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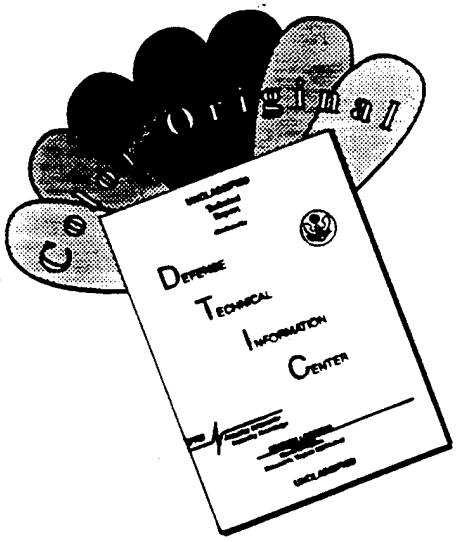

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As an external application to injured or developing systems, steady voltage gradients can alter the dynamics of such processes in a predictable and polarized way. We have exploited this in ways designed to enhance the regeneration of nervous tissue with small current regulated implantable devices. We have facilitated regeneration of nerve fibers within the lesioned adult mammalian spinal cord following acute application of DC fields. Further tests proved that a recovery of function in a normally permanent sensorimotor defects secondary to severance of the relevant intramedullary spinal cord tracts could be induced. This research employed the cutaneus trunci muscle reflex (CTM) of the adult guinea pig where functioning of skin musculature is dependent on long afferent projections within the ventrolateral spinal cord. Evaluation of skin movement and electrophysiological evaluation of the reflex proved that up to 25% of electrically treated animals recovered reflex functioning while 100% of the sham treated population remained permanently impaired. Further development of devices designed to affect both ascending and descending nerve fiber projections have been tested to be effective in restoring variable levels of functional recovery in clinical cases of naturally produced, and neurologically complete paraplegia in canines. We believe this technique provides a relatively simple medical intervention to recover lost functions following severe traumatic CNS injury.

*Bioimplants *Functional Recovery 21
*Central Nervous System Trauma
*Electric Fields

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FOREWORD

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INTRODUCTION

The behavioral loss following severe neurotrauma results from the inability of nerve fibers within the central nervous system (CNS) to regenerate significantly to form new, functionally meaningful connections as well as the physiological dysfunction of intact, yet demyelinated axons within white matter[1,2,3]. At present there is no known approach to altering the biology of this injury to significantly change the axis of behavioral deficits. For example, the administration of "high dose" methylprednisilone following spinal trauma must be made within the first 8 hours of injury and is only effective in neurologically "incomplete" cases[7]. In spite of other advancements[8], we should view the opportunity for significant recovery following severe CNS neurotrauma as limited, and the quality of life for those affected as impoverished.

Our approach to an experimental treatment involves the imposition of a weak (300-400 μV/mm) electric field across the long axis of the spinal cord and its injury site. This procedure originally emerged from well described growth and directional responses of neuronal processes to applied DC electric fields in culture.

When exposed to weak extracellular voltage gradients, nerve fiber responses include: an increase in the growth rate of regeneration when fibers face the cathode of the applied field; a decrease in growth rate when fibers face the anode of the applied field; resorption of neurites into the cell body when they project toward the anode; an inhibition of axonal degeneration (retrograde "dieback") when transected fibers face the cathode; an increase in retrograde degeneration when transected fibers face the anode; an increase in axonal branching when regenerating axons face the cathode; an increase in neurite outgrowth from cultured ganglion on the "cathodal" side; an induced oriented neurite growth towards the cathode and away from the anode in these same ganglion; an increased rate of growth (2-3 fold) when fibers turn towards the cathode and away from the anode; an increase in growth cone filapodia and cytoplasmic spines in the presence of a cathodal field; the respecification of oriented growth of neurites along the DC potential gradient and away from cues provided by "contact guidance" mechanisms. These observations have recently been reviewed[9,10,11,12,13,14,15,16] and summarized in several reports in addition to the responses of several non-neuronal cell types (embryonic fibroblasts, myoblasts, neural crest) to applied DC fields[13,14,17,18].

Lastly, observations of growth responses to applied DC fields are strengthened by the fact that developing neurons reside in a weak, polarized extracellular voltage gradient during ontogeny[19]. This may help explain the curious and striking responses of neurons and their processes to artificially applied weak extracellular gradients of potential.

Axonal Regeneration in the Mammalian Spinal Cord.

