Ineffectiveness of enzyme therapy on regeneration in the transected spinal cord of the rat

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The potential clinical significance of the experiments of Matinian and Andreasian prompted repetition of their experiments. A laminectomy was made at T-5 or T-8 in 92 young female rats, and a fine wire probe was passed beneath the intact cord. The cord was then transected and the probe lifted through the incision so as to ensure the completeness of the transection. Rats were injected with various enzymes of American or Russian manufacture, including trypsin, hyaluronidase, elastase, elastase plus trypsin, or vehicle. At autopsy 4 to 6 months later, all rats were paraplegic. The stumps of the spinal cord were separated by cysts and a fibroglial scar. Nerve fibers did not cross the lesion site, nor was there conduction of nerve impulses across the site of injury. There were no significant behavioral, histological, or electrophysiological differences between any of the treatment groups.

Transection of the spinal cord was then performed in additional animals using Matinian and Andreasian's original surgical method (without passing a probe beneath the intact cord). Most of these rats began walking within 2 to 3 weeks, without enzyme therapy. Histological analysis revealed intact nerve fibers in the ventrolateral region of the spinal cord, indicating that the spinal cord had not been completely transected. It is concluded that: 1) enzyme treatment neither facilitates nerve regeneration nor permits functional restitution in rats with spinal cord transection; and 2) previously reported findings were apparently the result of incomplete spinal transection.

KEY WORDS • spinal cord transection • enzyme therapy • experimental paraplegia • nerve regeneration

In most mammalian species, transection of the spinal cord renders the animal permanently paraplegic. Within a few days postoperatively, nerve fibers begin to regenerate into the site of injury, but these incipient regenerative efforts soon cease. Studies by Windle and his colleagues on cats with spinal cord transection indicated that the phase of active regeneration could be extended for several months by the administration of steroid hormones or Piromen (a pyrogenic polysaccharide of bacterial origin which apparently facilitated regeneration by reducing connective tissue and glial scar formation). Nevertheless, despite histological and electrophysiological verification of nerve fiber regeneration and impulse conduction across the site of transection, none of the animals showed recovery of posture or locomotion. Subsequently, attempts were made to depress scar tissue formation after cord transection by the intrathecal administration of trypsin in dogs and rats. These brief reports indicated that the treatment may have diminished the density of the collagenous scar, but since they were not followed by more complete reports on functional changes resulting from the reduced scar formation, we assume that the enzyme treatment did not ameliorate the paraplegic condition.

In spite of these disappointing results, there are scientific reasons for believing that a solution to the problems associated with central nervous regeneration can be achieved. Björklund and Stenevi have demonstrated regenerative capacity of the catecholaminergic pathways of the brain. They described axonal regeneration after transection of these pathways. Furthermore, their studies have shown not only that the regenerating nerve fibers functionally innervated peripheral tissues implanted into the brain, but
that the rate and degree of regeneration were enhanced by the administration of nerve growth factor.\textsuperscript{2} Collateral nerve sprouting, a form of regeneration in which intact nerve fibers grow new processes in response to injury of adjacent nerve fibers, has been demonstrated in the spinal cord by histological, electron microscopic, and biochemical techniques.\textsuperscript{10,16,18}

It is difficult at present to assess many of the studies on experimental paraplegia because inadequate experimental control procedures were often employed. In the rat and cat, for example, a subtotal transection of the spinal cord results in an initial phase of paraplegia which may last up to 1 month. After this period, the animal recovers posture and locomotion to a variable degree. In fact, as little as 5% to 10% of spinal cord substance need be left intact for such partial functional recovery to occur.\textsuperscript{5,6,23} It therefore seems likely that many reports of functional recovery in rats were the result of an incomplete spinal transection. It is essential that the histological and electrophysiological procedures be adequate to assess the degree of completeness of the spinal transection.

In 1976, an English translation of a 1973 monograph by Matinian and Andreasian\textsuperscript{17} was published in which enzyme therapy was reported to be efficacious in the treatment of experimental paraplegia in rats. Trypsin, hyaluronidase, elastase, or Pyrogenal were administered percutaneously at the site of injury to rats of which the spinal cord had been severed at T-5. They reported that the treated animals recovered the ability to support their weight on their hindlegs and to walk (albeit with an abnormal gait), and that control animals remained paraplegic. In addition to their clinical observations (which are summarized in Table 1), the authors presented histological evidence of nerve fiber regeneration and electrophysiological evidence of impulse conduction between the sciatic nerve and cerebral cortex. It is clear from the data shown in Table 1 that the effects are both statistically and biologically significant; to obtain satisfactory functional recovery in 27% to 47% of paraplegic rats is indeed a major achievement.

