Restoration of Blood-Nerve Barrier in Neuropathy is Associated with Axonal Regeneration and Remyelination

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Abstract. We investigated the temporal course of blood-nerve barrier (BNB) breakdown during the evolution of tellurium neuropathy, ricin neuropathy, and Wallerian degeneration following nerve transection or nerve crush. Blood-nerve barrier permeability was assessed with a 4,000-molecular weight fluoresceinated dextran from three days to 19 weeks after onset of neuropathy. Blood-nerve barrier breakdown was present during the first two weeks in all four models of neuropathy. Restoration of the BNB to the dextran began within four weeks and was complete by 14 weeks in tellurium neuropathy, a model of demyelinating neuropathy characterized by rapid remyelination, and after nerve crush, a model of Wallerian degeneration characterized by rapid axonal regeneration into distal stump. In contrast, there was persistence of BNB breakdown beyond 14 weeks in ricin neuropathy, a model of neuropathy with no axonal regeneration or remyelination, and after nerve transection, a model of Wallerian degeneration characterized by minimal axonal regeneration into distal stump. We conclude from these data that alterations in the BNB over the course of neuropathy differ among various types of neuropathy, and that these alterations are dependent on the form of nerve fiber injury. The lack of regenerating or remyelinating axons in ricin neuropathy and after nerve transection may be responsible for the persistent BNB breakdown found in these neuropathies.

Key Words: Axonal regeneration; Blood-nerve barrier; Neuropathy; Remyelination; Ricin; Tellurium; Wallerian degeneration.

INTRODUCTION

Breakdown of the blood-nerve barrier (BNB) due to increased permeability of endoneurial blood vessels occurs during the course of many neuropathies (1, 2). This BNB breakdown leads to an outpouring of osmotically active molecules into the endoneurium. The increased osmotic pressure in the endoneurium in turn leads to an efflux of water from the blood to the endoneurium and the formation of endoneurial edema (1, 3). Although it has been suggested that this increased BNB permeability may facilitate axonal regeneration by making plasma proteins more accessible to the regenerating axons (4), there are now several studies indicating that endoneurial edema may have detrimental effects on nerve fibers. Endoneurial edema has been associated with increased endoneurial fluid pressure (3), decreased nerve blood flow (5–7), endoneurial hypoxia (8), demyelination (6, 9), axonal atrophy (10) and axonal degeneration (11). If the BNB breakdown associated with neuropathy contributes to nerve fiber injury, then the length of time that this barrier defect persists during the course of a neuropathy may be an important factor in determining the degree of nerve fiber injury and the potential for axonal regeneration.

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Ricin, a toxic lectin found in the castor bean (Ricinus communis), can produce a peripheral neuropathy (12, 13). When injected into a peripheral nerve, ricin is rapidly taken up by axons and transported retrogradely to the axons’ cell bodies, where it inhibits protein synthesis and selectively destroys those neurons whose axons were exposed to the neurotoxin (12–15). We recently found that this ricin-induced axonal degeneration is accompanied by a breakdown of the BNB that begins within two days of the ricin injection and is still present 18 weeks later, even though the process of axonal degeneration is completed within the first few weeks after the ricin injection (16). The conspicuous absence of regenerating axons or remyelinating axons in ricin neuropathy suggested to us that the persistent BNB breakdown in ricin neuropathy may be due to the absence of regenerating or remyelinating axons (17). To test this hypothesis, we evaluated the sequential changes in the permeability of the BNB over the course of four neuropathies characterized by axonal regeneration (nerve crush), remyelination (tellurium neuropathy), and neither axonal regeneration nor remyelination (nerve transection or ricin neuropathy). The data from these four models of neuropathy indicate that the presence of regenerating or remyelinating axons is associated with restoration of the BNB during the evolution of neuropathy.

MATERIALS AND METHODS

Animal Models

Long-Evans rats were used in all experiments. The Long-Evans strain was chosen to maintain consistency with our previous studies of tellurium neuropathy and ricin neuropathy, which also used this strain of rat (16, 19–21). The rats were bred from stock originally obtained from Charles Rivers Breeding Laboratories (Wilmington, MA). The animals were weaned at 17 days of age, fed rat chow and water ad libitum and housed in solid-bottomed plastic cages with cyclic fluorescent lighting (12 hours light; 12 hours dark) and constant ambient temperature (28°C).

