Human Neuroma-in-Continuity Contains Focal Deficits in Myelination

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Abstract

Functional recovery does not occur in 10% of patients with neonatal brachial plexus palsy. In these patients, resection of a neuroma-in-continuity (NIC) and surgical nerve reconstruction are required. The formation of a NIC seems to prohibit functional recovery, but the underlying biologic mechanisms for this failure are poorly understood. We systematically analyzed a large series of NIC tissue samples from 17 neonatal and 3 adult patients using an array of immunohistochemical techniques. In a large proportion of patients (74%), the NIC contained multiple focal globular areas with markedly diminished myelination. These focal myelin deficits (FMDs) contain Schwann cells that enwrap axons in an apparently normal configuration but do not form myelin. Biomathematical analysis of a 2-cm neuroma predicted a higher-than-95% probability that an axon would encounter 10 FMDs. Axon segments in FMDs also had disturbed nodes of Ranvier (i.e., FMDs contained significantly fewer clustered Na\(^+\)1.6 channels and decreased Caspr and ankyrin G). These observations indicate that axons in NIC course through multiple FMDs and that this may be the pathobiologic basis for conduction blocks in patients with neonatal brachial plexus palsy. These observations indicate the need for novel strategies to promote functional recovery after neonatal brachial plexus palsy by improving myelination in the NIC.

Key Words: Myelin, Neuroma-in-continuity, Peripheral nerve injury, Schwann cell.

INTRODUCTION

Neonatal brachial plexus palsy (NBPP) is a peripheral nerve traction injury. The resulting nerve damage varies depending on the angle, duration, and magnitude of forces acting on spinal nerves C5 to T1. Severe lesions result in lifelong functional impairment of shoulder, elbow, and hand functions. The prevalence of NBPP is 1.6 to 2.9 per 1,000 births. Although complete spontaneous recovery occurs in most patients with NBPP, surgical treatment is indicated in approximately 10% of patients (1). The initial clinical presentation of NBPP correlates poorly with the severity of the injury. A major challenge in the treatment of NBPP lies in the distinction between axonotmetic lesions, which recover spontaneously, and neurotmetic lesions and root avulsions, which do not (2, 3). Typically, a neuroma-in-continuity (NIC) of the superior trunk of the brachial plexus is found during surgical exploration (4). The NIC usually undergoes resection, followed by nerve grafting with the autologous sural nerve. In most patients, this results in recovery of function, which is, however, never complete (5–7).

Resection of the NIC in NBPP does not significantly diminish clinical motor activity (4), and neurolysis does not show improvement in function (5, 6). Apparently, the formation of a NIC prohibits functional recovery, but the reason for this is poorly understood. Neuroma tissue is characterized by excessive fibrosis, which is generally considered an impediment to axonal regeneration (8, 9). We have previously shown that the inhibitory axon guidance molecule semaphorin 3A is upregulated in NIC and contributes to its growth-inhibitory properties (10). However, impaired axonal regeneration through the NIC cannot fully explain the absence of functional recovery. A histopathologic study of neuroma tissue obtained during NBPP surgery showed that the nerves distal from the NIC contain thousands of axons (11)—a number that should be sufficient for a significant degree of spontaneous recovery of function (12). These histologic data correlate well with electromyographic studies. At age 3 months, axonal continuity toward the biceps muscle was present in more than 90% of patients who had no signs of clinical recovery (13). These histologic and electrophysiologic data do not constitute preliminary signs of impending recovery. In a study of 10 patients, NIC was not resected in 5 patients because intraoperative electrophysiologic recordings showed neural continuity across the neuroma, but these patients did not recover well (14). The lack of functional recovery in the presence of axonal continuity may be partially explained by “axonal misrouting.” Random innervation of target muscles leads to cocontraction of antagonists (i.e., biceps and triceps muscle), resulting in an inability to flex the elbow voluntarily (15). Another possible explanation is that the formation of a NIC affects the function of traversing axons. Elec-
trophysiologic studies provide evidence that a conduction block occurs in patients with NBPP (16, 17).