However, there are certain disquieting features to the report. The recovery of hindlimb function after treatment of rats with spinal cord transection with Pyrogenal (the Russian equivalent of Piromen) is surprising, since Windle\textsuperscript{21} never observed functional recovery after treatment of similarly treated cats with this drug. Second, the photomicrographs were poorly reproduced so that it is impossible to assess critically the histological observations. Despite these reservations, the clinical implications of the work have stimulated considerable interest,\textsuperscript{1} and have justified careful and thorough verification of the experiments.

**Materials and Methods**

**Surgery and Postoperative Care of Rats**

Female Wistar rats weighing 90 to 110 gm were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg). A longitudinal skin incision was made along the spinous processes from T-1 to T-7, and the trapezius muscle was exposed. A bilateral incision was made through this muscle at a distance of 2 to 3 mm from the spinous processes and muscle attached to the spinous processes was resected close to the spines to expose the tips of the spinous processes. The vertebral musculature was separated from the spinous and laminae with a blunt probe, and retracted. All remnants of muscle were then scraped from the laminae of two adjacent vertebrae, and the interlaminar and interspinous ligaments trimmed away with fine scissors. The rat was hyperflexed and the exposed laminae and spinous processes removed with microscissors. The dorsal half of the spinal cord was thus exposed for the length of one and one-half to two laminae. Bleeding was controlled by pressure and saline rinses without the use of Gelfoam or of cotton packs. The dura mater was then incised, and a fine wire hook with a blunt end was introduced subdurally and gently maneuvered so that it lay ventral to the spinal cord. A small fragment of razor blade in a holder was then placed next to the wire hook which thus served as a guide; a complete transection was effected in one movement by drawing the blade through the cord next to the wire hook. The hook was then drawn up through the lesion to demonstrate that the transection was complete. Bleeding was controlled by rinsing with saline, and hemostasis achieved within 3 to 5 minutes.

The wound was closed by suturing the muscles with 4-0 silk thread and the skin with stainless steel clips. After surgery, the rats were given a single intraperitoneal injection of 2 ml of 5% glucose and an intramuscular injection of penicillin (25,000 units in 0.1 ml). Penicillin injections were continued for 14 days postoperatively. The urinary bladder of every rat was

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**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>No. of Rats</th>
<th>Recovery of Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete (%)</td>
<td>Partial‡ (%)</td>
</tr>
<tr>
<td>CONTROL</td>
<td>94</td>
<td>0</td>
</tr>
<tr>
<td>LIDASE</td>
<td>63</td>
<td>33.3</td>
</tr>
<tr>
<td>TRYPsin</td>
<td>41</td>
<td>34.1</td>
</tr>
<tr>
<td>TRYPsin first then ELASTASE</td>
<td>40</td>
<td>47.5</td>
</tr>
<tr>
<td>PYROGENAL</td>
<td>33</td>
<td>27.4</td>
</tr>
</tbody>
</table>

*Data are adapted from Tables 3 and 4 of the monograph by Matinian and Andreasian.\textsuperscript{17} Lidase is a Russian product that contains hyaluronidase activity. Pyrogenal is the Russian equivalent of Piromen, a pyrogenic bacterial polysaccharide.

†This designation indicates very weak to unsatisfactory recovery.
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evacuated by gently compressing the abdomen at the time of surgery and twice daily thereafter until bladder control became automatic. Rats were housed individually in a room maintained at 24°C. The bedding (cedar shavings) in each cage was changed daily.

**Treatment and Observation of Living Rats**

**Enzymes: Trypsin.** Crystalline bovine trypsin, dialedyzed and lyophilized, and free from chymotrypsin activity was secured from Sigma Chemical Company and from the Worthington Laboratories. In addition, trypsin similar to that used by Matinian and Andreasian\(^{17}\) was obtained from the USSR. The specific activity of each of these preparations was determined by incubating an appropriate concentration of enzyme for 3 minutes at 35°C in 40 mM Tris-HCl (pH 8.1) and 1 mM \(p\)-toluenesulfonyl-L-arginine. The change in optical density at 247 nm was determined spectrophotometrically and compared to the Worthington preparation as standard. The specific activities (units/mg protein) were: Sigma trypsin 23 ± 3 (mean ± SEM), Worthington trypsin 204, Russian trypsin 57 ± 0.1. Since we followed Matinian and Andreasian's procedure of injecting 0.35 mg trypsin per kg body weight (regardless of enzyme activity), the rats treated with Sigma trypsin received less than half the activity of those treated with Russian trypsin, while those treated with Worthington trypsin received almost four times the activity of those treated with the Russian trypsin. In this way, the effect of a wide range of dosages was assessed.

