DAMAGE AND REPAIR OF THE PERIPHERAL MYELIN SHEATH AND NODE OF RANVIER AFTER TREATMENT WITH TRYPsin

RILEY C.-P. YU and RICHARD P. BUNG E

From the Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri 63110. Dr. Yu's present address is Department of Anatomy, University of Texas Medical Branch, Galveston, Texas 77550.

ABSTRACT

Cultures of whole fetal rat sensory ganglia which had matured and myelinated in culture were treated for 1–3 h with a pulse of 0.2% trypsin. The tissue was observed during the period of treatment and during subsequent weeks using both light and electron microscopy. Within minutes after trypsin addition the matrix of the culture was altered and the nerve fascicles loosened. Progressive changes included the retraction of Schwann cell processes from the nodal region, the detachment of the myelin-related paranodal Schwann cell loops from the axon, and lengthening of the nodal region as the axon was bared. The retraction of myelin from nodal regions led to the formation of dramatic myelin redundancies; this pattern stabilized several hours after trypsin withdrawal. Breakdown of the altered myelin segments was rare. There were no discernable changes in neurons or their processes after this exposure to trypsin. The partial repair which occurred over a period of several weeks included the reattachment of paranodal Schwann cell loops to the axolemma and the insertion of new myelin segments where a substantial length of axolemma had been bared. The significance of these observations to the characterization of the Schwann cell-axolemmal junctions on myelinated nerve fibers is discussed. The dramatic degree of myelin change that can occur without concomitant myelin breakdown is particularly noted, as is the observation that these altered myelin segments are, in part, repaired.

The newer cytological techniques which have allowed recognition and definition of the diverse forms, functions, and responses of cell-to-cell junctions have now been applied to the specialized sites of attachment between the Schwann cell plasma membrane and the axolemma near the node of Ranvier (reviewed in references 2, 14). This recent work augments a considerable earlier literature concerning this distinctive region. In much of the older literature the adhesion between the cellular elements of this region was emphasized. It is of particular historic interest that shortly after his initial description of the nodal interruptions of myelin, Ranvier himself suggested that the nodal region functioned as a necessary attachment site between the myelin sheath and the axon (18). He emphasized the necessity for adhesion at this site for he supposed that in a nerve frequently carried in the vertical position (such as the sciatic) the myelin segments would slip along.
the axon to the more dependent portions of the nerve if they were not held in place by periodic special attachments to the axon surface. Soon thereafter the nodal regions were recognized by Lilie (13) as necessary points of high density current flow during the process of saltatory conduction, and Ranvier's quaint idea was forgotten.

Although early histologists recognized that the region directly adjacent to the node of Ranvier possessed unique staining properties (see review by Ramón y Cajal [17]) the detailed structure of this region was not understood until the nature of the myelin sheath itself was defined, and the nodal region examined in detail with the electron microscope (reviewed in references 3, 8, 14, 19, 20). Electron microscope studies indicated that in the paranodal region (adjacent to the bared axolemma of the node) the spirally disposed, compacted myelin lamellae terminated sequentially with each compacted membrane pair separating to enclose a small amount of Schwann cell cytoplasm. In longitudinal sections through the paranodal region, the helix of cytoplasm thus enclosed was apparent as a series of pockets; where these touch the axon the Schwann cell membrane appeared attached to the axolemma by a series of periodic densities (reviewed in references 3, 14). It should be noted that this general description is now known to apply primarily to the smaller myelinated fiber; special nodal features of larger myelinated fibers (over 4 μm) are discussed in detail elsewhere (1, 16, 20).

Against this background our fortuitous observation that trypsin treatment of myelinated nerve tissue cultures greatly altered the myelin-axon relationship seemed worthy of substantive study. It is the purpose of this report to describe the dramatic morphological alterations which are observed in nodal regions of small myelinated nerve fibers of sensory ganglion cultures during and after a course of treatment with trypsin, and to describe the subsequent repair processes that are observed to occur. Preliminary reports of this work have been presented (5, 7).