The aim of this study was to test the hypothesis that the absence of spontaneous recovery of arm function in patients with NIC is at least in part caused by impaired myelination of axons. During reconstructive surgery, a large series of NIC tissue was resected from patients with NBPP, and a smaller number of NIC tissue were resected from adult patients with a traumatic brachial plexus lesion. The NIC were systematically investigated using an array of immunohistochemical techniques. We found that most NIC samples show focal myelin deficits (FMDs), which typically are spherical areas virtually devoid of myelin. These areas are present throughout the neuroma. Many FMDs are filled with motor axons that are enveloped by Schwann cells that do not myelinate. Biomathematical analysis indicates that more than 95% of axons that traverse a 2-cm NIC encounter 10 or more demyelinated areas. The current observations provide the first cell biologic evidence that impaired spontaneous functional recovery after NBPP may at least in part be caused by focal myelination deficits in the NIC.

MATERIALS AND METHODS

Human Tissue

We investigated the NIC material of 20 patients (17 patients with NBPP and 3 patients with adult traction brachial plexus lesion of the superior trunk). Patient material was obtained and anonymized as stated in the 2011 Code of Conduct for Responsible Use of Human Tissue and Medical Research. The NIC was identified during nerve reconstructive brachial plexus surgery. The severity of the lesion of each exposed spinal nerve was classified as avulsion, partial avulsion, neurotmesis, intraforaminal neurotmesis, axonotmesis, or normal, based on CT myelography results, intraoperative morphologic characteristics, direct nerve stimulation, and frozen section examination. A lesion was considered a NIC when the following features were present: normal appearance of the spinal nerve at the intraforaminal level, a clear increase in cross-sectional diameter at the juxtaforaminal level, abundant epineural fibrosis, loss of fascicular continuity, increased consistency, and increased length of nerve elements with concomitant distal displacement of trunk divisions. On direct electrical stimulation of the spinal nerve proximal to the neuroma, contractions of related muscles were not strong enough to move the limb (18). Resected neuroma tissue and proximal and distal stumps were snap-frozen within 15 min of resection and stored at −80°C. In addition, after grafting, leftover sural nerve segments, which would otherwise have been discarded, were used as controls. Sural nerves were kept in Ringer solution and subsequently snap-frozen and stored at −80°C. The mean age of the 17 patients with NBPP was approximately 5 months (mean ± SD, 141.5 ± 6.8 days; n = 17). Neuroma material originated from the superior trunk of the brachial plexus. The proximal nerve stump consisted of a portion of the spinal nerve C5 or C6 located directly adjacent to the neuroma tissue. The age and sex of all 20 patients are provided in the Table.

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The table presents human case material from patients with NIC. Patients have been anonymized by letters (a) to (t). Corresponding age at surgery, sex, and days after the initial trauma are provided. For all available tissues, we scored the presence of FMDs in a cross-section (0, no; 1, yes). Subsequently, the number of FMDs was counted.

