Chapter 16

THE POTENTIAL FOR SPINAL CORD REGENERATION IN THE RHESUS MONKEY

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THE STUDY of spinal cord function after injury dates back 4,000 years. Egyptian physicians accurately described and carefully recorded both the symptoms and dire prognosis of paraplegia and quadriplegia in *Edwin-Smith Surgical Papyrus* (1) written approximately 2000 B.C. Unfortunately, until recently both the symptoms and the prognosis have remained nearly the same as they were in ancient Egypt. Despite great strides having been made in rehabilitative medicine in this area, the outlook for return of lost cord function has remained bleak. However, within the last sixty years there have been some exciting advances made in understanding the central nervous system, its pathophysiology, and its capacity for healing and regeneration. Ramon y Cajal was the pioneer in this field as he was in so many others related to the nervous system. In the classical work *Degeneration and Regeneration of the Nervous System* (2) published in 1928 he noted that the spinal axons of small mammals did regenerate when the cord was transected, but that they rapidly became entangled in a glial scar and were unable to bridge the gap of the injury. He and his pupil, Tello, explored experimentally the use of sciatic nerve grafts into the spinal cord and brain and in a few suitable cases were able to show sprouting of the central nerve fibers using the sheath cell component of the graft as a guidance system. The problem of axonal guidance systems was again addressed by Sugar and Gerard in 1940 (3). They implanted sciatic nerves immediately postinjury into the rat's severed spinal cord. Thirteen of their rats were said to regain motor function. This was confirmed by stimulating the motor cortex of the rats, which resulted in hindlimb movement. More recently the histologic and electron microscopic observations of Kao and his associates have demonstrated the pathophysiology of axonal terminal club formation and cavitation in the transected spinal cords of dogs (4, 5, 6); they have emphasized the importance of delayed debridement and peripheral nerve grafting in order to allow maximum "lysosomal spinal cord autotomy" prior to implantation of the peripheral nerves. This coupled with Blakemore's demonstration that central axons can be myelinated by Schwann cells (7) provide the scientific rationale for an in-depth look at the regenerative capabilities of the spinal cord in sub-human primates.

Simultaneous with the advancements in microsurgical techniques, which made Kao's work possible, there has been an increasing interest in investigating the effect of electromagnetic radiation on tissues. In 1961 Becker published his pioneering work on bioelectrical factors in amphibian limb regeneration (8). More recently Wilson has applied a pulsed electromagnetic field to the hemisected, nongrafted, spinal cord of cats (9). Wilson found less glial scar formation and more generating axons traversing the site of the cordotomy in the treated animals than in the controls. These results have encouraged us to test this mode of therapy in the grafted animal. The rhesus monkey was chosen because, unlike the dog, reflex walking is not seen after paraplegia. Moreover, preliminary studies have been carried out in the lower mammals, namely the rat, the dog, and the cat, making a spinal cord more similar in structure and function to man's the next logical step.

Methods and Materials

Total of nine rhesus *(Macaca mulatta)* monkeys were studied from a clinical, vascular, anatomical, histological, and electrophysiological point of view: two served as controls for the physiological and anatomical data; seven were test animals (see Table 16-1). Each of the test animals underwent a two level laminectomy at T12-L1 or L1-L2, followed by a crush injury to the cord delivered with a needle holder. The holder's 3 mm blade was applied circumferentially around the cord and closed to its minimum interblade distance for five seconds. Care was taken to include the entire diameter of the spinal cord within the blades. All surgical procedures, including the crush injury, were done using the Zeiss operating microscope. One, two, or three weeks later, depending into which treatment group the animal fell, the laminectomy was reopened. Using the microsurgical technique described elsewhere (10), the dura and pia were opened and the necrotic cord removed. Care was taken not to damage the anterior spinal artery or the smaller pial vessels. Six of the seven animals underwent a second surgery at one to three weeks after the crush for debridement of the necrotic tissue and in four of these six animals the gap was grafted with autologous peripheral nerve using two to three segments of the peroneal nerve placed longitudinally in the .05-1 cm gap left by the debris removal (Fig. 16-1). One of the seven animals did not have the damaged cord removed and thus served as a control for the other six.

Five of the seven animals, including four that were nerve grafted and one that was not, were treated with pulsed electromagnetic energy (EME) for thirty minutes twice a day (Table 16-1). The EME treatment was delivered by a Diapulse® Unit (Fig. 16-2). At the settings used, this instrument delivered an average of 25.3 watts for 65 microseconds (peak emission 975 watts) with an interval delay of 2,400 microseconds at a frequency

TABLE 16-I
SPINAL CORD REGENERATION IN THE RHESUS MONKEY:
TREATMENT, EVALUATION AND RESULTS

Monkey	*Pre- and Post-op Angio*	*Crush*	*Debridement*	*Graft*	*EME**	*SER's†*
896	+	+	2 wks	2 wks	+	–
704	+	+	3 wks	—	+	+
710	–	+	3 wks	3 wks	+	–
239	+	+	3 wks	—	–	–
707	+	+	—	—	+	–
649	+	+	1 wk	1 wk	+	+
884	–	+	3 wks	3 wks	–	–

Monkey	*Cortical Stimulation*	*Sacrificed*	*Autorad.*	*H.R.P.‡*	*Clinical Improvement*
896	–	13 mo.	–	–	–
704	–	9 mo.	–	+	–
710	–	8⅓ mo.	–	–	–
239	–	8 mo.	–	–	–
707	–	9 mo.	–	–	–
649	–	8 mo.	not done	not done	–
884	–	7½ mo.	–	–	–

* Electromagnetic energy treatment.
† Somatosensory evoked responses.
‡ Horseradish peroxidase.

