of the axon and myelin sheath, their degradation, and eventual removal from the nerve fascicle. Degenerating fibers were replaced within basal laminae by columns of Schwann cells whose processes initially contained myelin debris and, subsequently, lipid vacuoles. There were no detectable differences in the type or timing of pathological events in the distal stumps of control and AETT-intoxicated animals (Fig. 8).

Axonal regeneration: Regenerating myelinated and unmyelinated nerve fibers were found distal to the focal nerve crush lesion 2 to 15 weeks following surgery. No qualitative morphological differences were evident at any timepoint between the nerves of control and AETT-intoxicated rats.

DISCUSSION

This study has shown: (a) the number of SLIs visible per internode is proportional to the internodal length, (b) the number of SLIs visible per internode
Fig. 3. Isolated bubble (b) located deep within juxtanodal myelin of a large-diameter teased nerve fiber. Dissected from tibial nerve branches to the calf muscles of a rat intoxicated with AETT. n: node of Ranvier. Buffered glutaraldehyde and osmium fixation. Bright-field micrograph. ×2,700.

Fig. 4. Cross section of lumbar ventral root of an intoxicated rat. Several large-diameter fibers display prominent vacuolation, the vacuoles having developed deep within the myelin sheath. Axons (arrows) within the myelin bubbles are shrunken. Other fibers are demyelinated (d) or remyelinating (r). One-micrometer epoxy section stained with toluidine blue. Bright-field micrograph. ×510.
of larger-diameter fibers increases as a prelude to AETT-induced demyelination, (c) myelin bubbles first form juxtapodally or internodally deep within the sheath of fibers of large-diameter and long internodal length, and (d) Schwann cell responses during various types of traumatic injury appear morphologically unaffected by AETT.

One major conclusion from these observations is that AETT-induced demyelination is not associated with major dysfunction of the Schwann cell soma. During intoxication, Schwann cells underwent normal remyelination following both AETT-induced demyelination and the focal demyelination found in the perineural window. Furthermore, in denervated distal nerve stumps, Schwann cells carried out their usual functions of participating in the disposal of myelin and axonal debris, and aligning into bands of Büngner. During axonal regeneration following a crush lesion, Schwann cells responded as usual to develop myelinated and unmyelinated fibers. These observations suggest that AETT intoxication does not seriously interfere with Schwann cell functions such as: (a) mitosis following axonal or myelin loss, (b) resorption of myelin debris during Wallerian degeneration, (a) alignment of daughter cells into columns following axonal loss, (d) association with regenerating axons and receipt of the signal for myelination, (e) elaboration of short internodes of myelin, or (f) deposition of basal lamina during remyelination and nerve fiber regeneration (14). Thus, these observations challenge the long-held view (e.g., see 1, 21) that primary demyelination of the entire internode is necessarily indicative of Schwann cell somal dysfunction.

The pattern of AETT-induced demyelination most closely resembles that produced by hexachlorophene (2,2'-methylenebis(3,4,6-trichlorophenol)) and triethyltin compounds, with the reservations that, in these two experimental conditions, demyelination of entire internodes has not been demonstrated as a rule, and intramyelinic phagocytes are rarely as prominent as they are in the AETT-induced lesion. Nevertheless, AETT, triethyltin, and hexachlorophene all cause intramyelinic splitting and bubbling of CNS and PNS myelin, without apparent damage to the myelinating cell soma (reviewed in 16). The vacuolated myelin sheath is believed to house edema fluid containing sequestered sodium chloride, but the biochemical events which precipitate this pathological change are hypothetical (3). It has been known for some time that low concentrations of hexachlorophene and triethyltin uncouple and inhibit oxidative phosphorylation, and Cammer (2) has recently demonstrated that both AETT and diacetyl tetramethyl tetralin, its putative metabolite, also exhibit the same property. She

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Fig. 5. Single internode displaying a series of myelin bubbles separated by intact SLIs. The demyelinating process is most advanced in the juxtapodal region (j), where intramyelinic phagocytes have denuded the axon. Demyelination progresses in a pincer-like movement (arrows) toward the Schwann cell soma (s). Teased fiber from same source as indicated in Fig. 3 legend. Bright-field micrograph. ×165.

Fig. 6. Single internode displaying residual myelin bubbles, phagocytic cells (p) laden with myelin debris, and juxtapodal demyelination (d) and early remyelination (r). Teased fiber from same source as indicated in Fig. 3 legend. Bright-field micrograph. ×160.
Fig. 7. Cross section of thinly remyelinated fiber in perineurial window placed in peroneal nerve 2 weeks earlier at the commencement of intoxication with AETT. Electron micrograph of a thin epoxy section stained with uranyl acetate and lead citrate.