F, female; M, male.
Immunohistochemistry

Neuroma, proximal and distal stumps, and sural nerve tissue were cut into transverse 20-μm cryosections. Sections were immersion-fixed in 4% paraformaldehyde in 0.1 mol/L of phosphate-buffered saline (pH 7.4) for 15 minutes at room temperature. Immunohistochemistry (IHC) was performed in Tris-buffered saline (pH 7.4) containing 0.2% Triton X-100 and 5% fetal calf serum with the following primary antibodies: mouse anti-neurofilament (NF; 1:1000, 2H3 ascites; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); rabbit anti-S100 (1:400; Dako, High Wycombe, United Kingdom); rat anti–myelin basic protein (MBP; 1:400, MAB386; Millipore, Temecula, CA); mouse anti-SMI32 (1:1000, SMI32; Steinberger Monoclonals Inc, Lutherville, MD); rabbit anti–Na(v)1.6 (1:200, ASC-009; Alomone Laboratories, Jerusalem, Israel); sheep anti-Casp9 (1:100, AF7548; R&D Systems, Minneapolis, MN); mouse anti-Kv1.2 (1:100, 75-008, Neuromab; University of California, Davis, CA); mouse anti–ankyrin G (1:100, 75-008, Neuromab); rabbit anti-Krox20 (1:200, PRB-236P; Covance, Princeton, NJ); goat anti-Oct6 (1:100, sc-11661; Santa Cruz Biotechnology, Dallas, TX). Antigen-antibody binding was visualized using secondary antibodies (1:1000; Jackson ImmunoResearch, West Grove, PA).

In Situ Hybridization

RNA probe generation and in situ hybridization (ISH) have been previously described (19). In short, digoxigenin-labeled sense and antisense probes were generated from linearized full-length human MBP complementary DNAs by in vitro transcription. Hybridization specificity was verified by comparison with a sense probe hybridization control. We first tried to combine the protocols for ISH and IHC to examine MBP and MBP messenger RNA (mRNA) localization in the same tissue section; however, the ISH procedure has a negative impact on MBP staining. Therefore, ISH and IHC for MBP were performed on adjacent sections.

Epon Embedding and Staining of Myelinated Axons

A freshly obtained central portion of neuroma tissue was fixed in 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 mol/L of Na-cacodylate buffer (pH 7.4). Tissue was postfixed in 1% OsO4 solution in 0.1 mol/L of Na-cacodylate buffer (pH 7.4) and subsequently dehydrated stepwise (by increasing concentrations of ethanol and acetone) and embedded in Epon at increasing temperatures. Semithin sections 1 μm thick were cut on an ultramicrotome with a diamond knife and stained with Toluidine blue.

Image Quantification

Images were obtained using either a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany) fitted with an Evolution QEI digital camera (MediaCybernetics, Silver Spring, MD) or a Leica Microsystems camera (Leica Microsystems, Wetzlar, Germany). Confocal images were obtained with Leica TCS SP5 (Leica Microsystems). Focal myelin deficit quantification was performed by manually outlining the total nerve portion, fascicles, and FMDs of sections triple-stained for MBP, S100, and NF of 20 patients for which nerve portions were available, using Photoshop CS4. Subsequently, the corresponding surface areas were measured, and the respective percentage of fascicles was calculated. Finally, the number of FMDs per section was counted, and the mean proportion of fascicle surface area with FMDs was determined.

Three NIC characteristics were quantified according to a semiquantitative scoring system to obtain a correlation with FMD load. Transverse nerve sections were attributed points as follows: 1, total sectional surface (1, <5 mm²; 2, 5–10 mm²; 3, >10 mm²); 2, presence of intact fascicles (0, no; 1, yes); 3, presence of thickened fibrotic epineurium (0, no; 1, partially surrounded; 2, completely surrounded). A final score was calculated by applying the following formula: Scoreintactness fascicle × Scorethickness epineurium). Scores were binned to form 3 groups: Group 1, 0 to 1 point; Group 2, 2 to 3 points; Group 3, 4 to 5 points. A high score indicates that a nerve section has a large surface area with at least 1 intact fascicle and thickened epineurium. A low score means that a nerve section has a small surface area without a thickened epineurium, no intact fascicles, or both.

Quantification of Na(v)1.6, ankyrin G, Kv1.2, Caspr, Oct6, and Krox20 expression was performed with Image pro Plus software. First, FMDs were outlined manually. Subsequently, the Na(v)1.6 signal was quantified using fixed thresholds. Na(v)1.6, Oct6, and Krox20 signals inside FMDs were compared with the predicted amounts of signal based on directly surrounding myelinating tissue.