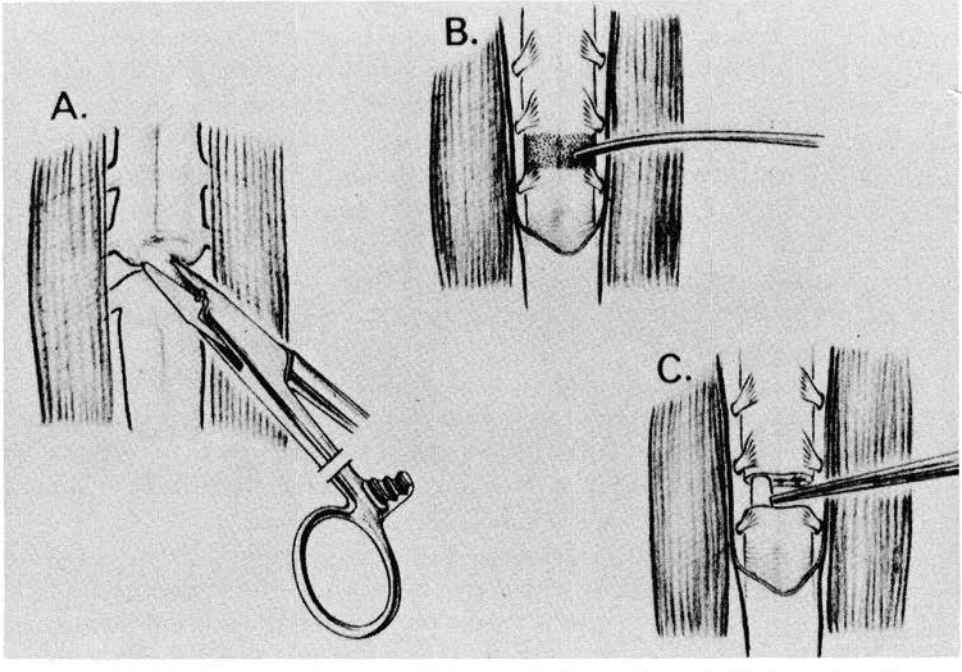

Figure 16-1. (A) Crush injury, (B) Subpial removal of necrotic cord, (C) Insertion of nerve graft.

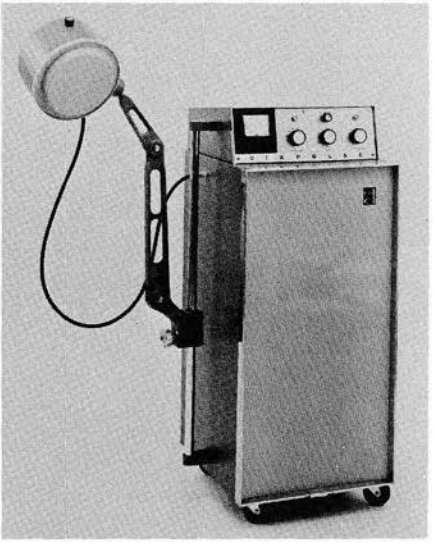

Figure 16-2. Diapulse unit.

of 27.12 megacycles. In the unanesthetized animal this causes no temperature elevation (11). The animals were then evaluated radiographically, neurologically, physiologically, histochemically, and histopathologically (Table 16-1).

Radiologic Evaluation

To evaluate potential major vessel damage by the original crush injury and the subsequent surgery, precrush and postsurgery spinal cord angiography was done on five of the seven paraplegic animals.

Neurologic Status

The animals were examined weekly for return of function in their lower extremities.

Physiological Status

Four days before kill, the afferent and efferent systems were studied using cortical somatosensory evoked responses (CSER), spinal cord somatosensory evoked responses (SSER), both above and below the lesion, and direct cortical motor stimulation with EMG recordings of the limbs (Fig. 16-3, 16-4). Both control and test animals were studied by the same investigator (D.R.) without the knowledge of previous treatment. With the animal under Ketamine® anesthesia, constant temperature monitoring and in a stereotactic head holder, bilateral craniotomies were performed exposing both the motor and sensory cortices. Two level laminectomies were

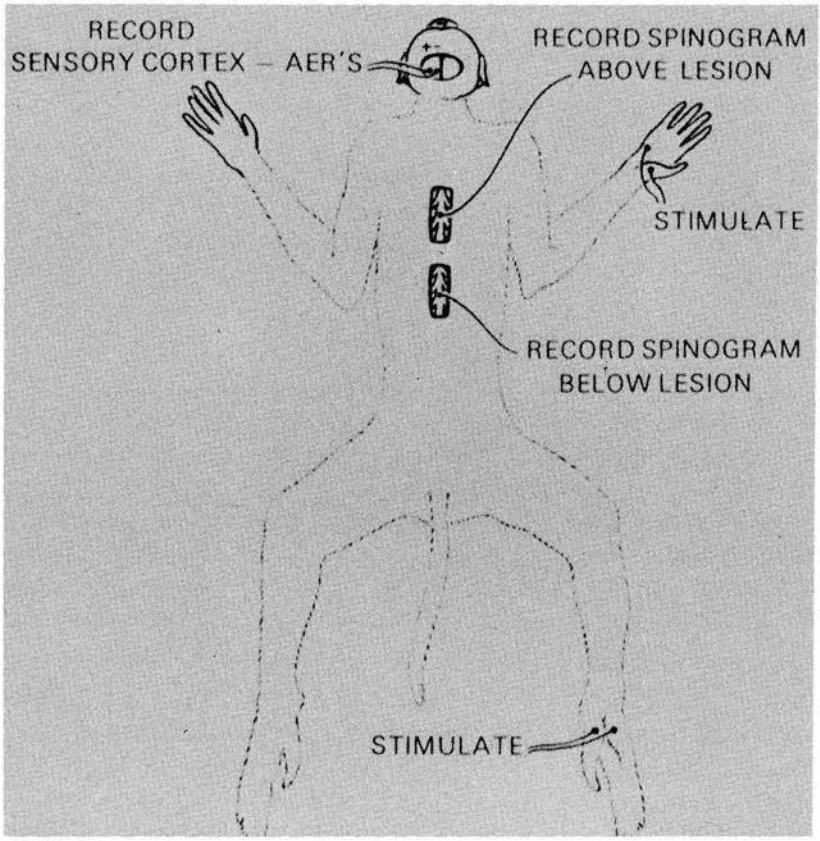

Figure 16-3. Schematic representation of stimulation and recording sites for SSERs and CSERs.

done both above and below the crush lesion at a distance of three to four inches. The dura and pia were opened at all sites using standard microsurgical techniques. Using Ag/AgCl ball electrodes attached to a Nicolet® stimulator, the motor cortices of both forelimbs and hindlimbs were sequentially stimulated fifty times at a strength of 11-14 milliamps, a rate of 15.1/sec, and a stimulus duration of 100 microseconds. EMGs of the upper and lower extremities were recorded using needle electrodes attached to a Nicolet CA-1000 recording unit.