Ricin Model: Twenty-day-old rats were deeply anesthetized with a mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg) and their left posterior tibial nerve exposed through a skin incision. Ricin (RCA 400, Sigma Chemical Co., St. Louis, MO) was injected into the left posterior tibial nerve at the level of the belly of the gastrocnemius muscle using our adaptation (16) of the intraneuronal pressure microinjection method of Wiley and Oeltmann (18). Twenty-five rats from eight litters received an intraneural ricin injection, four rats received an intraneural injection of physiologic saline, and four rats received an intraneural injection of saline followed by complete transection of the nerve at the injection site. The ricin was dissolved in physiologic saline and delivered with a hand-drawn glass micropipette. Each rat was given 350 ng ricin in a total intraneural injection volume of 1 μl. All individuals involved with the ricin microinjection were gloved and used extreme care in handling the toxin; instruments and containers were decontaminated with a commercial hypochlorite bleach solution. A small amount of 0.1% fast green dye was added to the injection mixture so that any leakage from the injected nerve would be more easily identified (18). The skin incision was closed with silk sutures. Permeability of the BNB was assessed in the left and right proximal sciatic nerves two days to 19 weeks after the ricin injection.

Tellurium Model: Rats were intoxicated with tellurium using a model previously characterized in our laboratory (19, 20). In this model, demyelination is limited to the first seven days of the intoxication; remyelination begins within seven days and proceeds rapidly such that the remyelinating nerve fibers are almost indistinguishable from age-matched controls by 30 days after onset of neuropathy (21). Twenty rats from four litters were intoxicated with tellurium; eight age-matched rats from four litters served as controls for the tellurium model. Intoxicated rats were started at 20 days of age on a 1% tellurium diet. The tellurium diet was made by mixing 1 gm tellurium powder (60 mesh, 99.999% pure; Alfa Products, Danvers, MA) with 87 gm milled rat chow (Purina pelleted rodent chow) and 12 gm corn oil. The corn
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...oil, which was added to retard separation of the mixture, causes neither pathological changes in nerve fibers nor breakdown of the BNB in sciatic nerve (19, 20). The intoxicated rats were fed the tellurium chow ad libitum for seven consecutive days, after which they were returned to unadulterated rat chow. Age-matched control rats received only unadulterated rat chow. Permeability of the BNB was assessed in the right proximal sciatic nerve of control and intoxicated rats three days to 16 weeks after commencing the tellurium diet.

**Nerve Crush Model:** Eighteen rats from three litters were deeply anesthetized with ether at 20 days of age. The left proximal sciatic nerve was exposed through a skin incision and crushed for 30 seconds at the sciatic notch with jeweler's forceps. The skin incision was closed with silk suture. Permeability of the BNB was assessed in the left sciatic nerve distal to the crush ("distal stump") and in the right sciatic nerve distal to the sciatic notch seven days to 14 weeks after nerve crush.

**Nerve Transection Model:** Ten rats from two litters were deeply anesthetized at 20 days of age with ketamine/xylazine. After exposing the left proximal sciatic nerve through a skin incision, two sutures 3 mm apart were tied tightly around the nerve and the nerve completely transected between the sutures with scissors. To retard axonal regeneration within the distal stump, the sutured end of the proximal nerve stump was turned proximally and sutured to the belly of an adjacent muscle, and the sutured end of the distal stump was turned distally and sutured to an adjacent muscle (22). The skin incision was closed with silk sutures. Permeability of the BNB was assessed in the distal stump of the left sciatic nerve and in the uncut right sciatic nerve distal to the sciatic notch 11 days to 18 weeks after nerve transection.