Probability That Axons Will Encounter an FMD in a 50-μm Section

For our binomial model, we converted the volume fraction measured in a 20-μm section into a probability that an axon will traverse an FMD in a 50-μm section. Because FMDs are approximately 50 μm in diameter, they can be fitted completely into a 50-μm section; this simplifies our binomial model. An FMD volume fraction of 2.8% was measured in 20-μm sections by dividing FMD outlines by fascicle outlines. The volume of a 50-μm-diameter sphere is two thirds the volume of a cylinder of the same diameter and height; therefore, 2.8% divided by this fraction yields a 4.2% probability that an axon will traverse an FMD in a 50-μm section.

Statistical Analysis

Data from FMD quantification were tested for normality using D’Agostino-Pearson normality test. When data were normally distributed, 1-way analysis of variance was applied; when data were not normally distributed, Kruskal-Wallis test and Dunn post hoc test were performed. Na(v)1.6, ankyrin G, Kv1.2, and Caspr signal data were tested for significance by paired Student t-test. A p value less than 0.05 (indicated by an asterisk) was considered significant.

RESULTS

FMDs Are a Characteristic Feature of NIC

In general, neuroma material is composed of seemingly healthy nerve fascicles and areas of substantial internodal scarring and cellular disorganization. All neuromas are...
surrounded by an abnormal epineurium displaying variable degrees of thickening. In 74% of patients, IHC for MBP revealed a striking pattern of circular or oval areas with focal deficits in myelination randomly present in fascicles with intact continuity in their course through the NIC (Figs. 1A–C; Table). Focal myelin deficits were found in the NIC of 5-month-old babies and adult patients (Table). The pattern of myelination in NIC tissue was also compared with that in leftover sural nerve segments. Focal myelin deficits were exclusively observed in NIC and not in the sural nerve (Figs. 1D–F).

**FMDs Contain Axons and Schwann Cells of Normal Anatomic Configuration**

To investigate whether FMDs were caused by the absence of myelinating Schwann cells, we stained the sections with the Schwann cell marker S100 and for MBP (Figs. 1A–C, G–J). Focal myelin deficits contain S100-positive Schwann cells that envelop axons, as shown by NF staining. The S100-positive Schwann cells located in FMDs displayed a normal 1:1 relationship with axons (Figs. 1G–J) but did not contain MBP. To exclude the possibility that clusters of axons in FMDs are entirely composed of c-fibers, which are unmyelinated by definition, we stained the sections for the motoneuron marker SMI32 (Figs. 1K–N). This staining shows that FMDs contain many SMI32-positive motor axons. Taken together, these observations indicate that FMDs contain numerous axons of motoneurons that are enveloped by Schwann cells, which do not form myelin.

**FMDs Can Be Visualized Independent of MBP IHC**

To determine whether lower MBP levels are associated with decreased MBP transcript expression levels in Schwann cells in FMDs, we combined MBP IHC with MBP ISH (Figs. 1O–R). Because of the incompatibility of the 2 protocols, we applied these 2 staining procedures on adjacent sections and subsequently identified FMDs with deficits in MBP (Fig. 1O) and MBP mRNA (Fig. 1P). In most FMDs, a clear overall decrease in MBP mRNA expression was identified. Some FMDs contained a small number of Schwann cells that displayed a clear ISH signal without apparent MBP expression (Fig. 1P, white arrows). All FMDs were filled with axons (Fig. 1Q).

To further confirm that axons in FMDs are not myelinated, we used classic osmium staining on nerve tissue from 4 patients (Figs. 1S, T). This IHC-independent method showed that FMDs are indeed deficient in myelin and contain axons of a wide variety of diameters (<1 to 6 μm).