Cortical somatosensory evoked responses and spinal somatosensory evoked responses were recorded using Ag/AgCl ball electrodes placed bilaterally on the sensory cortices of both the forelimb and the hindlimb and on the dorsal columns at least 3-4 cm above and below the crush lesions. The stimulus was delivered via needle electrodes inserted into the area of the median nerve in the forelimb and the peroneal or sciatic nerve

in the hindlimb. Stimulus strength was determined by adding two milliamps to the minimal current at which a muscle twitch was seen (t + 2). The stimulus was delivered using a Nicolet constant current stimulator at a rate of 0.5/sec, a duration of 100 microseconds, a filter band pass setting on the Nicolet CA-1000 of 5-3,000 Hz, and a sensitivity of 250 microvolts. Fifty stimuli were averaged over a period ranging 100-200 milliseconds. Each electrode's impedance was carefully checked and maintained below 2.0 kohms. In addition to the t + 2 milliamp stimulus, which ranged between 3 and 6 milliamps, each hindlimb was also stimulated with the maximal current output of the stimulator, or 19.9 milliamps. The two control animals differed from the test animals in that following the above series of tests a microscopic subpial transection of the cord was carried out and the entire series repeated at eight hours after transection.

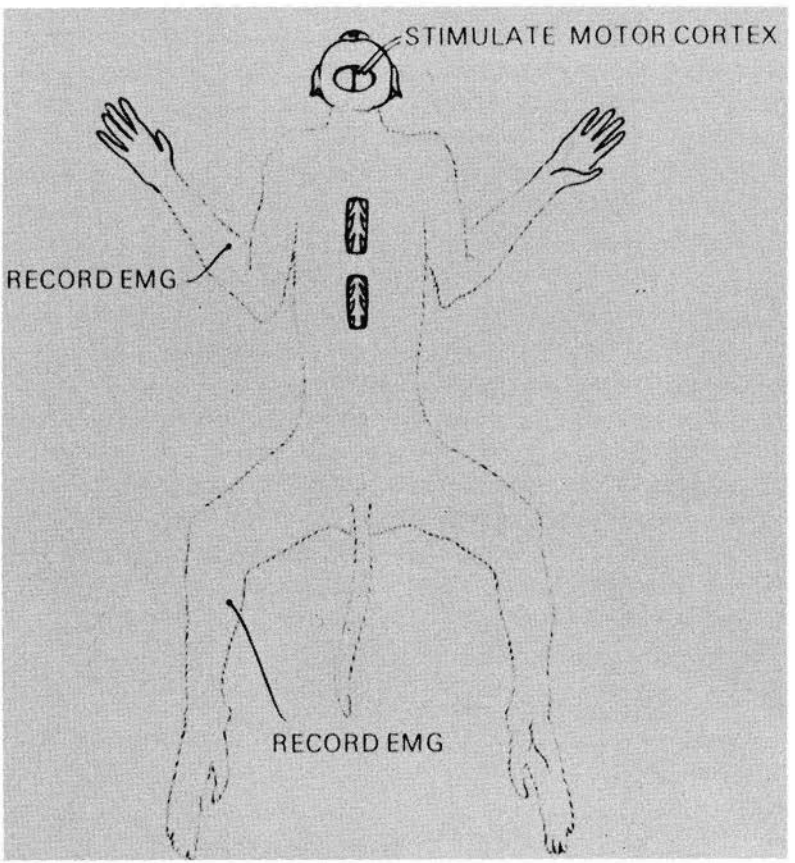

Figure 16-4. Schematic representation of stimulating site of the motor cortex and recording site for EMGs.

Histochemistry

Ascending and descending pathways were examined by determining the fate of horse radish peroxidase (HRP) injected into the dorsal columns below the lesion and radioactive leucine injected into the motor cortex respectively. Immediately following collection of the neurophysiological data, a 25% solution of HRP Sigma Type VI dissolved in distilled H_2O was injected with a Hamilton syringe into the lumbar cord several centimeters distal to the transection site. Each animal received four injections (two on each side) of four microliters each. The anatomical integrity of the corticospinal tract was tested by injecting tritiated leucine at a concentration of 20 microcuries/microliter of sterile saline into the hindlimb area of the motor cortex. A glass micropipette attached to a pressure injection system delivered five injections of five microliters to each hemisphere.

Three days following the injections the animals were killed by exsanguination. Four liters of 0.9% saline were perfused directly into the ascending aorta after a window had been cut in the right atrium of the heart. Following the washout, six liters of 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1*M* phosphate buffer, pH 7.4, and three liters of 10% sucrose in 0.1*M* phosphate buffer at 4°C were perfused. The brain and spinal cord were immediately removed and placed in the sucrose solution. Frozen transverse sections cut 50 microns thick 3-4 cm above and below the lesion, sections through the lesion, as well as brain stem and coronal longitudinal sections were obtained and collected in phosphate buffer. The standard HRP reaction was then carried out using the method of Mesulam (12). Tissue sections for autoradiography were also taken above and below the spinal lesion as well as in the brain stem and cortex. They were embedded in gelatin or paraffin. Frozen sections were cut at 50 micra in the former and routine sections at 10 micra in the latter. After thorough drying, the sections were defatted/deparaffinized in xylene for twenty-four hours and dehydrated. Under safe-light conditions the slides were dipped in Kodak® NTB-3 liquid emulsion and after drying were boxed with Drierite® and stored in the refrigerator at 4°C for three weeks. Autoradiographs were developed with Dektol® (one part stock to two parts water) at 19°C for 1.5 to 2 minutes, fixed and washed. Sections were stained with cresyl violet, dehydrated, and coverslipped.