MATERIALS AND METHODS

Sensory ganglia were removed from 17- to 19-day old rat fetuses and explanted into Maximow double cover slip assemblies on collagen-coated glass cover slips and maintained as previously described (3). More recently ganglia have been maintained in Aclar plastic inserts enclosed in modified culture dishes, as described in detail elsewhere (6). These dishes were incubated in a sealed container with a water-saturated atmosphere containing 5% CO₂ in air and ganglia were fed twice weekly with a complex medium containing (by volume) 62.5% Eagle's minimal essential medium (9), 24% human placental serum, 9.6% 9-day chick embryo extract (50% in salt solution), 2.4% of a 20% glucose solution, 0.6% of 200 mM L-glutamine, and 0.4% of a 400 μg/ml solution of Achromycin. After 6 wk or more of maturation, well-myelinated cultures of the type previously described in detail (3) were selected for experimentation.

Before experimentation, cultures were washed free of medium proteins with Earle's balanced salt solution (BSS). They were then incubated with a solution of 0.2% bovine crystallized trypsin (Mann Research Labs. Inc., New York) for 1-3 h, and subsequently returned to normal culture medium. The trypsin was added to regular culture medium made protein-free by omitting serum and embryo extract. Because reduced calcium levels in the medium are known to alter the nodal regions in this preparation (2), the trypsin solution contained normal amounts of calcium (and magnesium). The same protein-free medium was used to incubate sister cultures as controls. With the use of an inverted Zeiss microscope, continuous observation and sequential photographs were made directly on the living culture during the course of the trypsin pulse and thereafter during the period of reversal. Cultures were fixed during the course of trypsin treatment at 1 and 3 h and at 1, 2, 3, 7, 18, and 35 days after reversal from the treatment.

For whole-mount light microscopy, cultures were fixed with 2% OsO₄ in BSS, stained with Sudan Black B in 70% ethanol, and mounted whole with glycerin jelly. For light and electron microscopy of sectioned material, cultures were retained in the Aclar plastic insert (6) and were first fixed in BSS containing 2% glutaraldehyde, rinsed with BSS, and then further fixed with 2% OsO₄ in BSS. Dehydration in ethanol and embedding in Epon were also carried out directly in the plastic insert. Semithin sections (1-2 μm) were cut with glass knives and stained with Toluindine Blue; thin sections were cut with a diamond knife, double stained with 25% methanolic uranyl acetate for 10 min followed by lead citrate for 1 min, and studied and photographed in a RCA EMU-3G or Philips 300 electron microscope.

OBSERVATIONS

Light Microscope Observations on Living Tissue

Cultures selected for experimentation were of the type previously described in detail (3) and the various cell types present in this culture system have also been described (3). The neuronal somata were grouped centrally and were often contained within a more or less complete connective tissue...
capsule. Neuronal somata contained central nuclei and normally distributed cytoplasmic components. The outgrowth of nerve fibers from the explant was generally gathered into fascicles containing both myelinated and unmyelinated fibers. The highly refractile myelin sheaths often displayed some degree of “normal” irregularity along the length of the internode, particularly near the nodal region (Fig. 1a). Each myelin segment displayed a spindle shaped Schwann cell nucleus near its midpoint; a distinct nodal interruption delineated its length (Figs. 1a and b).

