Lesioned corticospinal tract axons regenerate in myelin-free rat spinal cord

(central nervous system/regeneration/neurite growth/inhibitors/oligodendrocytes)

T. Savio* and M. E. Schwab†
Institute for Brain Research, University of Zurich, August-Forel-Strasse 1, CH-8029 Zurich, Switzerland

ABSTRACT In the adult central nervous system (CNS) of higher vertebrates lesioned axons seemed unable to regenerate and reach their former target regions due to influences of the CNS microenvironment. Evidence from in vitro and biochemical experiments has demonstrated the presence of inhibitory substrate components in CNS tissue, in particular in white matter. These CNS components, which strongly inhibit neurite growth, were identified as minor membrane proteins of defined molecular mass (35 and 250 kDa) in oligodendrocyte membranes and CNS myelin. Oligodendrocyte development and myelin formation can be prevented by x-irradiation of newborn rats. Here we show that in myelin-free spinal cord corticospinal tract fibers transplanted at 2 weeks of age show reelongation of many millimeters within 2-3 weeks after the lesion. In normally myelinated controls, regenerative sprouts grew less than 1.7 mm caudal to the lesion.

Absence of regeneration of lesioned axons in the central nervous system (CNS) of higher vertebrates has been known for a long time (1). Nevertheless, when allowed to regrow into transplanted peripheral nerves, many types of CNS neurons show an intrinsic capability to regenerate and elongate their lesioned processes (2, 3). In vitro, neurons show a clear preference for peripheral nerve tissue if cultured in the presence of sciatic and optic nerve explants or on frozen sections of the peripheral nervous system or the CNS (4-6). For CNS tissue, gray matter is clearly preferred over white matter (6, 7). These effects are substrate effects rather than the result of a lack of trophic factors. Experiments with dissociated cells in culture and with tissue fractions as substrates have shown that differentiated oligodendrocytes and CNS myelin are, indeed, inhibitory substrates for axonal growth (8). This inhibitory effect is due to defined membrane proteins called neurite growth inhibitors (NI-35 and NI-250) that are present in oligodendrocyte membranes and CNS myelin (9, 10). They are tightly membrane-bound and act in a contact-mediated fashion.

The potent effect of these inhibitory myelin proteins in various in vitro assays led to the question of their relevance for CNS axonal regeneration in vivo. We, therefore, studied the regrowth of corticospinal tract (CST) axons after spinal cord lesions in the rat under conditions of suppressed myelin formation. Earlier lesion experiments in normal rats have shown that, after postnatal day (P) 4-6, no growth of CST axons can be seen caudal to the lesioned segments (11). Similar results were obtained in hamsters and cats (12, 13). We have, therefore, transplanted the rat spinal cord at P12-17, a time when (i) CST fibers have reached their target regions (14-16), (ii) no regeneration or growth of new axons after lesion occurs (11), and (iii) myelin formation in the CST is well under way (14, 17, 18). Regrowth of CST fibers over significant distances was seen within 2-3 weeks in myelin-free, but not in control, spinal cords.

MATERIAL AND METHODS

X-Irradiation. Newborn Lewis rats shortly after birth were anesthetized by cooling on ice and covered with a protective lead shield. A slit of 5 × 12 mm in this shield was adjusted in correspondence with the thoracic and lumbar spinal cord. A 5500-rad dose of x-rays (1 rad = 0.01 Gy) was administered under the following conditions: 50 kV, 15 mA, a 20-cm focus distance, and a 0.25-mm aluminum filter as modified from refs. 19 and 20. In some rats the irradiation was repeated at P3 or P4.

The efficacy and selectivity of the treatment was checked by immunostaining of longitudinal spinal cord sections for galactocerebroside (antibody O1) (21, 22) and for myelin basic protein (MBP), as markers for differentiated oligodendrocytes and myelin, and for glial fibrillary acidic protein (GFAP), a marker for astrocytes. P30 rats were fixed by perfusion with buffered 4% (wt/vol) paraformaldehyde (pH 7.2). Cryostat sections (25 μm) were incubated for 30 min with undiluted mouse monoclonal antibody O1 (hybridoma supernatant). For labeling with antibodies to MBP (Boehringer Mannheim) or GFAP (Dakopats, Copenhagen), sections were pretreated with 95% ethanol/5% acetic acid (vol/vol) for 30 min at 4°C. Bound antibodies were visualized with fluorescein-conjugated anti-mouse or anti-rabbit immunoglobulin (Cappel Laboratories).

In Vitro Experiments. X-irradiated and normal P30 rats were killed with an overdose of ether. Spinal cords were rapidly removed and frozen in liquid nitrogen. Cross sections (20 μm) from the lumbar spinal cord of x-irradiated or normal rats or from the nonirradiated cervical spinal cord were cut on a cryostat, mounted on glass coverslips (10 mm in diameter), dried, and placed into wells of 4-well culture dishes (Greiner, Nütingen, F.R.G.). After washing the sections with medium, neuroblastoma cells (NB-2A) were added (50,000-100,000 cells per well) in 100 μl of Dulbecco’s modified Eagle’s medium with 10% (vol/vol) fetal calf serum, incubated for 1-2 days at 37°C, fixed in 4% (wt/vol) formaldehyde, and stained with cresyl violet (6).

Lesions. The CST was transected in P12-17 normal and x-irradiated rats. The skin was longitudinally cut on the back. The spinal cord was exposed at the middle to lower thoracic level by a laminectomy and opening the dura. The dorsal one-half to two-thirds of the cord was bilaterally transected by cutting transversely with iridectomy scissors, and a staple-shaped stainless steel wire was placed into the lesion and left

Abbreviations: CNS, central nervous system; CST, corticospinal tract; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; P, postnatal day.