Histopathology

Routine slides were taken in coronal longitudinal sections through the lesion and stained with H and E, Laphams, Masson's, and either De Meyer's silver in the case of the paraffin sections or Bodian in the case of the frozen sections.

Results

Neurology

None of the animals recovered the use of their lower limbs during the period of follow-up, which in the longest lived survivor was thirteen months.

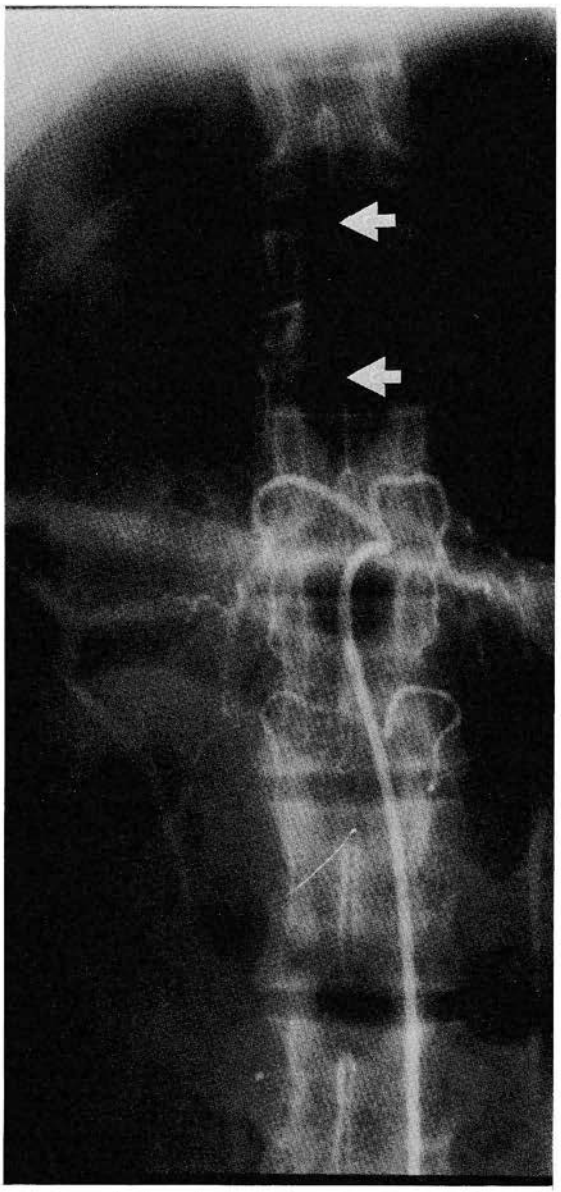

Figure 16-5. Preoperative spinal angiogram showing the anterior spinal artery intact (see arrows).

Radiology

In each of the five animals studied, the integrity of the anterior spinal artery was maintained through the crush injury and the subsequent surgery (Fig. 16-5, 16-6). No other abnormalities could be detected by this technique.

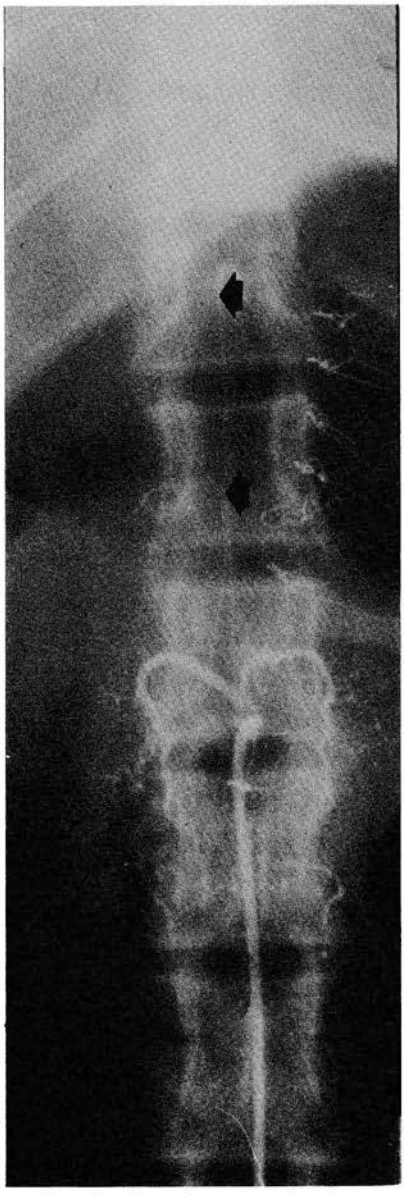

Figure 16-6. Post-nerve graft spinal angiogram showing anterior spinal artery intact. Arrows indicate the anterior spinal artery.

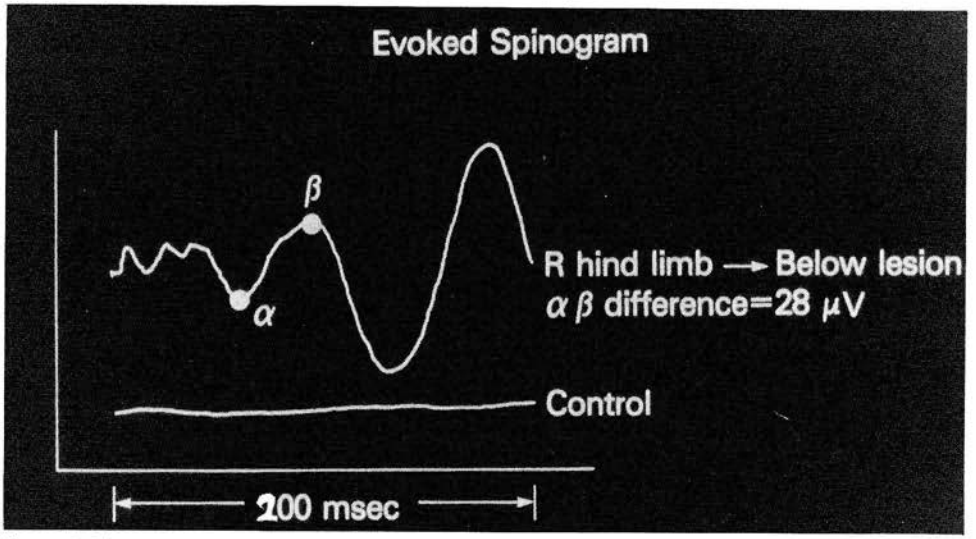

Figure 16-7. Top tracing: SSER measured rostral to the lesion nine months after transection of the cord (Monkey 704). Bottom tracing: Control noise level of the recording machine.