For a decade, we have been testing the responses of *completely transected ascending (sensory) nerve fibers* in adult guinea pig to the imposition of distally negative applied fields. We developed an implantable current regulated circuit to accomplish this - complete with "wick" electrodes to eliminate the possible confusion produced by effects mediated by electrode products[13]. To precisely determine the original plane of transection we developed the use of a "u" shaped marker device made of tantallium that was inserted into the hemisection[20].

Tract tracing was accomplished by anterograde labeling of ascending dorsal columns with horseradish peroxidase (HRP), the site of application on the order of 2-3 vertebral segments caudal to the original transection (approximately mid thoracic)[20,21]. Our three main observations in electrically treated animals (made approximately 50 days post-transection) were: 1) a strikingly elevated number of ascending HRP loaded fibers terminated deep within the caudal aspect of the lesion, many terminating *at the exact plane of the original transection*, 2) a smaller number of ascending fibers in only field-treated animals had circumnavigated the lesion by projecting around its lateral or ventral border into the *rostral spinal cord segment.* 3) the character of the anatomical response to the field was dependent on field strength. Few fiber termination's were within the lesion at the lowest magnitude of field (1 μA units), more numbers and deeper penetrations in the intermediate field strength (5 μA units) but without evidence of fiber projection into the rostral segment of the spinal cord. Such deviating fibers were observed in only the experimental group using 10 μA applied current (ca. 100μV /mm). All of these groups were compared to a comparable number of sham treated control animals that were implanted with non-functional (internally short circuited) implants.

Field Induced Recovery of Function in Canine Paraplegia

Severe traumatic spinal cord injury in the canine produces the same clinical deficits observed in Humans: loss of function, both motor and sensory, below the level of the lesion, spasticity, incontinence, and atrophy of the affected hind limbs. These problems produced by neurological "complete" trauma show very little response to conventional medical and surgical intervention (including acute administration of steroids)[14,22]. We employed a clinical protocol to isolate such severely injured animals from possible "incomplete" injuries presenting at the clinic. This included tests for superficial and deep pain cognition, standard reflex testing (to identify upper motor neuron sequelae), a regimen of evoked potential tests (including SSEP, MEP, and SEP), studies of ambulation, and tests for the loss of proprioceptive placing. To meet study criterion, a dog was required to be negative on all measures of neurological functioning. We further subdivided incoming candidates on the basis of injury type (fracture dislocation or severe traumatic disc herniation) and time post injury (hours to 1 month post injury, 1 month-1 year post injury , and >1 year). The acute traumatic disc herniation group was extremely interesting due to its direct comparison to the human injury mode - namely severe compression of the spinal cord followed by extreme central hemorrhagic necrosis[14,22]. One should not confuse this injury (which involves acute direct explosion of disc material into, sometimes laceration of, the spinal cord) in canine chondrodystrophic breeds with human "slipped" discs - which is rarely a spinal cord injury[14,22].

Since the resolution of behavioral deficits in a clinical injury might involve interaction of the applied field with ascending and descending tracts, we developed a methodology called "oscillating field stimulation" (OFS) born of an interesting observation by McCaig[16]. He noted that when neurites in culture experience an imposed DC field, growth responses towards the cathode may be immediate, while degenerative changes in neurites facing the anode take on the order of 45 minutes. The differing latency in the response to either polarity of the applied field may allow a window of opportunity where field imposition produces growth towards the cathode, with relatively short exposure - but less than the time necessary to induce degenerative changes at the anode. We developed an implantable circuit which would reverse the polarity of application every 15 minutes (described in reference 13). The current regulating and timing circuitry to accomplish this have been published elsewhere[13] (see methods below). Active OFS units and indistinguishable "sham" units

were produced in our laboratory, coded and provided the clinic for use in a randomized, blinded trial of their effectiveness in naturally produced canine paraplegia. All neurological exams were video taped at prespecified times: prior to surgery, just post-surgery, at the 6-8 week and 6 month recheck. Some neurological evaluations (deep pain and ambulation) were scored at the end of the experiment by comparison of video records for all dogs in the trial by a panel of investigators blinded to their status. Briefly our results were; 1) a strong trend for recovery of function *in all neurological categories* was evident in the experimental group *with no reverse trends*, compared to the sham-treated canines. 2) Only 15% of OFS treated dogs failed to recover in some category of evaluation compared to 60% of the sham-treated controls. 3) Approximately one-third of the OFS treated dogs recovered in all 4 categories of behavioral evaluation - *none of the sham treated animals did.* 4) The combined neurological score was significantly different between these groups at 8 weeks ($p < 0.033$) and at the 6 month ($p < 0.035$) recheck period.