At the time of surgery, trypsin solution (0.35 mg/kg in 0.5 ml phosphate buffer, 0.05 M, pH 7.4) was administered at the site of transection (before the wound was closed). Two hours later, the same dose was injected intramuscularly into the gluteal region. On subsequent days, the same dose of trypsin was injected percutaneously into the site of the lesion by the best visual estimate. This was followed 2 hours later by an intramuscular injection of the trypsin. Control rats received 0.5 ml of vehicle according to the same schedule. This treatment regimen was begun on the day of the operation and was continued for 15 days thereafter. Because of the instability of enzymes in solution, the lyophilized enzyme powder was dissolved in the phosphate buffer just before injection.

**Enzymes: Elastase.** Elastase was obtained from Sigma Chemical Company in the form of aqueous suspension containing 15 units/mg protein of \(\times 2\) crystallized enzyme. In a series of rats with spinal cord transections, 5 mg/kg (equivalent to 75 units/kg) of elastase was injected at the site of transection as described above. A second dose containing the same amount of enzyme was given intraperitoneally 2 hours later. These injections were also given for 15 days. A similar schedule was followed for another series of rats in which vehicle alone was injected (0.5 ml carbonate buffer, pH 8.8).

In a series of rats in which a combination of trypsin and elastase was used, trypsin from the Russian source was first injected at the site of transection (0.35 mg/kg in 0.5 ml phosphate buffer) and followed 30 minutes later by 5 mg/kg of elastase (also in 0.5 ml) injected into the same region. This treatment regimen was followed for 15 days postoperatively.

**Enzymes: Hyaluronidase and Lidase.** We used chromatographically pure hyaluronidase from bovine testes having an activity of 3000 units/mg protein, obtained from Sigma Chemical Company, and crystalline lidase, similar to that used by Matinian and Andreasian,\(^{17}\) obtained from the USSR. Since the lidase was accompanied by no statement of activity or purity, its specific activity was determined spectrophotometrically.\(^{4}\) The hyaluronidase from the Sigma Chemical Company contained 396 units/mg protein activity, while the lidase contained 75 units/mg protein activity. Single injections of 32 units/kg of hyaluronidase or lidase were made at the site of transection in rats for a period of 15 days. The enzymes were made up in 0.5 ml of 0.9% sodium chloride, and control rats were injected with 0.5 ml of 0.9% sodium chloride.

**Clinical Examination**

The rats were observed postoperatively for return of sensory and motor functions over a period of 4 months, after which time the acute electrophysiological studies and the terminal histological evaluation were performed. The clinical evaluations were made during the week of surgery and every 2 to 3 weeks thereafter. For sensory functions, responses to heat and cold were tested by dipping the feet of the rat in water at 55°C or at 5°C while holding the rat in such a manner that its hindlegs hung free. The time required for withdrawal was also noted. Response to pain proved to be a more reliable test and served as the standard method for testing sensory modalities. In this test, the skin below the transected region was pinched with a mouse-tooth forceps at several points on the back, hindlimbs, and tail. A response was positive if the rat squealed or consistently turned its head toward the site of stimulation.

Functional recovery in motor ability was assessed by determining the ability of a rat to use its hindlimbs in walking, by its performance in climbing a tilted wire screen (30° incline), and by its ability to walk along the edge of a table without falling off. Two other reflexes were tested in assessing functional recovery. The righting reflex was tested by placing the rat on its back and determining how long it took to right itself. Function of the peroneal nerve was assessed by determining whether the rat could spread its toes when lifted by the tail.\(^{12}\) The atrophy and tone of the hindlimb muscles was judged by palpating the muscles and by determining the degree of resistance offered by passive movement of the limbs.

All of the above clinical observations were on the scale ranging from 0 (absent) to ++++ (full ability). Variations in this scale can be described as ±, doubtful; +, slight; ++, moderate; and ++++, good.
Evoked potentials were recorded from the somatosensory cortex after stimulation of the sciatic or radial nerves of enzyme-treated and vehicle-treated rats. The animals were anesthetized intraperitoneally with chloral hydrate (400 mg/kg), and the cisterna magna was drained of cerebrospinal fluid by cisternal puncture to minimize cerebral edema. Craniotomy or laminectomy was then performed, the dura removed, and the left or right cerebral cortex exposed. The brain surface was covered with mineral oil and the temperature maintained at 37°C. The animal's head was fixed rigidly in a stereotaxic frame by means of ear bars and a mouthpiece. The stimulating electrodes were blunt-tipped, chlorided silver wires, and the recording electrodes were bipolar or monopolar platinum wires. The electrodes were placed on the surface of the cortex with the aid of a binocular dissecting microscope. The recording electrodes were connected to a preamplifier, and the potentials photographed with a Polaroid or kymograph camera. Sites on the cortical surface that responded to stimulation of the radial or sciatic nerves were mapped in the manner described by Hall and Lindholm. Stimulating pulses (2 Hz, 0.3 msec) were delivered by a square-wave generator. The stimulation voltage was gradually increased from 0.1 V in increments of 0.2 V until the threshold was reached. In general, the threshold was at 0.3 to 0.4 V. The precise localization of the recording electrode was verified by moving it 1 to 2 mm from the site at which the threshold had been achieved and observing whether the response was intensified or abolished.