Neurophysiology

Two of the animals, #649 and #704, showed evidence of afferent neural processing across the lesion site. Compare Figures 16-7 and 16-8 with Figure 16-9. This spinal activity, however, was not seen at the cortical level. No concurrent motor activity was seen below the level of the lesions when the motor cortices of these animals were stimulated. We noted in our control animals, as have others, that post transection SSERs distal to the lesion are larger in amplitude than pretransection recordings at the same level.

Histochemistry

Although there were two animals with evidence of afferent signal processing through the lesion level, one animal, #649, had a cardiac arrest and expired at the end of lengthy neurophysiological testing and therefore no histochemical data is available on this animal. Animal #704 had evidence of intracellular HRP activity 3-4 cm above the lesion (Fig. 16-10). None was seen in the brain stem. However, this animal died sometime within the first twelve to fourteen hours after the HRP injection. The time factor might account for the lack of brain stem labelling. The other animals showed no evidence of HRP being transported cephalad to the lesion site.

Autoradiography

No evidence of tritiated leucine was found below the lesion site, confirm-

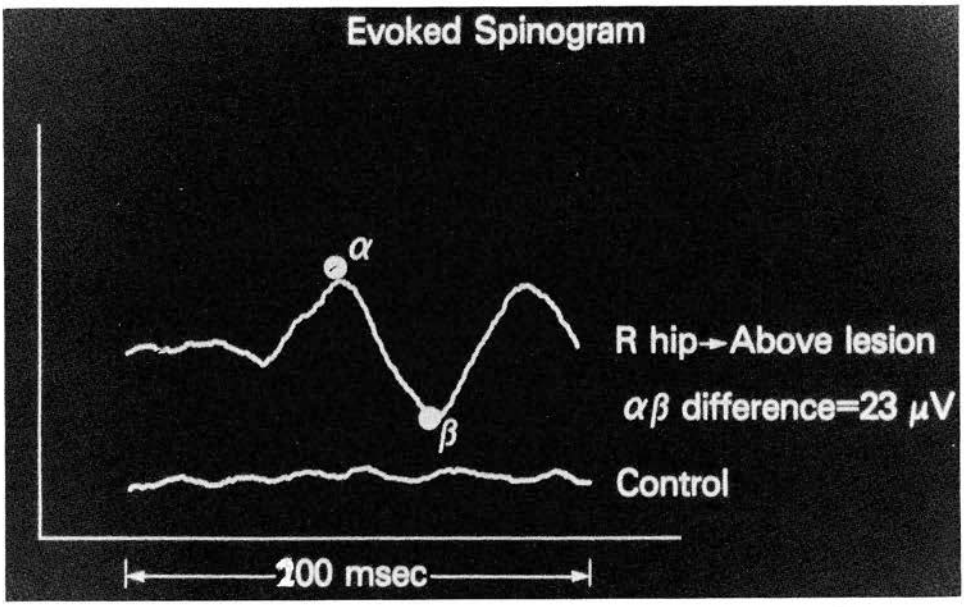

Figure 16-8. Top tracing: SSER measured rostral to the lesion nine months after transection of the cord (Monkey 704). Bottom tracing: Control, background electrical noise level of the recording machine.

ing the lack of efferent signal processing below the lesion and the failure of any clinical recovery of neurologic function.

Histopathologic

Animals #649 (crush, debridement, and nerve graft at one week, and EME) and #704 (crush, debridement, and EME) demonstrated evidence of axons bridging the gap of the lesion (Fig. 16-11, 16-12). In animal #896 (crush, debridement, and nerve graft at two weeks, and EME) there is also evidence of the axons bridging the lesion site. This animal appeared to show less cyst formation at both the distal and proximal sites of the graft (Fig. 16-13) than those grafted at three weeks. This is contrary to the findings in the dog (10), which suggests that maximum cyst formation and therefore the most opportune time to debride and graft takes place at one week. Animal #707 (crush injury and EME) showed no difference in histologic appearance (Fig. 16-14) from animal #239 (crush and debridement) (Fig. 16-15). Both had large cavity formation. This was in contrast to animal #704 (crush, debridement, and EME) which showed narrowing at the lesion site, but no cyst formation (Fig. 16-12).

Discussion

Abortive attempts of the mammalian spinal cord to regenerate post injury have been known since the 1920s (2). There appear to be three

major obstacles to successful completion of these axonal sproutings: (1) spinal cord autotomy and the subsequent cavity formation at the injured site; (2) the lack of a guidance system for the budding axons; and (3) glial scar formation. Kao et al. have described in detail the mechanism of spinal cord cavitation following spinal cord transection (4). Briefly, it appears that, after cord transection, the rostral and caudal spinal cord stumps are characterized by microscopical myelin cysts distended by fluid under pressure, each containing a swollen axon filled with many lysosomes and other axoplasmic organelles. As these microcysts rupture, the lysosomes with their lysosomal hydrolases are released into the extracellular space, destroying more of the spinal cord tissue, leading to cord cavitation. This process in the dog continues for approximately one week, provided that there is no further injury to the cord during that time, which could result in a retriggering of the whole process (6). Thus if one of the main obstacles to axonal outgrowth, namely the formed cavity, could be removed at approximately one week and a guidance system such as that found in peripheral nerves in the form of the bands of Bünger be inserted at the same time, two of the three obstacles could be, if not totally eradicated, at least lessened. In