In summary OFS stimulation appears to be an effective technique to accomplish recovery of function in clinical cases of spinal trauma if applied within 1 month of the injury (most convincing results were achieved when the application was within 2 weeks of injury[22]. Further advancement must depend on a deeper understanding of these mechanisms of action at least at *the cell and tissue level of inquiry*. **The current USAMRMC embodies just such an important evaluation of these relevant anatomies following DC stimulation using a defined guinea pig spinal cord lesion as well as further canine clinical trials intended to determine if a greatly increased magnitude (3 fold) of oscillating field stimulation will a) extend the effective time frame of acute treatment and b) lead to a greater level of behavioral recovery in all cases.**

BODY

EXPERIMENTAL METHODS AND RESULTS OBTAINED WITHIN 1 YEAR
(AUGUST 1994 - JULY 1995)

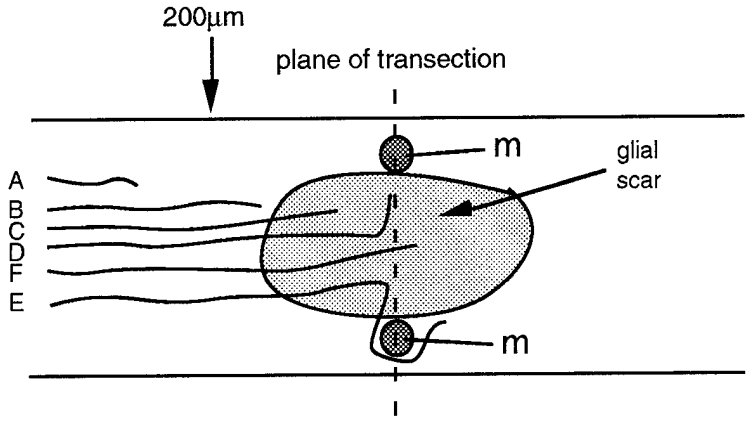
I. Overview: Applied Fields Both Influence Axonal Regeneration and Retrograde Dieback

We intend to further document axonal regeneration in response to the applied field by tracing dorsal column projections with a rhodamine labeled dextran (Flouroruby). The termination of labeled axons at planes caudal to the plane of the dorsal hemisection has been evaluated. Tract

tracing of originally severed ascending sensory fibers was accomplished with both conventional epifluorescence microscopy and with a BioRad MRC 1000 laser scanning confocal microscope using a Krypton/Argon emission source. The excitation wavelength was usually 569 nm while emissions were collected at 585 nm, and acquired to the computer using COSMOS® software. The digitized images were transferred to a Silicone Graphic's Indigo2 workstation. Three dimensional reconstruction and pseudocolor rendering was performed using Voxelview® software.

Results:

Termination of dextran filled ascending axons



Animal groups	A >200µm	B <200µm	C penetrates glial scar	D to plane of lesion	F through lesion	E around lesion
Experimental (n=14)	2/14 (14%)	1/14 (7%)	3/14 (21%)	8/14 (57%)	5/14 (36%)	4/14 (29%)
Control (n=14)	6/14 (43%)	6/14 (43%)	2/14 (14%)	0/14 (0%)	0/14 (0%)	0/14 (0%)

TABLE 1

Table 1 (above) displays the number of animals in the experimental group (field treated; 0.4-0.5 mV/mm using indwelling current regulated stimulators[21,28]), and the sham treated control group (implanted with non-functional stimulators). Also shown is the number of animals in which *the most*

rostral extent of dextran traced ascending axonal projections was observed. These proportions do not add to 100% since some spinal cords are represented in more than one group. For example, fibers in one spinal cord could be traced to the plane of transection [column D], but also around the lesion through undamaged parenchyma [column E]. The plane of transection was established by the marker holes (m) left in the tissue by an indwelling device that provides an index of the exact plane of the original dorsal hemisection (as in Borgens et al.[15,20,28]).

Sham-treated control cords were unremarkable, and contained well marked ascending tracts which terminated well caudal to the formed glial scar. Field treated spinal cords contained similar columns of axons that a) penetrated the caudal segment of the glial scar to the exact plane of transection, b) circumvented the scar through undamaged parenchyma - showing terminal endings typical of growth cones in the rostral segment of the cord, and c) penetrated the glial scar at the plane of lesion into the rostral segment. Regeneration around the scar was accomplished by large diameter axons (ca. 3-10 μm) which ultimately branched numerous times into fine submicron processes. Regeneration through the glial scar was only accomplished by such fine processes.

