FINE STRUCTURAL ASPECTS OF DEMYELINATION
IN VITRO. THE EFFECTS OF GUILLAIN-BARRÉ SERUM

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INTRODUCTION

The striking similarity of pathological alterations accompanying the Guillain-Barré syndrome (GBS) and experimental allergic neuritis (EAN) has been well recognized since the work of Waksman and Adams in 1955 (1). Both conditions may be characterized by segmental demyelination of peripheral nerves associated with mononuclear cell infiltrates (see the recent review by Asbury et al, 1969) (2). This similarity has been confirmed at the ultrastructural level (3, 4) and has suggested that the idiopathic disease of humans (GBS) might have a pathogenesis in common with the immune mediated disease of animals (EAN).

While the mechanisms by which myelin damage is produced in vivo in these disorders are unknown, most investigators have focused on the roles of serum factors and immunocompetent cells. At the ultrastructural level Lamport (5) in studying EAN, and Wiśniowski et al (6), who examined peripheral nervous system lesions in experimental allergic encephalomyelitis (EAE), stressed the invariable presence of invading mononuclear cells in areas of myelin breakdown. In contrast, Ballin and Thomas (7) and Schröder and Krüke (8) could not always detect such a relationship between cells and demyelination in EAN.

The availability of myelinated cultures of peripheral nervous tissue offered a method of studying certain aspects of this controversy under better controlled experimental conditions. In 1969, Arnason et al (9), using the light microscope, reported the demyelination of peripheral nerve cultures exposed to the cells derived from the buffy coats of patients with GBS, while the sera of the same patients were much less effective. In contrast, Cook et al (10) have shown, by light microscopy also, that serum from two-thirds of GBS patients, in the presence of complement, causes segmental swelling and fragmentation of

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myelinated nerve fibers. The present study was undertaken to characterize the
demyelination observed by the latter authors at the fine-structural level and to
attempt to answer the following questions:
1. Was the phenomenon observed with the light microscope a primary de-
myelination, or was it secondary to axon damage?
2. What was the precise pattern of myelin breakdown?
3. Did the changes seen in tissue culture resemble the demyelination observed
   in vivo in GBS, despite the absence of host cells in the experimental model?

MATERIALS AND METHODS

Cultures of mouse dorsal root ganglia were obtained from 18 day old fetuses according
to the methods of Peterson and Murray (11) and Murray (12). The cultures were main-
tained in vitro for 20-30 days at which time optimal myelination had usually been achieved.
They were then exposed to one of three feeding media all containing complement (10).
The first group was exposed to sera of known myelinotoxicity from 6 GBS patients, diluted
with an equal volume of feeding medium. A second group of cultures, used as a control, was
exposed to serum from normal individuals in the same proportion. The third group of
cultures, also a control, was exposed to feeding medium alone. A total of 26 cultures, 17
exposed to GBS serum, 11 to normal serum, and 8 exposed to feeding medium alone, was
studied.

At various intervals, between 20 and 72 hours after treatment, coverslips bearing the
cultures were removed from the Maximow assemblies and washed for five minutes in
 Locke's balanced saline solution. The cultures were then fixed for one hour at 4°C in 1.5%
glutaraldehyde in 0.067 M cacodylate buffer, pH 7.4, containing 1% sucrose. After fixation,
the cultures were washed at room temperature for 10 minutes (three changes) in 0.1 M
sodium cacodylate with 75% sucrose followed by a 5 minute wash (two changes) at 4°C with
0.06 M veronal acetate buffer, pH 7.4, containing 0.1% CaCl₂. Specimens were postfixed for
one hour at 4°C with 2% OsO₄, in 0.03 M veronal acetate buffer, pH 7.4, containing 0.05%
CaCl₂ and were then dehydrated in ethanol and embedded in Epon. After reorientation of
some blocks to permit transverse sectioning of the explant (13), thin sections were cut
with a diamond knife, stained with uranyl and lead salts, and examined in a Siemens 1A
electron microscope.

RESULTS

The findings to be described are in areas of the culture outside the central
necrotic zone which was present in occasional control and experimental cultures.
These central areas, where diffusion of media and gases is retarded, may show a
variety of degenerative changes in neurons, Schwann cells and all other cellular
elements. Outside these areas, the apparent condition of cells and their proc-
cesses was generally good.