Selected cultures were placed in a sealed chamber and were viewed and photographed on the light microscope stage under controlled temperature conditions during treatment. No changes were observed in control cultures incubated under these conditions. After trypsin addition progressive changes began within minutes. In the first 3-5 min, the substrate (reconstituted collagen) and its overlying connective tissue matrix underwent changes in their optical properties, as can be clearly seen in the series of light microscope photographs in Fig. 1. It appeared that the enzyme progressively altered the collagen substrate, as extended exposure (over 3.5 h) eventually caused the culture to loosen from its glass or Aclar base, with loss of the experimental material. 3 h of incubation with 0.2% trypsin neither significantly altered the neurons and the general organization of the explant region, nor caused notable changes in the more peripherally located Schwann cells. Many of the peripheral connective tissue cells (Fig. 1) and phagocytes were loosened, however, and many phagocytes were found floating free in the medium. There appeared to be a general loosening of the components comprising the nerve fascicles, and, attendant to this, a variety of vacuoles with diameters ranging between 4 and 8 μm were observed to form within the ensheathing connective tissue. This distinctive change was noted during the first 24 h after the trypsin pulse and then gradually disappeared. This alteration, which apparently resulted from an extracellular fluid accumulation, was not well preserved after fixation, and its exact nature was not determined.

The most striking change after trypsin treatment was observed along the myelinated fibers. Immediately after the optical change in the collagen substrate, the nodal region began to lengthen (Fig. 1). It appeared that the myelin sheath was pulled back from the nodal region as the gap slowly and steadily increased in length. The lengthening phenomenon at the nodal region progressed slowly for several hours, continuing after the culture had been returned to normal medium.

The distinctive effect of the enzyme at the node (Fig. 2) has been demonstrated in repeated experiments. After 3 h, almost every node observed in the light microscope was affected in the same pattern. Fibers located at a greater distance from the central explant, however, began to show the lengthening phenomenon before those in or near the explant. Consequently, the myelinated fibers in the distal outgrowth showed more extensive sheath retraction than did those more centrally located.

As myelin began pulling back from the node the small redundancies that normally occur along the mature myelin sheaths became exaggerated. The retracting sheath underwent an accordion-like folding and striking redundancies often appeared in the paranodal regions (Fig. 1). In most instances, the denuded axon spanning the lengthened node could be clearly visualized (Figs. 1 and 2b). These changes continued after trypsin removal, so that in the most severely affected outgrowth area as much as half the axon length was occupied by lengthened nodal regions and the remainder by the altered myelin segments. Accompanying these alterations there was also observed, in some internodes, an apparent splitting between the compacted lamellae of the myelin sheath. It is important to point out that despite the degree of alteration observed myelin segments did not progress to breakdown in the Wallerian pattern, which is the mode of breakdown after axonal loss (see reference 15).

Light Microscope Observations on Fixed Tissue

A series of cultures fixed as for electron microscopy but prepared as whole mounts during several weeks after the trypsin pulse were found most useful for analysis. 7 days after reversal, the paranodal areas began to appear less abnormal. Some shortening of the node and loss of extensive myelin redundancy in the paranodal area was noted. It should be emphasized, however, that the predominant picture continued to be markedly lengthened nodes, pleated myelin sheaths, and occasional splits, apparently within myelin lamellae. Few of these damaged internodes were observed to break down; they were retained and
gradually partially repaired over the subsequent 2 wk (see below).

In tissue fixed 18 days after reversal of trypsin treatment, nodal regions still clearly appeared abnormal. The nodal gap was smaller than earlier but remained greater than normal, and the paranodal myelin often remained bunched up to give the myelin sheath the appearance of an elongated dumbbell. Indications of some degree of repair were seen as a short sleeve of normal-appearing myelin sometimes extended from the paranodal redundancies to partially cover the nodal regions. Observations on the 18- and 35-day material indicated that, in regions where an extensive length of axon had been denuded, a thin newly formed sheath was sometimes interposed between the two older sheaths still bearing the identifying marks of trypsin damage (Fig. 3). These recently formed myelin segments represented about 5% of the internodes in the peripheral culture regions; they were less common near the explant. These preparations confirmed the impression that the neuron and its axon were resistant to tryptic digestion.