In other rats, a laminectomy was done to expose the cervical (C1-4) and lumbar (L1-5) spinal cord. The dura was removed and the exposed spinal cord covered with warm mineral oil. The sciatic nerve was stimulated as described above and action potentials recorded from the ipsilateral lumbar (below the level of transection) and cervical (above the level of transection) regions. In this way, transmission of nerve impulses across the site of transection was evaluated.

Histological Procedures

To avoid damage to the spinal cord during autopsy, the undisturbed spinal cord was prepared within the vertebral column. Rats were anesthetized and killed at selected postoperative time intervals by intracardiac perfusion with 15 ml of isotonic saline solution followed by 100 ml of Dubose-Brasil's alcoholic Bouin's fixative. The thoracic portion of the vertebral column was dissected from the perfused rat and decalcified for 5 days in two changes of formic acid-sodium formate buffer (pH 2.2, 4.5 M). The tissue was washed thoroughly and dehydrated through graded alcohol series and ether-alcohol. It was infiltrated in increasing concentrations of collodion (1%, 2%, 4%, 8%, 12%, and 15%), after which a block was formed by hardening the collodion in chloroform. The collodion block was infiltrated with paraffin wax (melting point 45°C), and serial longitudinal sections, 10 μm thick, were cut on a sliding microtome. Alternate slides were stained by the following five methods: hematoxylin and orange G-erythrosin for overall morphology; phosphotungstic acid-hematoxylin (PTAH) for reactive glia; Crossman-Mallory trichrome stain for connective tissue; Protargol for nerve fibers; periodic acid-Schiff (PAS)-gallocyanin for Nissl substance.

Results

Method of Transection

Two surgical approaches to spinal transection were attempted. Procedure 1: To ensure complete transection of the spinal cord, we performed a laminectomy, passed a fine probe beneath the intact spinal cord, and lifted the probe through the site of transection. Examination of five animals immediately postoperatively showed that the cord had been completely transected in every case (Fig. 1A and B). Procedure 2: To duplicate the procedure used by Matinian and Andreasian, a laminectomy was performed, the spine hyperflexed, and the spinal cord transected with a sharp blade. All 10 animals prepared in this way (and not treated with enzymes), were paraplegic postoperatively, but eight of them began to walk spontaneously within 2 to 3 weeks postoperatively. Histological examination of the spinal cord between 1 and 4 months postoperatively in these animals showed that fibers in the ventrolateral region of the cord had not been transected (Fig. 1C and D). The fibers in this region appeared normal, and did not have the tortuous appearance or the varicosities that are characteristic of regenerating nerve fibers (Fig. 1F). An additional group of five animals was autopsied immediately after operation; a ventrolateral fascicle of intact fibers was seen histologically in every case, thus showing that complete transection of the cord cannot be achieved consistently with this technique.

Since Procedure 2 did not achieve complete spinal transection consistently, we adopted Procedure 1 to assess the possible effect of enzyme therapy on axonal regeneration in the transected spinal cord.