Fig. 8. Cross section of a Schwann cell column displaying lipid vacuoles in a distal tibial nerve stump produced by transecting the nerve four weeks earlier, at the commencement of intoxication with AETT. Electron micrograph of a thin epoxy section stained with uranyl acetate and lead citrate. ×11,500.
has suggested that these demyelinating agents may damage the myelin sheath directly by increasing the proton and cation permeability of myelin lamellae, allowing the influx of cations, followed by that of water, via a mechanism similar in some respects to that involved in the uncoupling of mitochondrial oxidative phosphorylation (3). It is also recognized that certain demyelinating agents of this type inhibit a number of other enzymes, including carbonic anhydrase, ATPases, and some phosphatases and phosphodiesterases (3). Carbonic anhydrase has attracted special interest because of its presence in purified CNS myelin, where it could function by pumping ions and water from between myelin lamellae. While the enzyme activity of carbonic anhydrase is inhibited by hexachlorophene, it is insensitive to triethyltin, AETT, or diacetyl tetramethyl tetralin (2). These biochemical hypotheses, it should be noted, all predict that agents producing intramyelinic edema damage the myelin sheath directly, and are entirely compatible with our deductions (vide infra) from morphological observations regarding the pathogenesis of AETT-induced primary internodal demyelination.

The light microscopic appearance of increased numbers of SLIs in vulnerable internodes several weeks prior to the onset of myelin-bubble formation is a new observation which may provide insight into the pathogenesis of the AETT-induced lesion. Previous investigators have suggested that incises increase in number early in PNS and CNS segmental demyelination induced by diphtheria toxin (1, 9, 23) and in Wallerian degeneration of peripheral nerve (20). However, other authors have provided evidence that the apparent increase in number of SLIs is due to the dilatation of previously closed incises invisible by light microscopy (6, 7, 13, 22). Schmidt-Lanerman incises represent channels of cytoplasmic continuity between the adaxonal and abaxonal compartments of the Schwann cell. Although their function is unknown, these cytoplasmic channels may provide pathways for intracellular communication and transport of materials from the Schwann cell soma to the myelin sheath (10, 11). The proportional relationship between number of SLIs visible per internode and internodal length (and fiber diameter)—observed in this study and previously by Ghabriel and Allt (6)—is consistent with the concept that SLIs function as a supply pathway. Viewed from this standpoint, an increase in the apparent number of SLIs per internode during AETT intoxication may represent a physiological response of the Schwann cell to toxic attack on the myelin sheath. This type of response, which is predominantly displayed by large-diameter fibers, should be reversible if AETT treatment is withdrawn. Since large-diameter internodes are also the first to undergo demyelination, it is conceivable that the toxic attack on myelin outstrips the putative compensatory response of its Schwann cell soma, and the formation of bubbles in vulnerable parts of the myelin sheath then ensues. If the onset of myelin-bubble formation indeed represents a mismatch between available Schwann somal supply and excess somal demand created by toxin-induced inactivation of myelin components, then regions most removed from the source of supply (i.e., “distal” regions) predictably would be the first to manifest pathological alterations. This idea is consistent with the observation that, during AETT intoxication, the
earliest myelin splits are often found in inner myelin lamellae of paranodal, juxtanodal, and, less commonly, internodal regions. It is also relevant to note that biochemical and metabolic observations of comparable toxin-induced demyelinating conditions are consistent with the view that nervous tissue is capable to some extent of adapting to demyelinating agents by increasing the rates of synthesis of myelin components (3).

The internodal bubbling and loss of myelin seen with AETT intoxication is one of several types of primary demyelination induced by toxic chemicals. Some demyelinating agents, like alkyl tin salts, hexachlorophene, AETT, cycloleucine (1-aminocyclopentanecarboxylic acid), and certain halogenated salicylanilides, seem to leave the myelinating cell soma intact, while others, including lead, diphtheria toxin, Cuprizone® (biscyclohexanone oxalyhydrazone), telleurium, ethidium bromide, isoniazid, and actinomycin D, also damage the myelinating cell soma (reviewed in 16). In exploring the various pathogeneses of primary demyelination, it may be helpful to consider analogies between vulnerabilites of the myelinating cell and that of the neuron—a cell architecturally comparable to the myelinating cell in that each possesses an isolated somal synthesis center and a huge cytoplasmic/membranous process that must be metabolically supported by the soma. In the case of the neuron, toxic damage to the somal synthesis center, exemplified by the sensory neuronopathy induced by Adriamycin®, results in a rapid, Wallerian-like breakdown of the axon (4). By contrast, toxic damage to the axon process, seen with 2,5-hexanedione (12), is expressed by pathological changes commencing multifocally in the distal ends of long and large-diameter fibers, few or no morphological changes appearing in the neuronal soma. In parallel fashion to agents that produce neuronopathy, compounds like ethidium bromide seem to primarily damage the Schwann cell soma, causing demyelination and loss of Schwann cells from both myelinated and unmyelinated fibers (K. Suzuki, personal communication). By further analogy with agents that induce distal axonopathy, AETT may first induce pathological changes in the “distal” regions of the largest and longest myelin sheaths (i.e., inner myelin lamellae of large-diameter fibers), in the apparent absence of morphological damage to the Schwann cell soma. A similar idea of distal vulnerability of myelinating cells has been proposed by Hirano (10) in relationship to the accumulation of crystalline-filamentous arrays (Hirano bodies) in the Syrian hamster with hindlimb paralysis.

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