**Tracer Methods**

Permeability of the BNB was assessed in intoxicated and control rats with a 4,000-molecular weight dextran labeled with fluorescein isothiocyanate (FITC-dextran; Sigma Chemical Co., St. Louis, MO) using methods previously established in our laboratory (16). Neuropathic and control rats deeply anesthetized with ketamine/xylazine were injected intravenously with the FITC-dextran (0.75 mg FITC-dextran/g body weight) after tying off their renal arteries and veins bilaterally. Five minutes after injecting the dextran, 1 cm segments from the right and left sciatic nerves just distal to the sciatic notch or the site of nerve transection/crush were quickly excised and immediately frozen in liquid nitrogen. Longitudinal sections of the right and left sciatic nerves were cut on a cryostat at 10–12 μm and examined with a Zeiss fluorescent microscope for the presence of fluorescent material in the interstitium of the endoneurium and epineurium. The sciatic nerve's epineurium, which has no BNB, served as an intra-animal control in all models for the adequacy of the delivery of the FITC-dextran into the bloodstream and the ability of the FITC-dextran to extravasate through the normally leaky epineurial blood vessels. In the sciatic nerve transection and nerve crush models, the intact right sciatic nerve served as an intra-animal control for the efficacy of the normal BNB to exclude the FITC-dextran from the endoneurium. In the tellurium model, left sciatic nerve of age-matched control rats served as an inter-animal control for the efficacy of the normal BNB to exclude the FITC-dextran from the endoneurium.

**Morphological Methods**

Immediately after removing 1 cm segments of the unfixed right and left sciatic nerves for fluorescent microscopy, adjacent 1 cm segments from the proximal right and left sciatic nerves were excised, placed on a firm piece of paper, and immersed in buffered 4% glutaraldehyde (0.05 M phosphate buffer, pH 7.2). After 24 hour fixation, portions of the tissue were washed in buffer, osmicated in Dalton's chrome osmium (2%), dehydrated, cleared with propylene oxide, and embedded in Spurr's resin. Semi-thin sections for brightfield microscopy were stained with paraphenylene diamine; thin sections for electron microscopy were stained with uranyl acetate and lead citrate. To assess the degree of axonal regeneration in the models of nerve crush and nerve transection, additional frozen sections were cut from the blocks of sciatic nerve used for fluorescent microscopy and stained with the Bielschowsky method for axons.
RESULTS

Ricin Model: The ricin-injected rats showed paresis and atrophy of the distal musculature of the left hindlimb throughout the 19 week period. The un.injected right hindlimb showed no abnormality. The ricin-injected rats gained weight normally, and there was no indication of systemic toxicity. Semi-thin sections of the left, ricin-injected nerve confirmed the presence of Wallerian-type nerve fiber degeneration in less than a quarter of the fibers in proximal sciatic nerve during the first three weeks after ricin injection. Fourteen weeks after injection there was considerable nerve fiber loss but no evidence of regenerating or remyelinating axons. The contralateral uninject ed right sciatic nerve revealed no degenerative changes.

Fluorescence microscopy of frozen sections from proximal sciatic nerve of control rats after FITC-dextran injection revealed brightly fluorescent, extravascular material in the epineurium but none in the endoneurium. Left sciatic nerve proximal to the ricin injection revealed extravascular endoneurial and epineurial fluorescence in 12 of 13 rats at two days to four weeks, six of seven rats at 14 weeks, and five of five rats at 15 to 19 weeks after ricin injection (Fig. 1a). The uninject ed right proximal sciatic nerve revealed bright fluorescence in the epineurium but no extravasated FITC-dextran within the endoneurium at any time point (Fig. 1b). Rats receiving an intraneural saline injection with or without an accompanying nerve transection at the injection site in posterior tibial nerve did not show extravasated FITC-dextran within the endoneurium.

Tellurium Model: The tellurium-intoxicated rats developed hindlimb paresis within four days after starting the tellurium diet and gained less weight than the age-matched controls. The paresis resolved within two weeks of starting the tellurium diet.

There was extravasation of brightly fluorescent extravascular material in the endoneurium as well as in the epineurium of proximal sciatic nerve in all six rats examined between three days and two weeks after beginning the tellurium diet (Fig. 1c). However, at three weeks only two of four rats had endoneurial fluorescence, and at 12 weeks only one of five rats had endoneurial fluorescence. There was no evidence of endoneurial fluorescence at 14 weeks (two rats) and 16 weeks (three rats) after beginning the tellurium intoxication (Fig. 1d). Age-matched control rats had extravasated FITC-dextran only in the epineurium.