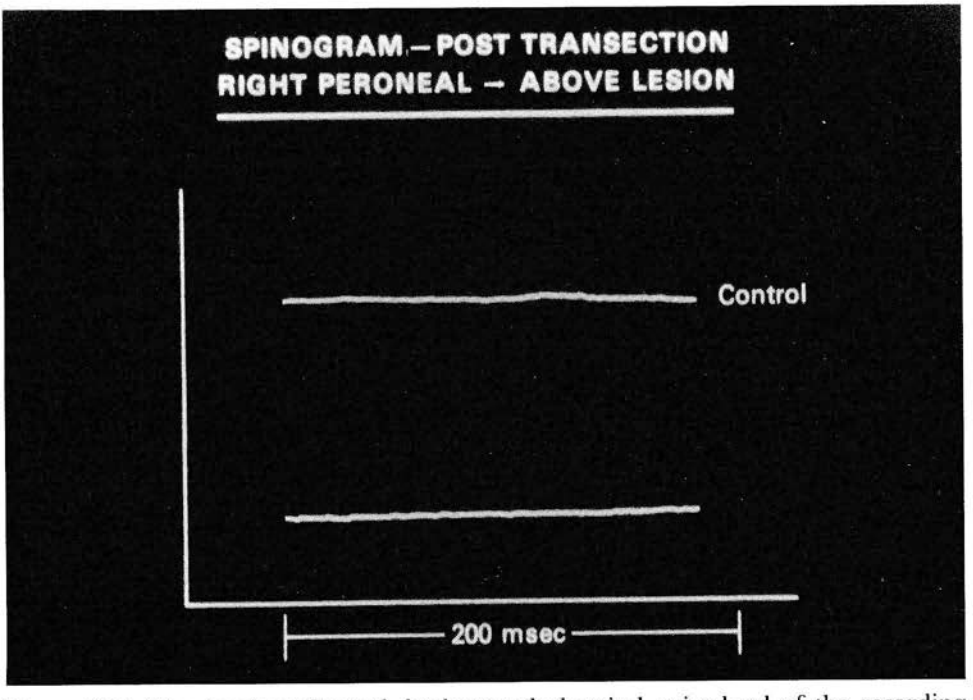

Figure 16-9. Top tracing: Control, background electrical noise level of the recording machine. Bottom tracing: SSER measured rostral to the lesion immediately after transection of the cord.

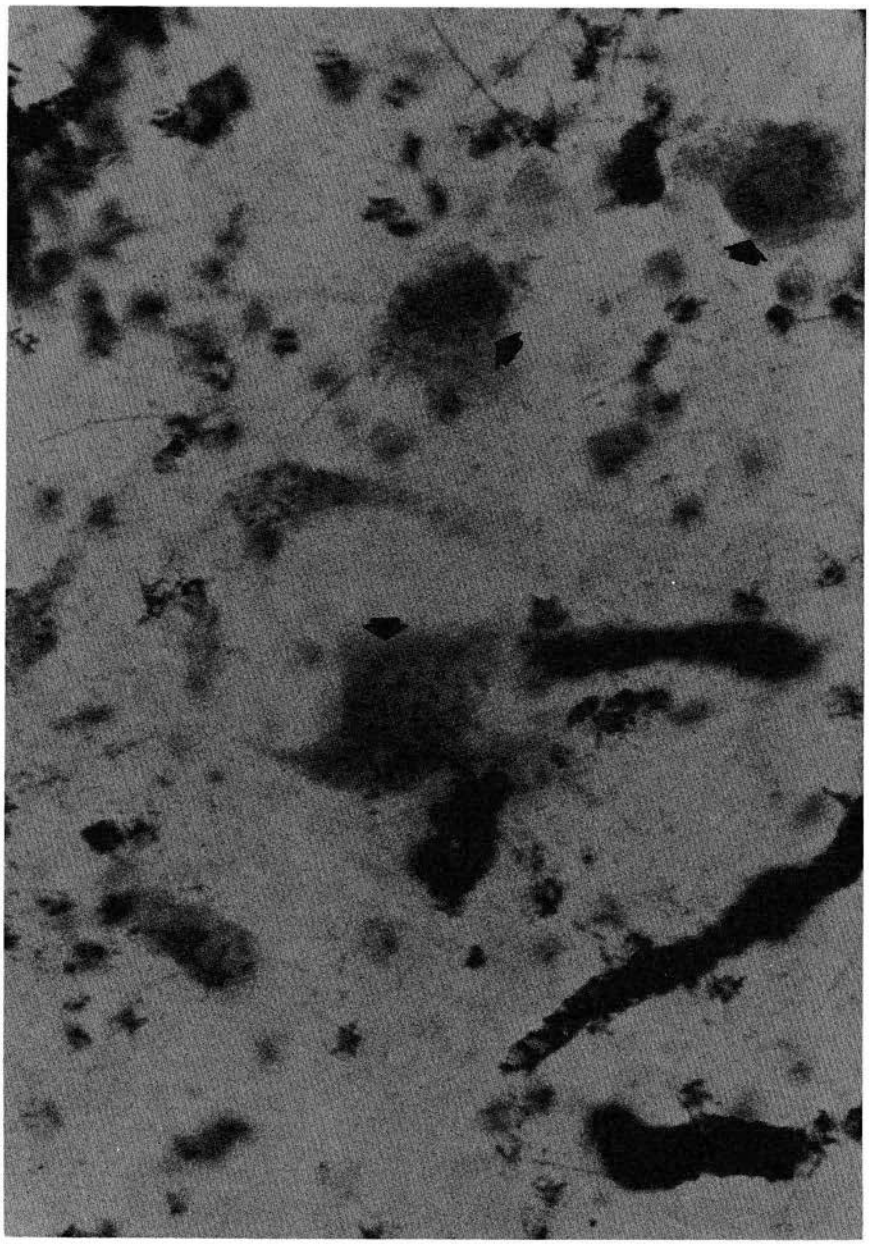

Figure 16-10. Positive intracellular HRP reaction within the neurons (arrows) in the gray matter of the spinal cord rostral to the lesion after injection of the HRP into the spinal cord caudal to the lesion in Monkey 704.