Clinical Evaluation

All the rats showed a total flaccid paralysis of both hindlimbs during the first 2 postoperative weeks. After this period, rats with subtotal transection (as determined by subsequent histology) recovered and demonstrated coordinated locomotor movements. On
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Fig. 1. A: Total transection of the spinal cord, saline treatment, 1 day postoperatively. Trichrome, x50. B: Total transection of the spinal cord, saline treatment, 1 day postoperatively. Protargol, x220. C: Subtotal transection of the spinal cord, saline treatment, 15 days postoperatively. Protargol, x110. D: Same as C, but at higher magnification (x 220). E: Dilated central canal proximal to the site of total transection, saline treatment, 4 months postoperatively. PTAH, x50. F: Fibers regenerating from dorsal roots (arrow) 4 months after total transection of the spinal cord, trypsin-elastase treatment. Protargol, x110.
the other hand, the rats with totally transected spinal cords displayed a persistent paralysis of the hindlimbs and a variable degree of hindlimb rigidity (regardless of whether they had been treated with enzymes or vehicle). All of these enzyme-treated and vehicle-treated paraplegic rats exhibited violent twitching hindlimb movements when heat or cold was applied to the hindlimbs. Since no vocal response occurred, these twitching movements apparently resulted from exaggerated spinal flexor reflexes at the lumbar level and not from restitution of the spinothalamic temperature pathway. When the skin of the leg or foot was pinched with a forceps, an exaggerated crossed-extensor reflex was also seen. A “pain” response (squealing) occurred only when a dermatome innervated by dorsal roots rostral to the level of the lesion was pinched. All of the enzyme-treated and vehicle-treated rats demonstrated these responses throughout the 4-month postoperative period, during which time they remained paraplegic. However, in those animals whose cords subsequently proved to have been subtotally transected, pinching of the skin caudal to the region of transection not only evoked a vocal response, but also caused the animals to turn their head in the direction from which the painful stimulus originated. This response was first noted at 1 to 2 weeks postoperatively.

Despite the intactness of the lumbar cord, as revealed by the hyperreflexic withdrawal and crossed-extensor reflexes, no rat with a completely transected cord could walk. The rat moved forward solely by the action of its forelimbs, and its hindlimbs were dragged along passively. The paraplegic rats were able to climb on inclined wire screen with little difficulty by the use of their forelimbs. When the hindlimb phalanges became caught in the meshwork of the screen, hyperreflexic movements occurred. However, these were never purposeful and lasted no more than a few seconds, after which the limb became flaccid again. Purposeful movement of the hindlimbs could not be elicited even by such severe stress as tilting the screen so as to face the rat in an extreme downhill direction.

Although rats with subtotally transected spinal cords could easily walk along the edge of a table and seldom fell off, those with transected cords invariably had great difficulty in moving along the edge without falling off. In rats with complete spinal transection the righting reflex was sluggish or in some cases absent, and the toe-spreading reflex was consistently absent. Wasting of the muscles was variable but never great.

**Electrophysiology**

In all rats with histologically proven total transection of the spinal cord, evoked potentials could be recorded from the sensory cortex after stimulation of the contralateral radial nerve, but not after stimulation of the contralateral sciatic nerve. Typical responses from a control rat treated with pH 7.4 phosphate buffer and two rats that received Russian trypsin are shown in Fig. 2. Stimulation of the radial nerve caused responses from somatosensory cortex within 10 msec in all animals, whereas stimulation of the sciatic nerve did not. Thus, the sensory pathway
between hindlimb muscles and the cortex was not intact in these rats. Figure 3 shows the results of similar experiments after treatment with other enzymes. In unoperated rats, stimulation of either the radial or the sciatic nerves evoked responses in the somatosensory cortex (not illustrated). In summary, stimulation of the sciatic nerve evoked no cortical responses in any of the rats with totally transected cords (regardless of treatment with enzymes).

Since all animals with histologically demonstrated subtotal spinal transection exhibited almost complete recovery of spontaneous locomotor ability, it was important to verify that these animals had intact and functional neural connections between the lumbar spinal cord and the cerebral cortex. Figure 4 illustrates the electrophysiological findings on two unoperated rats and on two with subtotal spinal cord transection. In all four rats, cortical potentials were evoked by stimulation of the sciatic nerve (Fig. 4A1 to D1). These responses were abolished when the spinal cord was transected acutely at the L1-2 spinal level (Fig. 4A2 to D2). However, an action potential was recorded from the caudal stump of the acutely transected cord in response to sciatic nerve stimulation (Fig. 4A3 to D3), indicating intactness of neural pathways between these sites. As would be expected, stimulation of the radial nerve in these animals, both before and after acute spinal transection at L1-2, elicited responses in the contralateral somatosensory cortex (Fig. 4A4 to D4).

Recordings from the dorsal surface of the cervical spinal cord are shown in Fig. 5. In the rat whose cord had been totally transected (as verified histologically) and treated with lidase, evoked potentials were elicited in response to stimulation of the radial nerve but not of the sciatic nerve. However, in the rat with a subtotally transected cord (as determined by subsequent histology), potentials were elicited by stimulation of either radial or sciatic nerves.