*Present address: Istituto Superiore di Sanità, Laboratory of Pathophysiology, Viale Regina Elena 299, I-00161 Rome, Italy.
†To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
for the entire survival time of the animal (23). This was done so that the precise position and depth of the lesion could be checked later. Collagen strips were placed on the exposed spinal cord with 400 μl of Bacitracin (Roche, Basel), and muscle and skin were separately sutured. By this operation all the fibers of the dorsal CST running in the medioventral part of the dorsal funiculus (the large majority of the CST fibers) as well as the fibers of the dorsolateral CST [very few fibers in the rat (24)] were transected bilaterally. Rats were allowed to survive 15–27 days after the lesion. In treated rats the lesion was placed at the rostral end of the x-irradiated region.

Tracing of the CST. At P37–41, 1 μl of a 5% (wt/vol) wheat germ agglutinin-horseradish peroxidase solution was injected uni- or bilaterally into the frontoparietal cortex to anterogradely label the CST fibers. Then 24 hr later, rats were fixed by perfusion with 1.25% (vol/vol) gluteraldehyde/1% paraformaldehyde, and complete series of longitudinal frozen sections (25 μm) of the caudal one-third (10–25 mm) of the spinal cord were cut, mounted, and reacted using tetramethylbenzidine as a substrate (25). Section series were evaluated under dark-field illumination in polarized light and reconstructed to determine the maximum distance at which labeled CST fibers could be observed caudal to the lesion site. All rats where the CST lesion was incomplete or doubtful, as judged from careful examination of the section series, were excluded from the analysis. Likewise, rats in which the spinal cord was lesioned completely or to such an extent that sprouting CST fibers could not reach the caudal part of the lesion were discarded.

RESULTS

Suppression of Myelin Formation by X-Irradiation. Experimental rats were x-irradiated over the thoracic and lumbar spinal cord at birth (P0), a treatment known to prevent oligodendrocyte development and myelin formation (19, 20). Immunostaining of spinal cord sections with antibody O1 (recognizing galactocerebroside, refs. 21 and 22) and antibodies against MBP as markers for differentiated oligodendrocytes and myelin confirmed the almost complete absence of these antigens (Fig. 1 a–d). In contrast, GFAP-positive astrocyte processes formed a dense network in the fiber tract region ("white matter") as in normal controls (Fig. 1 e and f). Schwann cell immigration (26), visualized by the antibody O1 or MBP, did not occur to a detectable extent over the time period observed. Biochemical experiments also showed the complete absence of the neurite growth inhibitors NI-35 and NI-250 in these spinal cords (N. Schaeren Wiemers, P. Caroni, and M.E.S., unpublished observation).

In Vitro Substrate Properties of Spinal Cord Sections. We first tested the substrate properties of myelin-free spinal cord tissue in vitro. Neuroblastoma cells were cultured on frozen sections from x-irradiated and normal P30 lumbar spinal cord as well as from cervical nonirradiated spinal cords for 2 days. On the sections of control lumbar spinal cords and of the cervical nonirradiated region, neuroblastoma cells selectively adhered to gray, but not white, matter (Fig. 2 a); in sharp contrast, cells were found in large concentrations also on the fiber tract regions ("white matter") on sections of irradiated spinal cords (Fig. 2 b). These results show that the nonpermissive substrate property of white matter can be reversed by suppression of oligodendrocyte development, confirming our earlier results with antimetotic agents and with antibodies against myelin-associated neurite growth inhibitors (6).

In Vivo Experiments: Lesions of the CST. By 2–3 weeks after the spinal cord lesion, vertebrae in the x-irradiated region sometimes appeared altered in their shape (see refs. 19 and 20), occasionally compressing the spinal cord below. The lesion itself appeared large and always showed a typical histological picture: the wire hole was surrounded by com-
as a result of dying back). A zone of sprouting was always present rostral to the lesion in controls and in x-irradiated spinal cords (Fig. 3a). Sprouted CST fibers could be seen to course around the lesion, either ventrally or laterally, as well as occasionally through tissue bridges (Fig. 3c). Only relatively few fibers reached the caudal part of the lesion area (Fig. 3a and c). These fibers, however, were easily recognizable as fine rows of reaction product. In all of the controls, none of the newborn animals, a proce-
noticeable, however. It may be due to suboptimal trophic factor supply, lack of very favorable substrates, intrinsic properties of the CST neurons, and probably a strong "barrier" effect of the very large lesions.

The pronounced difference in the reelongation of lesioned CST fibers between myelinated and myelin-free spinal cords is consistent with a role of myelin-associated neurite growth inhibitors in preventing axon reelongation under normal conditions. This interpretation is supported by experiments (27) in which a similar regeneration of CST axons has been obtained in normal spinal cords under the influence of an antibody that neutralizes the inhibitory activity of CNS myelin.

We thank Mrs. L. Schnell for technical advice. Drs. C. Bandlow, D. Cadelli, J. Kapfhammer, and P. Paganetti for critically reading the manuscript, Mrs. S. Kaufmann for secretarial help, and Mr. R. Schoeb and Mrs. E. Hochreutener for their help with the figures. This work was supported by the Swiss National Science Foundation (Grant 3.065-0.87), the Swiss Multiple Sclerosis Society, the Dr. E. Slack-Gyr Foundation, the American Paralysis Association, and Regeneron Pharmaceuticals, Inc., New York.