**Electron Microscope Observations**

**CONTROL CULTURES:** The ultrastructural organization was comparable to that described for the normal dorsal root ganglion in culture (3). In longitudinal sections, the nodal gap was not completely unsheathed but was surrounded with interdigitating Schwann cell processes extending from the adjacent internodes. These processes and the axolemma were covered with a continuous basal lamina. All of the cytoplasmic loops observed were apposed to the axon membrane at the paranodal region, as is characteristic for thin myelin sheaths (16). Frequently, from one to several electron-dense particles were present between each loop and the outer leaflet of axolemma (as in Fig. 8). Desmosome-like junctions were sometimes seen between adjacent loops. Occasionally exaggerated cytoplasmic loops with large granular inclusions were observed. The complex nodal anatomy noted by others (1, 16, 20) in larger myelinated nerve fibers in vivo was not observed along the uniformly small fibers of the culture system.

**EXPERIMENTAL:** Longitudinal sections through the nodal region were most extensively studied. Comparison of cultures fixed at 1 and 3 h indicated that trypsin first caused many perineurial cells and connective tissue components to loosen (Fig. 4), with some loss of the fasciculated organization of the nerve fibers. Some unmyelinated fibers were loosened from their normal Schwann cell investment but without change in their axoplasmic content. Along myelinated fibers the Schwann cell processes at the nodal gap were gradually pulled away and the basal lamina in this region, as well as elsewhere, was no longer demonstrable in the electron microscope. At this stage the axolemma of the nodal region appeared entirely naked with some debris, apparently resulting from the digestive process, occasionally scattered around the nodal region (Fig. 4). Initially, all the terminal loops of the myelin sheath maintained their position and the node retained its normal dimensions. It is our impression that the disappearance of the periodic densities between the axolemma and the Schwann cell cytoplasmic loops began at this stage.

Subsequently, the Schwann cell cytoplasmic...
loops became detached from the axon membrane and began to separate from one another. Concomitantly they decreased in size and increased in density (Fig. 5) a change also noted after loop detachment induced by low calcium levels (2). It appeared that as the loops loosened they retained, for a time, a tenuous connection to the axolemma resulting in a local projection toward the receding

**Figure 2** These light micrographs are of osmium-fixed Sudan Black-stained regions of the outgrowth 3 h after the onset of trypsin treatment. Fig. 2 a illustrates the uniform lengthening of the nodal regions and the tendency for bulbous myelin enlargements near the lengthened nodes. These altered nodal regions may be compared with the normal node region illustrated in Fig. 1 a. (Fig. 2 b) In this enlargement of an altered nodal region the axon (arrow) can be seen spanning the altered myelin segments. Fig. 2 a, × 270; Fig. 2 b, × 900.

**Figure 3** This photomicrograph is from an osmium-fixed Sudan Black-stained outgrowth region in a culture prepared 18 days after the reversal of a 3-h trypsin treatment. The small segment of myelin between the altered myelin segments has been formed during the repair processes which follow trypsin treatment. The fascicle in which this myelinated fiber courses also contains many unmyelinated fibers (which cannot be distinguished individually) and their accompanying Schwann cells. × 680.
FIGURE 4 These two electron micrographs illustrate changes in the nodal region after 2.5 h of trypsin treatment. In each case a short segment of axon has been denuded of its interdigitating Schwann cell processes and these can be seen retracting toward the region of compact myelin. No apparent separation of the terminating Schwann cell loops from the axolemma has occurred at this time. The loosening of the basal lamina material (as at arrow) is also illustrated. Some debris, apparently from the digestive process, occurs in the nodal region around the denuded axon in Fig. 4 b. In Fig. 4 a there is a branching of the axon at the node, a not infrequent occurrence in the proximal regions of the outgrowth. Fig. 4 a, $\times$ 21,500; Fig. 4 b, $\times$ 23,000.
FIGURE 5 This electron micrograph illustrates the substantial nodal changes that have occurred after 3 h of trypsin treatment. The terminating Schwann cell loops are much smaller in size than normal and have been pulled away from the axolemma. At certain points (e.g., arrows) these loops appear to retain a tenuous connection with the axon surface which results in a portion of the axolemma being everted toward the loop. Despite the detachment from the axolemma many of the loops retain their close proximity in a tight collar around the axon; the redundancy of myelin is observed proximal to this region. In many regions of the electron micrograph no basal lamina material is seen on the external Schwann cell surface. In other areas, as at the right, it appears loosened. $\times$ 5,400.
loop (Fig. 5). As the loops became completely detached from the axolemma, the node became lengthened (Fig. 5). There was some separation between the sheath and axon all along the inter-node but especially at the paranode. These spaces gradually expanded as the node increased in length and the loosened sheath began to pleat forming the characteristic redundancies (Fig. 6).