Among the control cultures, minor variations within a single culture were
observed but the ultrastructural findings were remarkably similar from culture
to culture (14). Among cultures treated with serum from patients with GBS,
however, substantial differences were found between cultures, as well as among
the various areas within a single culture. These differences could not be defi-
nitely attributed to the varying times after exposure at which the cultures were
examined.
A. Alterations in myelin sheaths and Schwann cells:

In cultures exposed to GBS sera many myelinated fibers appeared normal (Fig. 1). On the other hand, numerous altered sheaths were apparent, as well. A unique characteristic of these alterations was the regular separation and general loosening of the myelin lamellae which appeared in cross-section to start from the external mesaxon and extend inward along the intraperiod line, producing remarkably even spacing between two or more lamellae (Fig. 3). The sheath usually appeared normal otherwise (Fig. 2). These changes were usually limited to a single internode. Somewhat similar separations of the intraperiod line have been observed when peripheral nerves are exposed to hypotonic solutions (15, 16). However, the separations observed in the present study were much more regular, and always began with an opened external mesaxon which is not observed in hypotonic solutions. Occasionally, the separation extended all the way to the internal mesaxon at which point it communicated directly with the periaxonal space (Fig. 4). Similarly altered sheaths were also observed in longitudinal sections and, at the nodes of Ranvier, the lateral loops were separated from one another. Usually, the lateral loops retained their close apposition to the axolemma and the transverse bands remained intact (Fig. 5). Sometimes, however, the transverse bands appeared missing (Fig. 6). This regular separation of myelin lamellae was observed in cultures treated with sera from 3 of the 6 GBS patients, and was not observed in control cultures.

The changes described above were generally modest in size and extent and would be invisible in the light microscope. Other segmental changes, however, were much grosser and, no doubt, corresponded to the alterations detected in the living cultures by light microscopy. One of the most common among the gross changes was large, focal, intramyelinic splits of the intraperiod line (Fig. 7). These changes are not specific since intramyelinic splits have been described in a wide variety of disorders of both the central (17–26) and peripheral (27–28) nervous systems. Another gross change was an enormous distention of the periaxonal space (Fig. 8) often associated with outpocketings of the myelin sheath which were then apparent as so-called “myelin rings” or “voids” similar to those described in a variety of conditions (29–34). In extreme examples of these changes, the entire myelin segment may be involved and the myelin lamellae show a vesicular type of dissolution (Fig. 9) as described in various other conditions as well (3, 35–38). These changes were usually limited to a single segment. The altered myelin usually remained within the Schwann cell and the surrounding basement membrane. The large, focal, intramyelinic splits and the distention of the periaxonal spaces were observed in cultures treated with sera from all 6 GBS patients. Occasionally, milder examples of the same changes were observed in control cultures.

Vacuolization of Schwann cell cytoplasm was commonly observed in cultures exposed to GBS sera and occasionally similar but milder changes were observed in control cultures. These changes varied in degree, and were noted in lateral loops, inner loops, external cytoplasmic collars and especially in Schmidt-Lan-
Fig. 1. An apparently intact myelinated nerve fiber in a culture 50 hours after exposure to Guillain-Barré (GB) serum. ×25,000

Fig. 2. A myelinated fiber in a culture 50 hours after exposure to GB serum. The external mesaxon (arrow) is open. ×22,000
Fig. 3. A myelinated fiber in a culture 50 hours after exposure to GB serum. The external mesaxon (arrow) is open and the separation extends almost completely around the fiber between the cytoplasm and the outermost myelin lamella. × 24,000

Fig. 4. A myelinated fiber from a culture 50 hours after exposure to GB serum. All the lamellae appear separated and loosened. × 96,000
Fig. 5. A longitudinal section through the paranodal region of a myelinated axon (A) from a culture 48 hours after exposure to GB serum. The lateral loops are separated from each other but not from the axolemma where the transverse bands are visible. × 74,000

Fig. 6. A longitudinal section through the paranodal region of a myelinated axon (A) from a culture 72 hours after exposure to GB serum. The lateral loops are separated from each other, as well as from the axolemma and the transverse bands are not visible. × 105,000

In general, vacuolization was most pronounced in areas immediately adjacent to the myelin sheath and when severe, was always associated with alterations in the myelin sheath (Fig. 10). Vacuolization of Schwann cell cytoplasm is not unique since similar alterations have been described in other disorders of the peripheral nervous system (7, 39, 40).
Fig. 7. An altered myelinated fiber from a culture 24 hours after exposure to GB serum. Intramyelinic splits arising at the intraperiod line and resulting in electron lucent areas are visible. × 30,000

Fig. 8. An altered myelinated fiber from a culture 72 hours after exposure to GB serum. The perixonal space is distended and the surrounding myelin is distorted. The axon (A) is well preserved. × 21,000
Fig. 9. Disintegrating myelin from a culture 24 hours after exposure to GB serum. × 27,000

Fig. 10. A cross section of a severely demyelinated fiber in a culture 24 hours after exposure to GB serum. Only the axon (A) and basement membrane are relatively intact. The surrounding cell processes seem well preserved. × 30,000
In the most severely involved fibers, large axons devoid of myelin and enclosed within a markedly altered Schwann cell were observed. These axons were surrounded by numerous vesicles and several distorted remains of myelin lamellae (Fig. 11) or by a cytoplasmic process containing numerous dense lipid droplets. These extensive changes were found only in cultures treated with GBS serum and not in controls.