Histological Observations

The histological changes in all rats were similar regardless of whether or not they were treated with enzymes. The low-power photomicrographs (Fig. 6) show that 4 months postoperatively the site of the lesion was indicated by a scar of variable thickness, surrounded on either side by cystic spaces. The scar was primarily collagenous, as revealed by the trichrome stain. Although the scar was dense in some animals, it was quite attenuated in others; these variations were, however, unrelated to treatment, and the scar was similar in enzyme-treated and control animals. The PTAH stain revealed that glial fibers did not contribute materially to the scar. The cells within the scar were primarily fibroblasts and macrophages. Variable numbers of nerve fibers were seen, but for the most part these derived from dorsal roots. Figure 7A, for example, illustrates a myelinated nerve bundle within the scar; when traced in the silver impregnation preparations, (Fig. 7B) this bundle is clearly seen to originate from a dorsal root. Figure 7C illustrates that even a dense collagenous scar became well vascularized, and that the tortuous course of the blood vessels contributed to the disorganized, disoriented appearance of the scar. Also seen within the scar were aggregations of epithelial cells which had the appearance of glandular acini (Fig. 8F) and which were ependymal in origin. The central canal, in regions adjacent to the scar, became widely dilated (Fig. 1E) and the lining of epithelial cells began to proliferate (Fig. 7D). The proliferated cells gathered into small spherical clusters and migrated into the ad-
FIG. 4. Evoked potentials from the somatosensory cortex and dorsal surface of the lumbar spinal cord in two unoperated rats (A and B) and in two rats with subtotally transected spinal cords (C and D). In A₁ to D₁ are shown the evoked potentials in the cortex after stimulation of sciatic nerve. Between the recording in rows A₁ to D₁ and A₃ to D₃ the lumbar cord of each animal was transected acutely between L-1 and L-2. The sciatic nerve was again stimulated, and records A₃ to D₃ illustrate evoked potentials in the portion of the cord caudal to the acute transection in response to sciatic nerve stimulation. Subsequent stimulation of the radial nerve in these rats was still capable of evoking potentials in the somatosensory cortex (A₄ to D₄). Stimulation parameters were same as in Fig. 2.

FIG. 5. Evoked potentials recorded from the ipsilateral dorsal surface of the cervical spinal cord after total or subtotal transection. The rat with its spinal cord completely transected was treated with lidase (left), and the other animal received no enzyme treatment (right). Responses were recorded 4 to 5 months postoperatively. Note that stimulation of the radial nerve evoked responses from the dorsal surface of the cervical cord in both rats, whereas stimulation of the sciatic nerve evoked a response only in the rat with subtotal transection (three superimposed traces). Stimulation parameters were the same as in Fig. 2.

Adjacent scar tissue (Fig. 7E) where they could often be seen as isolated cell nests which concealed their origin from the central canal (Fig. 7F). These cell groups formed secretory acini which, by accumulation of fluid, gave rise to adenomatous cysts of various sizes (Fig. 7E). Sometimes the cells lining these cysts were more than a single cell layer thick, while at other times the cell layer was single and flattened.

The cystic dilations that surrounded the scar, and thereby separated the rostral and caudal segments of the spinal cord, were variable in dimension. In some animals they were small and did not completely separate the cut ends of the spinal cord, while in others they were massive and resulted in a separation of the cut ends of the cord for as many as three vertebral segments. The extent of cyst formation was unrelated to treatment, and was similar in enzyme-treated and vehicle-treated groups. The spinal cord at the margin of the cysts was devoid of axons (Fig. 8A), but contained proliferated glial fibers which encapsulated the stumps of the spinal cord (Fig. 8B). Some of the nerve cells in this region appeared quite normal with the

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Fig. 6. Photomicrographs showing the effect of vehicle and enzyme treatments on the lesion site in rats 4 months after total transection. C = cyst. Trichrome, ×50. A: Carbonate buffer treatment. Arrow indicates budding of acini from central canal. (See Fig. 7D for higher magnification.) B: Sigma trypsin. C: Russian trypsin. D: Lidase (Russian hyaluronidase). Note the extent of cyst formation (which prevented union of rostral and caudal segments of spinal cord) in all animals and the variable thickness of the connective tissue scar (which was found to be unaffected by any of the enzyme treatments).
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Fig. 8. Photomicrographs of the region adjacent to the lesion 4 months after total transection of the spinal cord. A: Russian trypsin treatment. Note that the nerve fibers do not reach the margin of the lesion which is bounded by a cyst (C). Protargol, ×110. B: Elastase treatment. Proliferation of glial fibers is seen at the margin of the lesion (at the very site shown to be devoid of nerve fibers in A). PTAH, ×110. C: Trypsin-elastase treatment. Neurons are seen undergoing retrograde changes in the spinal cord one vertebral segment from the margin of the lesion. PAS-gallocyanin, ×440. D: Sigma trypsin treatment. Arrows indicate degenerating neurons within developing microcysts. PAS-gallocyanin, ×440. E: Elastase treatment. Note the extension of necrosis into adjoining segments even at this late postoperative time. Trichrome, ×50. F: Sigma trypsin treatment. Acini of ependymal denervation in a strand of connective tissue are shown crossing the site of transection. Trichrome, ×440.
galloxyanin stain, while others showed chromatolytic and degenerative changes (Fig. 8C). As the neurons degenerated and were phagocytized, microcysts that contained neuronal debris could be discerned. The cellular nature of the debris could be appreciated only in sections stained by the trichrome method, which revealed remnants of the cell nucleus and nucleolus (Fig. 8D). With all other histological methods, the contents of the microcysts appeared hyaline and homogenous.