Figure 1 shows a series of confocal pseudocolor images obtained using a Z axis scan of the region of dense scar formed at the plane of transection 3 months post lesioning in an adult guinea pig. In Fig 1 A, a 3D reconstruction of a portion of the lesion is shown. The reconstruction was composed of 30 individual two micron thick optical scans along the horizontal longitudinal plane of the section. The hatched line indicates the plane of the original hemisection (determined by the use of the indwelling marker device[20]), the arrowheads *in the caudal segment of the hemisected spinal cord* indicate dextran filled axons projecting to this plane of transection, the arrows indicate axon profiles *within the rostral segment*. The region boldly circled is shown in high magnification in Fig. 1B, C, and D. In Fig. 1B, a single two micron thick optical section used in the reconstruction is shown. Note that regenerating, branching axons project to the edge of the large cyst in the right side of the photomicrograph, but these fibers cannot be traced *through* the dense ribbon of scar (marked S), in spite of the axon profiles clearly present within the adjacent rostral segment. By deleting optical planes that do not contain axon profiles (reducing the background " noise " produced of the numerous isodiametric glial cells and fibroblasts comprising the scar) prior to 3D reconstruction of digital images, axon profiles *can be seen crossing this region as well as branching within it (*arrowheads and

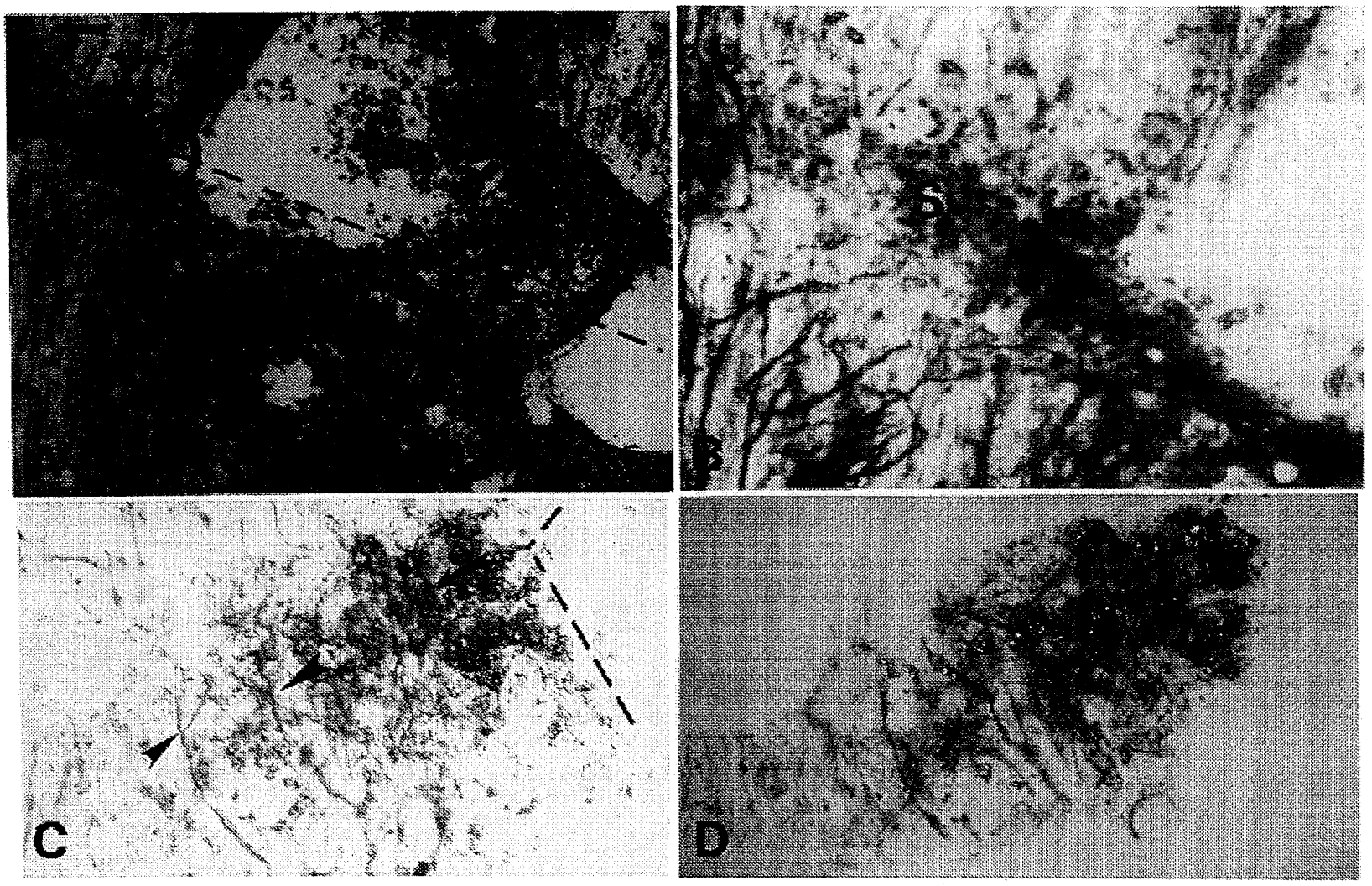


figure 1

arrows in Figs 1 C and D). These very small fibers were previously unobserved in earlier studies using high molecular weight horseradishperoxidase (HRP) tracers, and extend and confirm our earlier observations of spinal cord regeneration in the adult mammal in response to applied electric fields. This study has been completed during the first year of USAMRMC support resulting in a manuscript entitled "Mammalian Spinal Cord Regeneration in Response to an Applied Voltage Gradient Demonstrated by Tract Tracing And Confocal Laser Microscopy," which has been submitted to the *Journal of Neurocytology*.

These studies are both an extension of, and improvement on, our seminal observations in adult guinea pig SCI[21] in which we could not discriminate precisely between regenerative events or effects of the field on retrograde axonal degeneration.

II. Overview: Applied Fields May Reduce "Secondary" Injury Following Spinal Trauma

The notion that trauma to the brain and spinal cord results in a two stage injury process is accepted by most investigators in the field. The first stage is an instantaneous destruction of parenchyma followed by so called "secondary injury" pathology. This latter stage is believed to occur as a slow time course of auto destructive events - superimposed on the original degenerative phenomena caused by the initial insult. Secondary injury is correlated to several physiological and biochemical changes in the vicinity of the