**Quantitative Analysis of FMDs Indicates That Most Axons in a Neuroma Are Subject to Focal Demyelination**

To investigate the size and shape of individual FMDs in NIC, we stained the 20-μm serial sections for MBP, S100, and NF (Fig. 2). Individual FMDs often extended into directly

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**FIGURE 1.** Identification and histologic characterization of FMDs. Low-magnification images of representative sections of a NIC and its corresponding proximal stump (inset) processed for IHC for S100 (Schwann cells; (A) and MBP (B). (C) Merged image of (A) and (B). Myelin basic protein staining shows areas of FMDs (arrows). Focal myelin deficits are hardly present in the proximal stump. S100 (D) and MBP (E) immunohistochemical staining of a healthy sural nerve. (F) Merged image of (D) and (E). Note that FMDs are not observed in the healthy sural nerve. Immunohistochemical staining for S100 (G), MBP (H), and NF (I) demonstrates that FMDs contain Schwann cells and axons, but MBP expression is significantly reduced. (J) Merged image of (G) to (I). Hoechst nuclear staining (K) and immunohistochemical staining of a section of a NIC for motor axons in FMDs using anti-SMI32 (L) and anti-MBP (M) antibodies. Anti-SMI32 antibody detects numerous axons in FMDs. (N) Overlay of (K) to (M). Schwann cells in FMDs exhibit diminished expression of MBP mRNA (green) and protein (red). Myelin basic protein (O) and NF (Q) IHC and MBP ISH (P) on adjacent sections show that FMDs are associated with an overall decrease in MBP mRNA; however, ISH signal is still detected in occasional Schwann cells in FMDs (arrows). (R) An overlay of (O) to (Q). (S, T) A sample of an Epon-embedded semithin section stained with osmium exhibits clusters of axons (encircled with white dotted lines) lacking proper myelination. Axons in FMDs vary in diameter, and FMDs are surrounded by myelinated axons. Scale bars = (G–N) 50 μm; (O–R) 100 μm; (S, T) 30 μm.
adjacent sections but rarely extended into more than 2 to 3 sections. Focal myelin deficits are spherical, which can be deduced from their increase and subsequent decrease in size in adjacent sections. Focal myelin deficits appear and disappear seemingly randomly across fascicles. The mean ± SD diameter of FMDs is 51 ± 18 μm.

To quantify the presence of FMDs in the NIC of all patients, we performed IHC-based quantification with the MBP, S100, and NF of FMDs. The corresponding proximal and distal nerve stumps were also quantified where available. Focal myelin deficits with varying degrees of severity were identified in 14 of 17 patients with NBPP and in all adult patients (Fig. 3D; Table). The size of the total nerve and the number of fascicles and FMDs were quantified (data not shown). The mean proportion of fascicle surface area with FMDs was 2.8%. Proximal and distal nerve pieces had a significantly lower number of FMDs (mean ± SD, 7.0 ± 1.8 and 3.8 ± 2.6, respectively) compared with NIC (mean ± SD, 20.3 ± 5.5; p < 0.05; Fig. 3A).

Because FMDs were present in some proximal and distal nerve portions, an unbiased classification of nerve sections—dependent of their clinical classification as NIC, proximal stump, or distal stump—was made. The scoring system, based on the degree of epineurial fibrosis, provided an enhanced prediction of the amount of FMDs per group compared with the clinical classification of the tissue (i.e., NIC, proximal stump, or distal stump). Tissue with a larger cross-sectional area, an intact fascicle, and a thicker epineurium had significantly more FMDs (mean ± SD: Group 1, 2.2 ± 1.0; Group 2, 10.4 ± 2.8; Group 3, 28.4 ± 7.1; p < 0.001; Fig. 3B).