Even after 4 months postoperatively, necrosis of the spinal cord adjacent to the cysts was noted (Fig. 8E), and suggested the possibility that the degenerative changes had not yet ceased. Variability in the rate of formation in the experimental animals. Experiments and suggested the possibility that the degenerative spinal cord adjacent to the cysts was noted (Fig. 8E), and degenerative changes (Fig. 8C). As the neurons gallocyanin stain, while others showed chromatolytic and degenerative changes (Fig. 8C). As the neurons gallocyanin stain, while others showed chromatolytic and degenerative changes (Fig. 8C).

To compare the results of the various treatments in an unbiased fashion, all slides were coded and randomized and examined independently by two observers on a double-blind basis. The variability was seen equally in all control animals and enzyme-treated rats. None of the enzyme treatments had any effect on the histological composition of the lesion site. However, in animals with subtotal transection of the spinal cord, the cysts were smaller and fewer, and the cut ends of the spinal cord were better approximated.

Discussion

Effect of Enzyme Therapy

The present report describes observations made while attempting to duplicate the experiments of Matinian and Andreasian, who reported that enzyme therapy restored function in rats with transected spinal cords. However, our experiments on 92 rats revealed that none of our similarly prepared (enzyme-treated or vehicle-treated) rats showed any recovery from paraplegia, impulse conduction across the site of injury, or histological evidence of nerve fiber regeneration across the lesion. Partial functional recovery generally occurred if the spinal cord was incompletely transected; these animals recovered use of their hindlimbs to a considerable degree, and conduction of impulses across the site of injury was demonstrable. However, this recovery was not dependent on treatment with enzymes.

These negative results are not altogether surprising, since several previous publications have already appeared in which enzyme therapy was found to be without beneficial effect on regeneration of the central nervous system. In the earliest report, Guth, et al. showed that the application of trypsin and hyaluronidase according to Matinian and Andreasian's procedure was without histological or functional effect on the hemisected spinal cord of the rat. Subsequently, Knowles and Berry noted that trypsin and elastase or trypsin and hyaluronidase had no effect on regeneration after lesions of the cerebral cortex. Feringa, et al., very recently published a brief report indicating that these enzyme combinations had no effect on rats rendered paraplegic by midthoracic spinal transection. They suggested several reasons for the positive results of the Soviet investigators: "(1) that impurities contained in their enzyme preparations were the active ingredients; (2) that they had unique experimental animals; or (3) that other unknown factors are responsible." The first possibility is not tenable since in the present study we used both trypsin and lidase preparations that were identical to those used by Matinian and Andreasian. The second possibility is clearly too trivial to merit consideration; there is simply no evidence that the functional organization of the spinal cord varies significantly within species. Careful consideration of the surgical techniques and histological methodologies enables us to explain the cause of the discrepancy.

Surgical Procedures

Complete transection of the spinal cord of rats can be obtained consistently only when a fine probe is passed beneath the intact cord and lifted through the lesion after the transection is made. By this procedure, 116 of 120 animals showed complete transection of the spinal cord. Complete transection of the rat's spinal cord cannot be achieved consistently merely by direct incision with a fine blade. As has been noted by others, many of the animals so treated will have incomplete spinal transection, and animals with very little remaining spinal tissue will recover considerably from their initial paraplegia. As we and others have noted, visual inspection is inadequate to assess whether the cord has been completely transected; for example, Feringa, et al., attempted complete transection in 96 rats, and were successful in only 59 of them, despite careful surgical efforts to make the lesion complete and a thorough visual inspection of the lesion. We do not know the mechanism underlying the recovery from paraplegia after incomplete transection of the spinal cord. However, it seems clear that as little as 5% to 10% of residual pathways can and will allow restoration of considerable function within 2 to 4 weeks postoperatively. Recovery resulting from regeneration after complete transection of the spinal cord would be expected to take considerably longer. Matinian and Andreasian did not report the time at which locomotor function began to return, but they observed that bladder and bowel function returned in 6 to 16 days in the enzyme-treated animals and in 17 to 45 days in the control rats. A difference of this magnitude at so early a postoperative interval cannot be attributed to an effect of treatment on nerve regeneration, and suggests that the lesion may have been less extensive in the enzyme-treated animals. Furthermore, they reported that cortical potentials could be evoked by stimulation of the sciatic nerve...
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within 22 days postoperatively. The multiplicity and complexity of the factors involved in restoration of function after nerve injury (retrograde reaction, growth through the scar, inhibition of growth by disorganized scar tissue, maturation of regenerated nerve fibers, and establishment and maturation of synaptic connections) make it unlikely that regeneration of ascending and descending spinal pathways could occur so rapidly as to restore function within 4 weeks postoperatively.