Fig. 11. An altered myelinated fiber from a culture 50 hours after exposure to GBS serum. The axon (A) is surrounded by a cell process, vesicles and lamellae, but no intact myelin. X 20,000

Fig. 12. A myelinated fiber in a culture 50 hours after exposure to GBS serum. Loosened and degenerating myelin and irregular vesicles, as well as an apparently viable and separate cell process, surrounding the comparatively well preserved axon. X 20,000
Fig. 13. Higher magnification of a portion of Figure 12. Altered myelin is visible on either side of the viable cell process. An abrupt discontinuity of a major dense line is visible at the arrow. $\times 112,000$

Fig. 14. A separate cell process, surrounded by a basement membrane, is visible within the periaxonal space in the myelinated fiber from a culture 72 hours after exposure to GH serum. $\times 30,000$
Fig. 15. A cell process penetrates the basement membrane (arrows) and appears to surround the axon (A) in this culture 50 hours after exposure to GB serum. The bulk of the cell containing numerous membrane-bounded dense bodies including distorted myelin, is outside the basement membrane. × 20,000

Fig. 16. An altered myelinated fiber in a culture 50 hours after exposure to GB serum. The axon is distended and its organelles are disorganized. The myelin and Schwann cell, however, are well preserved. × 20,000
B. Invading Cell Processes:

Invading cells were usually absent from the affected areas described above. However, cell processes obviously originating in the culture were occasionally seen in association with altered myelin sheaths. These were seen between myelin lamellae (Figs. 12, 13) and within the periaxonal space where they sometimes were found to encircle the axon one or more times. Some invading processes were accompanied by a basement membrane (Fig. 14) indicating their Schwann cell origin, while others, with no such membrane were clearly identifiable as phagocytes (Fig. 15). Invading cell processes were observed only among the cultures treated with GBS serum.

C. Axonal Changes:

In both control and experimental cultures, non-myelinated fibers and ganglion cells appeared normal. Axons, even when completely denuded of myelin were rather well preserved. On rare occasions, however, definite alterations could be observed unassociated with damaged myelin sheaths. These axoplasmic changes included focal swelling (Fig. 16) associated with granular disintegration or focal accumulation of vacuoles, mitochondria and dense bodies. Similar changes were occasionally in control cultures but less frequently than in cultures exposed to GBS serum.

DISCUSSION

The results obtained in the present study may shed some light on the questions posed in the Introduction.

The answer to the first question as to whether these changes represent primary demyelination or Wallerian degeneration seems clear. For the most part, in areas outside the central necrotic zone, found in both treated and untreated cultures, the axons remained remarkably well preserved, even in areas of extensive demyelination. Rare axonal alterations were observed but these could be found even when the surrounding myelin and Schwann cell were apparently normal. These configurations were probably examples of early Wallerian degeneration presumably originating in the central necrotic zone. Thus, the changes observed in cultures exposed to GBS sera do represent a true demyelination, as was concluded by Cook et al (10) in their light microscopic study.

The second question regarding patterns of myelin breakdown is less simply answered. While we have considerable information regarding the various configurations of myelin disintegration observed after the application of GBS serum, the precise sequence of events is difficult to ascertain. It is tempting, however, to regard the opening of the external mesaxon and the subsequent progressive separation of myelin lamellae as an early or perhaps the initial sign of myelin breakdown. Such changes would then permit the rapid infiltration of myelin toxic factors allowing greater contact with the myelin surfaces. These alterations might then lead to the other configurations observed including splitting of the myelin lamellae at the intraperiod line, distention of the periaxonal space and the various abnormalities in Schwann cell cytoplasm. Finally, total
destruction of the sheath occurs accompanied by vesicular dissolution of the myelin resulting in a denuded axon. A difficulty with the above schema is that the regular lamellar separation regarded as an early change leading to myelin breakdown was observed in cultures exposed to sera from only half of the GBS patients used.

The only other fine-structural study of true demyelination of peripheral nerve in tissue culture, so far as we are aware, was made by Masurovsky et al in 1967 (41). These authors irradiated cultures of rat dorsal root ganglia and observed the subsequent destruction of myelin with concomitant sparing of the axon at least in the initial stages. The configurations associated with demyelination were quite similar to those observed by us. Our own results differ in that myelinated fibers alone were affected whereas irradiation also damaged the non-myelinated population. Furthermore, Masurovsky et al (41) reported no invading cell processes, such as we observed associated with demyelination. We regard the invading cell processes as either Schwann cells, perhaps reconstructing the damaged myelin sheath, or macrophages removing cellular debris.