Parallel with these nodal changes was intermittent splitting at the intraperiod line in various

![Figure 6](image-url) An electron micrograph illustrating an extensive myelin redundancy near an altered node. The loosened Schwann cell loops (13 on each side) are pulled away from the axolemma but remain as a collar around it, and the redundancy occurs in the more proximal part of the paranodal region. The cell nucleus in the lower right belongs to the Schwann cell related to this myelin internode, indicating the extent of the myelin retraction. × 52,000.
regions of the internodal myelin. As the lengthening process of the node came to a halt, some of the elongated nodes appeared as naked but normal axons (Fig. 7). These were distinguished from unmyelinated axons by the absence of any relation to a Schwann cell process. It should be noted, however, that in almost all nodal regions examined, the detached loop endings themselves always remained close to the axon surface. Thus, the loop region remained as a collar near the axon and the characteristic redundancies occurred proximal to this point.

3-5 wk after trypsin reversal, some small myelin segments with exceptionally smooth contours were observed, particularly in the culture periphery. These apparently resulted from the formation of new myelin segments in the distal axonal regions suggesting that trypsin treatment may have induced some new myelin formation in these well-matured cultures. Even at this time it was not difficult to identify those nodes which had been damaged by trypsin and subsequently partially repaired. They generally showed very irregular myelin, and many of them retained their characteristic abnormal redundancies near the zone of myelin termination. Due to the retention of considerable nodal lengthening, some of the nodal regions appeared incomplete (i.e., paranodes without a corresponding partner). In a few cases, a complete but asymmetric node was observed. On one side, these nodes showed a series of terminal loops varying in size and continuous with an abnormal myelin sheath and bulky redundancy. On the opposite side, a smooth myelinated internode terminated with normal cytoplasmic loops apposed to the axon. This configuration suggested that the formation of the latter internode had occurred after trypsin treatment. These newly formed myelin sheaths were produced by Schwann cells which had migrated to occupy the extensively denuded axon between retracted myelin segments. In paranodes related to marked myelin redundancies, and thus identified as having been substantially damaged by the earliest trypsin treatment,

![Figure 7](image_url)
the originally loosened and detached terminal loops were again observed to project normally onto the axonal membrane. When compared with nodes in control cultures, however, these cytoplasmic loops were frequently irregular in size and shape. The punctate dense regions which normally bind these loops to the axolemma were also reformcd (Fig. 8). The node was again at least partially surrounded by interdigitating Schwann cell processes extending from the internodal area, and these processes and the axolemma were again covered by a basal lamina.

DISCUSSION

In the present experiments trypsin has been used for the short-term treatment of mature, highly organized peripheral nerve tissue in culture. Although it seems most probable that the reactions observed result directly from proteolysis, we cannot rule out the possibility that hydrolysis products or other enzymes activated by the trypsin treatment may play a part in the reactions observed. Of particular interest are the observations that neurons and axons appear relatively resistant to damage by this trypsin exposure, and that the specialized contacts between Schwann cell and axolemma in the paranodal region of the myelinated nerve fiber are particularly susceptible.

The pattern of myelin change seen in these cultures is undoubtedly influenced by their histotypic organization (3). The neuronal somal region is often substantially encapsulated by connective tissue elements and the proximal larger fascicles are surrounded by substantial perineurial tissue. These components must be penetrated by the enzyme before its action on the myelin sheath itself. This seems the most likely explanation for the more intense effect noted in the more peripheral fibers and the delay in the onset of similar changes near or in the explant itself. This aspect of the trypsin response led us to construct the temporal sequence of changes from selected culture areas as well as from the total time of treatment.