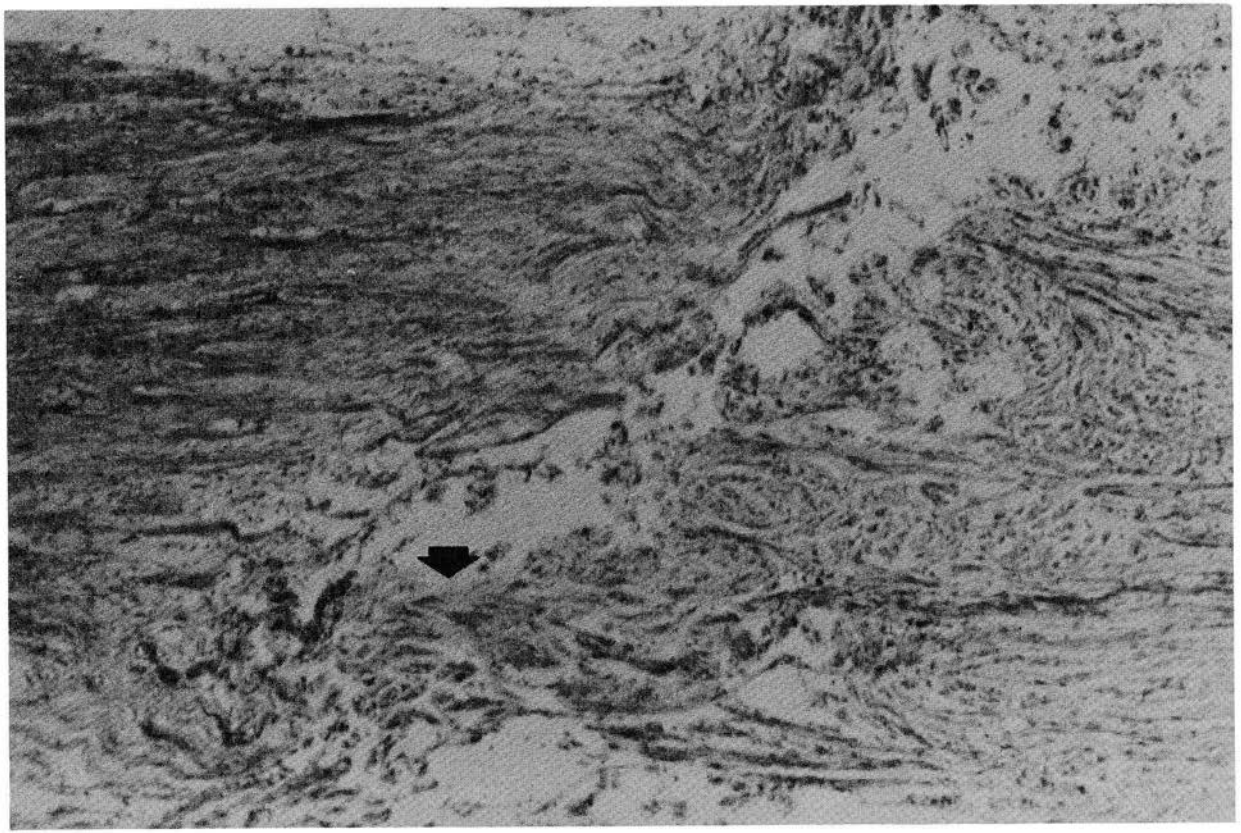

Figure 16-11. A longitudinal section of a nerve-grafted spinal cord showing the nerve graft — spinal cord junction. Nerve graft is to the left; spinal cord distal to the lesion is to the right. Axons are seen bridging the two segments (arrows). Hematoxylin and eosin stain has been used. All histological microphotographs in this report are longitudinal sections with the orientation of the animal's head to the left of the picture.

Figure 16-12. Monkey #704 had no nerve graft. Nerve fibers are seen bridging the lesion site.