The remaining question regarding the similarity of changes observed in vitro to those seen in vivo may, in a general way, be answered affirmatively. Many of the changes observed were distinctly similar, although not identical, to those previously reported in GBS and also in EAN and EAE.

To our knowledge, there are no other published fine-structural studies of the demyelinating effects of GB serum in vitro. We must, therefore, compare our results to the several in vivo studies reported. Finean and Woolf (39) were the first to describe the fine structural alterations in GBS in biopsy specimens of peripheral nerve. Our findings agree in that only myelinated fibers are the targets but we have not observed the severe axonal changes they report. The discrepancy may be explained by the fact that Finean and Woolf (39) examined the cutaneous nerve where Wallerian degeneration may follow severe, chronic disease resulting in axonal damage (see Asbury et al) (2). Our results compare more closely with those of Wiśniewski et al (3) and Carpenter (4) who studied spinal nerve roots and peripheral nerves in postmortem material derived from GBS patients. These authors did not, however, report the regular separation of myelin lamellae or myelin splitting. These same authors pointed out that mononuclear cells were nearly always found in areas of myelin destruction. This last observation contrasts with our findings and may best be explained by the experimental design whereby no host cells were present.

In a light microscopic study of the in vitro effects of EAN serum, Yonezawa et al (42) reported segmental demyelination, lysis of the myelin sheaths and phagocytosis after the application of sera from animals with EAN. These results were remarkably similar to those reported by Cook et al (10) in their in vitro studies of GBS sera. No ultrastructural studies on the effects of serum from EAN animals on nervous tissue cultures have been described. However, fine-structural studies of peripheral nerve lesions in animals with EAN and EAE show that the process is primary demyelination. Disagreement exists as to the prevalence of mononuclear cells in areas of demyelination. Lampert (5) and
Wiśniewski et al (6) both regard such cells as an absolute requirement. Ballin and Thomas (7) and Schröder and Krücke (8), on the other hand, conclude that myelin breakdown can occur even when such cells are not immediately present.

A detailed fine structural study of the effects of EAE serum on cultures of central nervous system tissue has been reported recently by Raine and Bornstein (26). Their results were similar to our own in that demyelination occurred without the constant presence of invading mononuclear cells. Damage to oligodendroglia and myelin breakdown with preservation of the axon were observed by these authors. The investigators of in vivo studies of EAE also question the necessity for the immediate presence of invading mononuclear cells for myelin disintegration (43, 44). Bubis and Luse (45), Lampert and Carpenter (36) and Lampert (37), on the other hand, among the first to study EAE in detail at the fine structural level, have emphasized the importance of the immediate contact between invading mononuclear cells and the myelin sheath in order for demyelination begin. According to Lampert (30, 37) regular lamellar separation identical to that which we observed in peripheral nerve fibers was a characteristic of EAE.

The alterations reported here occurred, either exclusively or to a much greater degree, among the cultures exposed to GBS serum. However, we do not mean to imply that these changes occur only as an effect of GBS sera. For it has recently been shown (46) that occasionally sera from patients with other disorders will produce changes in myelinated fibers in culture which, at the light microscopic level, are indistinguishable from those observed after the application of GBS serum. Moreover, the serum of one out of eleven normal individuals was found to have a similar effect. Only a careful comparison at the ultrastructural level of the patterns of myelin breakdown between those produced by GBS sera and by myelinotoxic non-GBS sera will permit us to make a reasonable judgment concerning the specificity of the morphological alterations reported here.

SUMMARY

Sera from patients with the Guillain-Barré syndrome and from normal individuals were applied to myelinated cultures of mouse dorsal root ganglia. From 20 to 72 hours after exposure, the cultures were examined with the electron microscope. Several alterations in the myelin sheath were observed in the GBS cultures. These alterations included opening of the external mesaxon, regular separations of the myelin lamellae, intramyelinic splits, distention of the periaxonal space and vacuolization of Schwann cell cytoplasm. Occasionally, viable cell processes, some of which were identifiable as either Schwann cells or phagocytes, were seen invading the damaged myelin sheaths. Most of the axons, even those completely denuded of myelin, were rather well preserved. The alterations in the myelin sheaths, therefore, represent a true demyelination rather than a reaction to axonal damage.
FINE STRUCTURAL ASPECTS OF DEMYELINATION in Vitro

ADDENDUM

Since the completion of this manuscript, we have received a communication from Drs. M. Dubois-Dalcq, M. Buyse, G. Buyse and F. Gorce confirming the presence of a complement-dependent demyelinating factor in 5 out of 6 cases of GBS. Ultrastructural studies of peripheral nerve cultures showed primary demyelination with splitting of myelin at the intraperiod line as well as lamellar disintegration and alterations of the Schwann cell cytoplasm.

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