Nerve Crush Model: After crushing the left sciatic nerve, the rats developed paresis and atrophy of the left distal hindlimb. Over the 14 week period, the rats progressively regained strength and size of the left hindlimb. Microscopy of sections of the nerve distal to the crush revealed large numbers of degenerating axons at two weeks after crush and large numbers of regenerating axons at the 14 week time point. Bielschowsky-stained sections of the nerves confirmed the efficiency of axonal regeneration within the distal stump after nerve crush (Fig. 2a, b).

Extravasated FITC-dextran was present in the endoneurium as well as in the epineurium in all six rats examined between three days and two weeks after nerve crush (Fig. 2c). However, only one of four rats had endoneurial fluorescence between three and six weeks after nerve crush, and none of eight rats had extravasated fluorescent material in the endoneurium at 14 weeks after nerve crush (Fig. 2d). No extravasated FITC-dextran was found in the endoneurium of the contralateral, uncrushed sciatic nerve at any time point.

Nerve Transection Model: After transecting the left sciatic nerve, the rats developed paresis and atrophy of the left distal hindlimb that did not resolve over the 14 week period. Microscopy of sections of the nerve distal to the transection revealed large
numbers of degenerating nerve fibers at two weeks after nerve transection. Bielschowsky-stained longitudinal frozen sections and plastic-embedded cross sections of the distal stump revealed only a very few regenerating axons at the 14 week time point (Fig. 3a, b), confirming the efficacy of the nerve transection model in preventing axonal regeneration within the distal stump.

Extravasated FITC-dextran was consistently present in the endoneurium and epineurium of the distal stump in four of four rats examined at early time points (11 days to eight weeks) and in six of six rats examined at late time points (14 to 18 weeks) after nerve transection (Fig. 3c, d). No extravasated FITC-dextran was found in the endoneurium of the contralateral, uncut sciatic nerve at any time point.
Fig. 2. Nerve crush. a and b. Longitudinally cut, Bielschowsky-stained frozen sections of sciatic nerve from a control rat (a) and a rat 14 weeks after nerve crush (b). The number of regenerating axons in sciatic nerve distal to the crush (b) approximates the number of axons in control (a) nerve. c and d. Longitudinally cut frozen sections of sciatic nerves removed five minutes after FITC-dextran was injected intravenously into rats one week or 14 weeks after sciatic nerve crush. Extravascular fluorescent tracer is present within the endoneurium at one week (c) after crush, but is restricted by an intact BNB to the epineurial tissue at 14 weeks (d) after crush. a and b, brightfield microscopy ×100; c and d, fluorescence microscopy, ×41.

The time interval within the course of each neuropathy during which BNB breakdown was demonstrated is schematically presented in Figure 4.

DISCUSSION

The finding of BNB breakdown in all four models within the first two weeks after onset of neuropathy is in keeping with previous studies documenting that BNB breakdown occurs within one to a few days after the onset of ricin neuropathy, tellurium neuropathy, and Wallerian degeneration following nerve transection or nerve crush (16, 20, 23–29). The mechanism of the barrier breakdown in these neuropathies is unknown, as is the route by which the molecules move through the endoneurial capillaries (2). Increased vascular permeability in neuropathy has been attributed to a variety of possible causes, including direct injury of endoneurial blood vessels or a response of these vessels to vasoactive substances released from endoneurial mast cells, inflammatory cells, degenerating axons, degenerating myelin, or regenerating axons (1).

It has been shown previously that BNB breakdown may persist for weeks during the course of a neuropathy (23, 24). Our data confirmed this finding and further revealed that the temporal course of this barrier breakdown was dependent upon
Fig. 3. Nerve transection. a and b. Longitudinally cut, Bielschowsky-stained frozen sections of sciatic nerve reveal a large number of axons in control nerve (a) and only a single regenerating axon in the distal stump 14 weeks (b) after nerve transection. c and d. Longitudinally cut frozen sections of distal stump of sciatic nerves removed five minutes after FITC-dextran was injected intravenously into rats 11 days or 14 weeks after nerve transection. Blood-nerve barrier breakdown is evidenced at both time points by extravasation of fluorescent tracer within the endoneurium. a and b, brightfield microscopy, ×100; c, fluorescence microscopy, ×62; d, fluorescence microscopy, ×41.