**Histological Methodologies**

The previous discussion leads to the suggestion that Matinian and Andreasian may have not completely transected the spinal cord in many of their animals. To validate the surgical procedure, one must examine the animals at short postoperative intervals to demonstrate physiologically the absence of impulse conduction across the site of injury and to verify histologically, in serial sections, that the transection was complete. It is especially important at these early postoperative times to avoid mechanical damage to the cord during autopsy. If but 10% of the spinal cord remained intact, this tissue could easily be torn during removal of the spinal cord from the vertebral column; in such an eventuality the incompleteness of the surgical procedure would not be noted. To obviate this possibility, we prepared histological sections of the spinal cord within the vertebral column. This procedure, which was also used by Feringa, et al., has the additional merit of maintaining the gross relationships of the osseous and neural elements, thereby enabling us to distinguish with certainty regenerated dorsal root fibers from intrinsic spinal fibers.

Matinian and Andreasian did not employ these precautions in preparing their specimens for histological examination. Nevertheless, we can make some tentative interpretations from Figs. 12, 13, 17, 19, 20, 21, 33, and 36 of their monograph. Several of the photomicrographs of animals that showed recovery of function reveal ventrally located, longitudinally oriented tissues that may well represent undamaged fascicle of nerve fibers. Figures 12A, 20A, and 36A are noteworthy in this regard. In Fig. 20A the huge cyst in the dorsal portion of the cord does not extend to the ventral region, indicating that the ventral part of the cord may well have been undamaged at the time of surgery.

Their Figs. 13A and 33 illustrate preparations from enzyme-treated rats that showed full recovery of function. However, the scar tissue is so dense that it is difficult to conceive that nerve fibers could have regenerated through so formidable a barrier. Indeed, Feringa, et al. reported a similar case of apparent regeneration which proved illusory when careful histological reconstruction of complete serial sections revealed a fascicle of nerve fibers that had been spared at the time of surgery. Unless complete serial reconstruction is performed in every case of apparently successful regeneration, one cannot rule out the possibility of incomplete transection of the spinal cord.

Finally, questions may be raised about the nature of the fibers in some of their illustrations. Those in their Fig. 21, for example, do not have the appearance of nerve fibers and may represent reticular fibers which are often revealed by silver techniques. Likewise, the nerve fibers in Fig. 19 do not appear to be "newly-formed" because they lack the varicosities that are characteristic of regenerated nerve fibers. In Fig. 17A, we see virtually no nerve fibers in a specimen from an animal that showed partial recovery of function. In this case, at least, there seems to be little correlation between the functional recovery and the presence of nerve fibers at the site of injury.

**Functional Recovery**

It remains difficult to explain, in the report by Matinian and Andreasian, why all of the control animals remained paraplegic while 27% to 45% of the enzyme-treated ones showed considerable return of function. In such large-scale (579 rats), long-term (4 to 6 months) experiments one must pay careful attention to experimental design. Animals must be assigned to various treatment groups in a controlled fashion to rule out unconscious bias in the selection of the animals, and, if more than one surgeon is employed, each surgeon must operate on some animals of each treatment group. The monograph does not provide sufficient information to assess the adequacy of these aspects of the experimental design.

**Conclusions**

We have been unable to obtain support for the contention that enzyme therapy facilitates spinal regeneration and functional recovery in spinal cord transected rats. The available data suggest that the contrary conclusions of Matinian and Andreasian were derived from experiments in which the spinal cord had not been completely transected. The variability in the histopathological picture after spinal transection does not enable us to state with certainty that the enzyme treatment had no effect on the density of the scar, but we can state that significant functional nerve fiber regeneration did not occur in animals with either dense or loose connective tissue scar. Since factors other than scar tissue formation influence the regenerative process, it is necessary to analyze the interaction of these factors if a rational approach to treatment of paraplegia is to be achieved.

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References


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