To estimate the potential functional significance of FMDs, we developed a model to calculate the mean number of FMDs an axon will encounter in a typical 2-cm-long segment of a nerve. In this model, 2 assumptions were made: i) FMDs are randomly present across a fascicle, and ii) axons progress through the nerveoma in a straight line. Under these assumptions, the number of FMDs traversed by an axon is binomially distributed (k ≥ FMDs encountered; n is the number of sections; p is the percentage of axons that encountered an FMD per 50-μm section). Because FMDs have a mean diameter of 51 μm, p equals 4.2% for a 50-μm section. A typical 2-cm neuroma would be composed of 400 sections with a thickness of 50 μm. On binomial distribution of these parameters, there is a higher-than-95% probability that an axon will traverse 10 or more FMDs (Fig. 3C). The probability of an axon encountering FMDs will increase if it traverses the nerve in a tortuous manner; thus, this calculation is likely an underestimation of the number of FMDs encountered by axons in the NIC. Figure 3E provides a schematic illustration of the relationship between axons and FMDs in a NIC based on the outcome of this biomathematical analysis.

**Multiple Components of Nodes of Ranvier Display Diminished Clustering in FMDs**

Saltyatory conduction in myelinated axons requires functional nodes of Ranvier. We hypothesized that nodes of Ranvier on axons in FMDs are disturbed. Therefore, we studied the distribution of the sodium channel Na(v)1.6 (a protein localized at internodes), the potassium channel K(v)1.2 (localized at juxtaparanodes), ankyrin G (which serves as an intracellular anchor for sodium channels at nodes of Ranvier), and the contactin-associated protein Caspr (present at paranodes). The localization of these 4 proteins was studied in myelinated areas of the NIC and in FMDs using triple staining for NF, MBP, and the nodes of Ranvier protein. Na(v)1.6 and ankyrin G were present in distinct clusters on myelinated axons in between myelin sheaths, whereas K(v)1.2 and Caspr were localized in the (juxta)paranodal region of the nodes of Ranvier (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A776).

In FMDs, the typical nodal and paranodal clustering of these protein components of nodes of Ranvier was visibly disturbed (Fig. 4). Quantitative analysis revealed a 2.0-fold to 2.5-fold decline in the signal for Na(v)1.6 and its intracellular anchor protein ankyrin G and a 6-fold decline in Caspr in FMDs compared with myelinated areas of the NIC (Figs. 4A–E, F–J, N–T). The localization of the potassium channel K(v)1.2 is disturbed in FMDs (Figs. 4J–M), but quantitative analysis does not demonstrate a significant decline in signal in FMDs (p = 0.06; Fig. 4O). It is possible that because of the spread of these markers nodes of Ranvier along nonmyelinated axons in FMDs, expression...
declines below the limit of detection. We therefore limit our findings to accumulations.

**Transcription Factors Are Not Differentially Expressed in Schwann Cells of FMDs**

The expression of 2 transcription factors (early growth response 2/Krox20 and POU class 3 homeobox 1/Oct6), which play a key role in the myelination program of Schwann cells, was compared for Schwann cells that populate FMDs and their surrounding myelinating Schwann cells. We used an IHC analysis of early growth response 2/Krox20 and POU class 3 homeobox 1/Oct6 in combination with MBP and DAPI. Quantitative analysis of both transcription factors did not reveal significant differences between Schwann cells that populate FMDs compared with healthy myelinating surrounding tissue.
FMDs and those surrounding myelinating tissue (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A777).

**DISCUSSION**

The degree of functional recovery after traumatic nerve injury depends on a range of factors. These include the age of the patient, severity of the lesion, distance of the lesion from end organs, and degree of misrouting (8, 20–22). The results of the present study show for the first time that focal deficits in myelination are present in 74% of NIC in patients in which spontaneous recovery after a closed brachial plexus traction lesion did not occur. The observed FMDs are typically spherical or ovoid and are present in fascicles filled with axons that exhibit an otherwise normal pattern of myelination. Focal myelin deficits contain Schwann cells that do not form myelin but enwrap axons in a normal 1:1 relationship. Focal myelin deficits are found in the NIC of 5-month-old babies and adult patients, indicating that the presence of FMDs is not related to age. As illustrated in Figure 3E, our mathematical model predicts that virtually all axons in a NIC have multiple focal deficits in myelination. The functional deficits found in patients with NIC may therefore at least in part be caused by a reduction in the propagation of axon potentials resulting from focal deficits in the myelination of axons.