Nerve crush

Tellurium neuropathy

Nerve transection

Ricin neuropathy

Duration of BNB Breakdown (Weeks)

Fig. 4. Schematic representation of the time interval within the course of each of the four neuropathies during which abnormal permeability of the BNB was demonstrated.
the type of neuropathy. We found in tellurium neuropathy, which is characterized by demyelination and remyelination, and after nerve crush, which is characterized by axonal degeneration and regeneration, that restoration of the barrier’s impermeability to the low molecular weight dextran began within four weeks and was complete within 14 weeks after onset of neuropathy. In contrast, we found in ricin neuropathy and after nerve transection, which are neuropathies characterized by axonal degeneration but no regeneration or remyelination, that the BNB remained abnormally permeable to the dextran beyond 14 weeks after onset of neuropathy. Thus, resolution of the barrier defect occurred in the two models of neuropathy characterized by significant axonal regeneration or remyelination, but not in the two models characterized by absence of regeneration or remyelination. These data support the hypothesis that persistence of the BNB breakdown associated with neuropathy is due to the absence of regenerating or remyelinating axons.

Seitz et al (29) have also presented data supporting the hypothesis that an absence of regenerating axons may lead to prolonged BNB breakdown. These investigators found in murine sciatic nerve that the BNB significantly regained its impermeability to macromolecular tracers by 30 days after a nerve crush accompanied by axonal regeneration, but not after a nerve transection unaccompanied by axonal regeneration.

The observation that restoration of the BNB occurred as quickly in tellurium neuropathy as after nerve crush suggests further that remyelination alone may be sufficient to restore the barrier’s integrity, since tellurium neuropathy is characterized by considerable demyelination and remyelination, but only minimal axonal degeneration and regeneration (19, 20, 30). It should also be noted that many of the regenerating axons in the nerve crush model were also myelinating. This raises the possibility that it was the process of myelination of the regenerating axons, rather than the process of axonal regeneration, which was important for barrier restoration. Further studies are needed to determine if regenerating axons that are not myelinating are also capable of restoring the BNB during the course of neuropathy.

Our study, which focused on restoration of the BNB during the later stages of neuropathy, did not address the question of what effect regenerating or remyelinating axons may have on the onset and degree of BNB breakdown during the initial stages of neuropathy. There are, however, data from previous studies suggesting that regenerating axons may also have an effect on BNB permeability during the early stages of Wallerian degeneration (4, 25). Mellick and Cavanagh (25) found in hen sciatic nerve that changes in BNB permeability during the first 32 days of Wallerian degeneration were dependent on whether the nerve was transected, crushed or tied. More recently, Sparrow and Kiernan (4), studying BNB breakdown during the first 21 days after transecting or crushing rat sciatic or hypoglossal nerves, reported that the extravasation of tracer was greater in the distal stump when regenerating axons were present (nerve crush) than when regenerating axons were absent (nerve transection). Furthermore, these authors found that the wave of increased vascular permeability in the distal stump of the nerve crush model advanced at the same rate as the regenerating axons (4). Based on these reports and our current observations, it appears that regenerating axons may initially play a role in enhancing BNB breakdown during the early stages of neuropathy and subsequently play a role in restoring BNB integrity during the later stages of neuropathy.

In summary, we have found that restoration of BNB impermeability during neuropathy is associated with axonal regeneration or remyelination. These findings support the hypothesis that regenerating or remyelinating axons are necessary for

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restoration of the BNB during the evolution of a neuropathy. In light of several recent studies implicating chronic endoneurial edema as a cause of nerve fiber injury (5–11), it seems reasonable to conclude that the potential of nerve fibers to remyelinate or regenerate after injury may not only be important in determining the nervous system’s ability to recover function, but also in determining the nervous system’s susceptibility to continued nerve fiber injury mediated by the consequences of persistent BNB breakdown.

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