This sequence of changes may be summarized as follows. A change in the refractility of the collagen substrate and a loosening of the matrix of connective cells over this substrate are noted within several minutes. Alterations in fascicle components follow, and within 15 min the nodal regions begin to lengthen. At the fine structural level it can be observed that this has been preceded by a loss of basal lamina material, and a retraction of the Schwann cell processes that normally interdigitate at the node. Thereafter the Schwann cell paranodal loops begin disengagement from the axon, and the myelin in this region begins to show abnormal redundancies. This process stabilizes only several hours after removal of the trypsin pulse and is slowly repaired over a period of several weeks. The repair process does not restore the normal contour of the myelin internode but involves a reattachment of Schwann cell paranodal loops to the axolemma and the introduction of new internodes in some regions where myelinated axons are denuded over a considerable length.

This response is in some ways similar to that observed in this culture system when calcium levels in the culture medium are lowered (2). The chief similarity is that after several days at calcium levels of 0.025–0.050 mM, nodal regions begin to lengthen and this has been shown to occur concomitantly with a detachment of paranodal Schwann cell loops from the axolemma. This is accompanied in low calcium conditions by substantial fluid accumulation between the Schwann cell and the axon. The response to calcium reduction differs, however, in that in the low calcium effect the nonloop Schwann cell processes covering the axolemma in the nodal region retain their affinity for the axolemma. Thus the axon remains ensheathed by interdigitating Schwann cell processes even as the paranodal myelin is being pulled back. The reaction to calcium reduction differs, however, in that in the low calcium effect the nonloop Schwann cell processes covering the axolemma in the nodal region retain their affinity for the axolemma. Thus the axon remains ensheathed by interdigitating Schwann cell processes even as the paranodal myelin is being pulled back. The reaction to calcium reduction differs, however, in that in the low calcium effect the nonloop Schwann cell processes covering the axolemma in the nodal region retain their affinity for the axolemma. Thus the axon remains ensheathed by interdigitating Schwann cell processes even as the paranodal myelin is being pulled back.
FIGURE 8 This electron micrograph was obtained 35 days after reversal of trypsin treatment. The type of redundancy observed at the lower left (r) indicates that this node had been altered by the trypsin treatment. The Schwann cell cytoplasmic loops are again normally apposed to the axolemma and densities can be observed between these two membranes at points of contact. A basal lamina is again observed at the external surface of the Schwann cell. Axon (a). × 60,100.
where excluded by regularly arranged bridging particles. The common gap junction differs, however, in that (a) it occurs as a patch rather than as a spiraled linear array, and (b) there are differences in the organization of particles within the membrane leaflets (see discussion in reference 14). But of particular interest in relation to the present study is the observation that intermediate or desmosomal junctions are generally susceptible to calcium depletion or trypsin digestion, and that gap junctions (and tight junctions) are not (10). Thus the susceptibility of the Schwann cell-axolemmal junction to these treatments is exceptional. How these distinctive properties may be related to the function of this region in the implementation of saltatory conduction is not known: some speculative suggestions are given elsewhere (2, 14).

The reaction of the myelin sheath and its related Schwann cell to trypsin treatment represents one of several instances where damage to the myelin sheath has occurred which has not led to myelin breakdown and disposal. The damaged myelin segment is then at least partially repaired. This is in contrast to other instances (such as in diphtherial toxin poisoning) (12, 15) where damage to a myelin segment progresses to complete breakdown and removal (see discussion in reference 5). Repair may then occur by remyelination of the vacated axonal region. It remains unclear why, in some instances, myelin damage progresses to breakdown, and in other instances the segment of myelin is repaired, even though this repair may not fully restore the normal anatomical configuration of the internode.

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