**Clinical Implications**

The key finding of this study is that multiple areas with FMDs are present in NIC in a large proportion of patients. This pathologic feature may contribute to the lack of spontaneous recovery after a brachial plexus traction injury in this group of patients. The focal nature of FMDs is a novel feature not previously observed in other peripheral demyelinating diseases. Genetic demyelinating disorders, such as Charcot-Marie-Tooth disease type 1, can display segmental demyelination (23–25). Immune-mediated demyelinating diseases, such as Guillain-Barré syndrome, are characterized by a heavy infiltrate of mononuclear cells (26). Both genetic and immune-mediated forms of peripheral nerve demyelination display patterns of demyelination that are distinct from FMDs found in NIC.

The deficits in myelination in NIC may, however, have significant clinical implications. We found fewer accumulations of Na(v)1.6 channels, Caspr, and ankyrin G on axons in FMDs compared with myelinated axons. The clustering of sodium channels on the axon membrane at the nodes of Ranvier of myelinated axons is essential for saltatory action potential propagation (27). Various demyelinating pathologies display disrupted sodium channel organization (28, 29). The distribution of sodium channels is also abnormal in multiple animal models of demyelination, including heterozygous transgenic mice with 2 extra copies of the proteolipid protein gene (Plp) (30) and Trembler-J mice (an animal model for Charcot-Marie-Tooth disease type 1) (31). The decreased density of clusters of Na(v)1.6 channels in FMDs suggests that saltatory conduction through the NIC is impaired, which might have a negative impact on muscle function.

It is technically challenging to demonstrate a conduction block in patients with obstetric brachial plexus injuries through nerve conduction studies because of the complex anatomy of the brachial plexus. Its small size in neonates, its highly fasciculated nature, and the potential presence of misrouted axons make it difficult to record supramaximal stimulus intensities proximal and distal to the lesion and to obtain consistent recordings of compound muscle action potentials of target muscles. Reliable measurements are virtually impossible when examination is performed in a conscious 3-month-old neonate; conduction studies on sedated patients before or during surgery have been more successful. An electrophysiologic study in 129 patients reports that 25% of cases of NBPP are exclusively caused by a conduction block (16). In a more recent study, a more-than-50% drop in compound muscle action potential was found during intraoperative measurement in 25% of patients with obstetric brachial plexus injury who were undergoing reconstructive surgery (17). In a histologic article examining acoustic neuromas from patients with severe hearing deficits, conduction blocks are implied based on local subtle deficits in myelination (32). Together, these studies suggest that action potential propagation could be impaired in NIC despite axonal continuity.

The decision for surgical intervention in NBPP should always be based on recovery of function because electrophysiologic data can be misleading (14,33). Andrisevic et al (17) developed a treatment algorithm, which they used in deciding to resect a NIC with less than 50% conduction and, subsequently, to perform nerve grafting. Although the applied algorithm is under debate (34), clinical observations point toward the existence of deficits in nerve conduction in NIC. In our study, there was no correlation between age at the time of operation and FMD load; therefore, there was no evidence that the total number of FMDs in neuroma declines across time. However, all patients were treated within a relatively short time window (88–190 days; mean, 139.2 days) such that a possible temporal correlation between FMD load and the age of the patient across a longer period cannot be excluded. Delaying surgical exploration in severe injuries should be avoided whenever possible as chronic denervation of end organs, and especially of the nerves, can negatively influence functional recovery (35). It is not clear whether FMDs contain newly regenerating axons or axons that did not rupture. Because FMDs are mainly observed in anatomically intact fascicles, it is possible that FMD pathology impedes the function of axons that remained in continuity. The apparent failure of Schwann cells to myeline axons in a NIC might be a target for the development of new treatment strategies. Brief electrical stimulation of a focally demyelinated rat nerve leads to a robust enhancement of the intrinsic myelination response (36). Whether this technique, which has also been used to promote axonal regeneration in compressed nerves (37), might be useful in the treatment of traction lesions remains to be established.