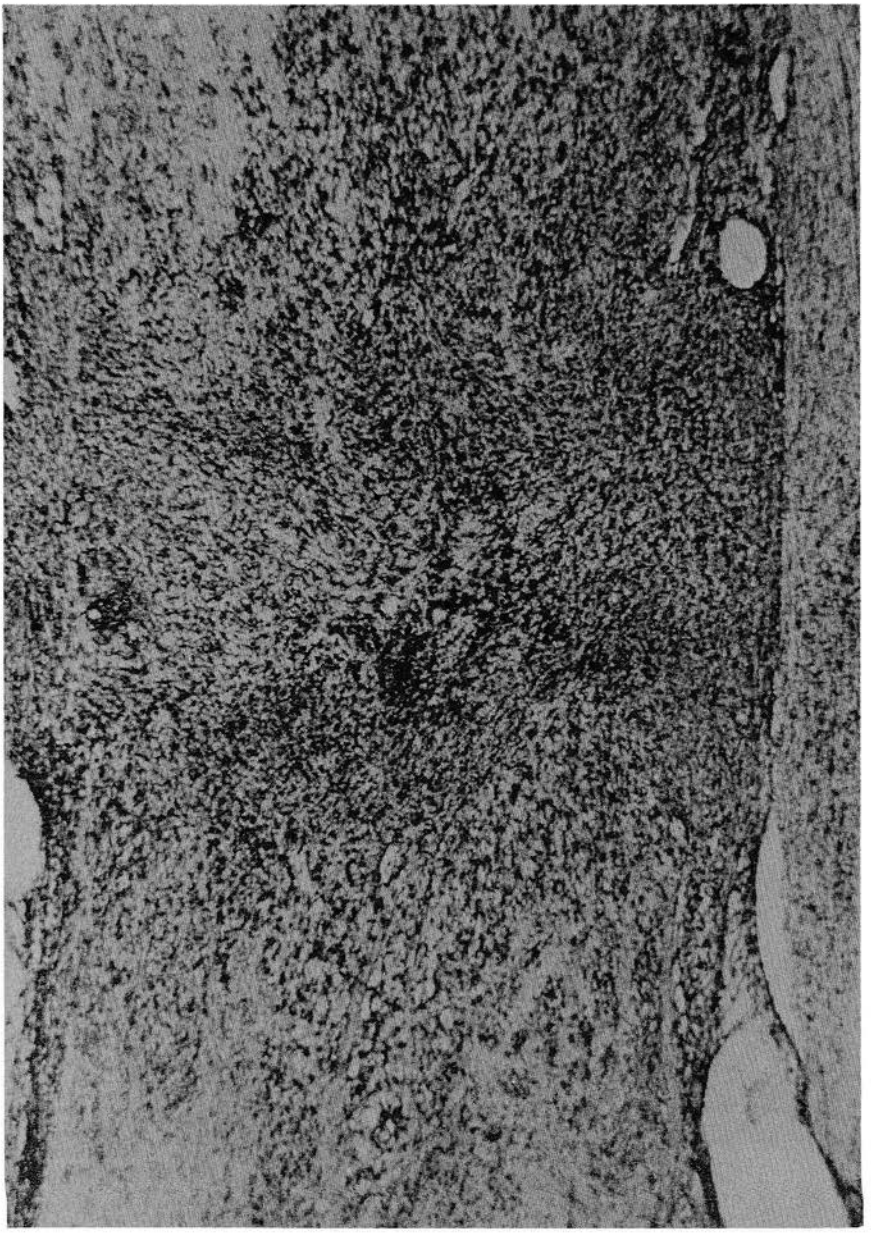

Figure 16-13. Monkey grafted at two weeks. Axons are bridging the centrally placed nerve graft. There is little cavity formation present.

Figure 16-14. Monkey treated with EME only. Note large cavity formation and total lack of continuity of the spinal cord.

Figure 16-15. Monkey treated with debridement only. Large cavity formation is again present, which disrupts cord continuity.

our study this was accomplished by using the microsurgical technique for the subpial removal of the necrotic debris and subsequent nerve grafting at one, two, and three weeks. Since the time sequence of lysosomal activity may well be species dependent, it was elected to use three different grafting times. The best histological evidence of a viable nerve graft was the one done at two weeks (Fig. 16-13). The animal that had positive SSERs was grafted at one week but histologically still had considerable cavitation present. Clearly, a larger series to study cavity development in the rhesus monkey is needed.

The third barrier to spinal axonal regeneration, glial scar formation, has been dealt with in several different ways over the years. Bacterial pyrogens in the form of Piromen in this country (13, 14) and Pyrogenal in the Soviet Union (15) have been used in spinal animals with mixed results. ACTH has been used with some beneficial effects (16). Trypsin (17), hyaluronidase, and elastase (18) have also been used to decrease scar formation but the results are not impressive. More recently Wilson et al. have applied pulsed electromagnetic energy to reanastomosed median nerves in rats and to hemisected spinal cords in cats (9). They observed that there was less fibrosis and scarring around and within the peripheral nerves from the animals exposed to the EME treatment than controls and that the treated peripheral axons regenerated at an advanced rate when compared to controls. In the hemisectioned animals they also found less glial scar in treated animals than in the controls and an increase in the regenerating neurons traversing the region of the hemicordotomy in these treated animals. In these two experiments it appeared that the EM energy decreased scar formation and also had some primary effect on rate of axonal regrowth. In our series the two animals that had evidence of physiological recovery both had been treated with pulsed EM energy, one with a graft and one without. The animal without the graft also had evidence of HRP transport across the injured site.

It is hypothesized that electromagnetic energy affects a biological system by repolarizing partially depolarized cell systems such as exist in injured tissues. This may be linked at the ionic level to the maintenance of the cell membrane potential by the so-called "sodium pump." The lack of extensive glial scar formation in treated animals may therefore be due to the possibility that in actively regenerating axonal tissue the glial scar has no cavity within which to accumulate. This is supported by the fact that both of the monkeys with evidence of axonal regrowth across the gap had the necrotic debris and supposed cystic cavitations removed surgically and then were subjected to treatment. The monkey treated with EME without the necrotic debris first being removed showed both cavitation and scar formation. The monkey treated with debridement alone and no EME was no different from the monkey treated without debridement and with

EME. This suggests that in the subhuman primate both debris removal with or without peripheral nerve grafts and pulsed EME give maximal advantage to the regenerating axons; obviously further data are needed to test this pilot observation.

The preservation of the major vascular supply channels to the cord as shown on angiography supports the well-established notion that traumatic paraplegia is primarily a neuronal and not an ischemic injury, and while smaller capillaries were in all likelihood altered, the potential for revascularization was present. In this regard there is no essential difference in the response of the brain and spinal cord to mechanical trauma. Although the number of our animals is limited, several interesting observations can be made. First, in the rhesus monkey the optimal time for nerve grafting after traumatic paraplegia appears to be one to two weeks. In the two grafted at three weeks, neither histological, histochemical, or physiological evidence for regeneration was present. Second, of the two animals with positive spinograms one had removal of debris, nerve grafting, and EME treatment. Unfortunately, no HRP or autoradiography was performed. The second had removal of debris, no grafting, and EME treatment and was the only animal with a strongly suggestive positive HRP response in addition to the evidence for signal processing through the lesion zone. Obviously the debris removal and EME treatment were common to both. Third, our main intention to test the concept of delayed nerve grafting in a subhuman primate was not fully achieved due to the difficulty in nursing such animals. We believe that further investigation in less valuable and more readily available mammals should be carried out to further elucidate the following:

(1) Electromagnetic energy and its efficacy in decreasing glial scar formation and enhancing axonal growth.
(2) Timing of debris removal and nerve grafting.
(3) The role of nerve grafts versus prosthetic guidance systems.

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