**Mechanisms of FMD Formation**

To understand the presence of FMDs, we sought to explore different possible mechanisms of myelin deficiency, including an immune-mediated mechanism, deficits in (de)differentiation of Schwann cells, and relationship with degree of fibrosis. Although a number of demyelinating neuropathies have been described, the spherical focal nature of the unmyelinated areas we observed here is remarkable. Some cases
have been reported with plaque-like demyelination in peripheral nerves (38). In this report and in other cases of acquired demyelinating disease, immune cells mediate the process of demyelination (39). We did not observe CD68-positive macrophages in our series of NIC (data not shown). Although we cannot exclude that these immune cells have reseeded from the NIC at this relatively late time point after injury (5 months), we have no evidence that the formation of FMDs is the result of an immune system-mediated process. We did not observe morphologic features (e.g. onion bulbs) indicative of active remyelination and demyelination. This suggests that demyelination plays no role in the pathogenesis of FMDs but that myelin defects probably represent a failure to myelinate axons after the initial nerve trauma.

The gross pathologic features of NIC might provide some insights into the mechanism of FMD development. We observed that the size of the neuroma, combined with the presence of a continuous epineural fibroblast sheet, correlated with the load of FMDs in NIC. This suggests that fibrosis is a direct cause of FMDs or that fibrosis and FMDs are caused by the same process. It is possible that extensive fibrosis and an increase in nerve diameter cause compression, which can result in focal deficits in myelination, as is commonly observed in compression neuropathies such as carpal tunnel syndrome (40,41). Demyelination has also been demonstrated in animal models of nerve constriction (42, 43). Recently, polymorphisms in the 3’ untranslated region of the collagen gene COL5A1 have been associated with an increased risk of developing carpal tunnel syndrome (44). Therefore, it is possible that the extensive fibrotic response observed in NIC is directly or indirectly involved in the pathogenesis of FMDs. It cannot be excluded that other mechanisms related to nerve compression (e.g. ischemia) are also involved (45, 46). Animal models for inducible focal nerve demyelination could provide further insights into the pathophysiology of FMDs (47, 48). Conversely, axonal signals initiate the myelination program of enveloping Schwann cells (49, 50). Therefore, it is possible that focal areas of axonal dysfunction cause FMD formation.

Myelin formation by Schwann cells is tightly controlled by transcription factors that dictate their state of differentiation and myelination (51). POU class 3 homeobox 1/Oct6 has been identified as a key transcription factor for promyelinating Schwann cells (52). Conversely, early growth response 2/Krox20 is expressed by mature myelinating Schwann cells (53). To determine whether these transcription factors are dysregulated in FMDs, we performed IHC for Krox20 and Oct6. However, the expression levels of Oct6 and Krox20 inside and outside FMDs did not change (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A777). Interestingly, c-Jun, a negative regulator of myelination (54), is also widely expressed by Schwann cells in neuroma tissue. Similar to Krox20 and Oct6, c-Jun was not found to be differentially expressed in FMDs (data not shown).

In conclusion, the discovery of FMDs in NIC sheds a new light on the neuropathology underlying the absence of functional recovery in a subset of patients with NBPP and adult traumatic brachial plexus injury. The presence of FMDs indicates that either dysfunctional Schwann cells or focal areas of axonal dysfunction are a central element of the pathobiology of NIC. Focal myelin deficits are found in relatively intact fascicles, where they may impede action potential propagation of otherwise intact axons. More work is needed to elucidate the mechanism by which FMDs are formed. The discovery of FMDs may inspire novel strategies for treating traumatic brachial plexus injuries aimed at improving the myelinating properties of Schwann cells in the NIC.

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