lesion, including the loss of free radical scavengers, the accumulation of lactate and peroxides within the lesion, and post traumatic ischemia[23,24] all of which appear to be involved with progressive histological dissolution of tissue at the site. *A particular cellular species directly implicated in this process is the macrophage,* and the hyperbolic invasion of injured CNS parenchyma by these phagocytic cells leads to extensive destruction and cavitation of the tissue[23,25]. Quantification of the density of macrophage invasion following contusion injury to the spinal cord demonstrates as much as 30% of the lesion volume at 2 weeks post injury can be accounted for by these cells[23,24]. The role of inflammatory processes and the particular role of the macrophage have been recently reviewed[1,5,24,25,26,27]. It may be possible that the voltage gradient applied *soon after injury* may create conditions where macrophage infiltration of the lesion could be impaired or redirected in some predictable way. Since these cells constitute the single largest immigrant to the lesion site, they should be considered as a possible target of the applied field. This

can be determined by identification of macrophages with immunohistochemical technique followed by computer assisted sampling and cell counting, comparing control and experimental (electrically-treated) animals.

Results: During year 1 of USAMRMC support, we have developed a novel analytical procedure for assessing the degree of macrophage infestation in 3 week old compression lesions of rat spinal cord in 12 animals. In a (see below) second study we have increased the number of control animals since rigorous quantitation of macrophage number in subacute compression injuries in rodent spinal cord has not been adequate[25].

Our new approach to quantification employs the use of the ED 1 monoclonal marker for macrophages (Serotech) coupled to a computer assisted means of counting these HRP marked cells in *over 75% of the lesioned* region of spinal cord. This technique provides not only an estimate of macrophage number per unit area of lesion, but as well the area of damaged parenchyma containing marked macrophages.

The use of a monoclonal marker for macrophages (ED-1 marking) is now routine in this laboratory and clearly marks activated macrophages. We digitize color images of the lesion using both RastorOps® and Media Grabber Software; sample over 75% of the lesion at 20X on a widefield Olympus VAN OX laboratory microscope, and count labeled macrophages with IP LAB Spectrum Software, converting the pixel counts to macrophages/cm^2 of lesion. (Prior to the study we determined the mean number of pixels/macrophage at 20x by averaging counts of 100 cross sections of individual macrophages marked by this immunocytochemistry.) The IP Lab Spectrum® program allows multiple methods for the transformation of the anatomical data for actual counting, two methods of which were useful to our analysis of HRP labeled macrophages: 1) a graded color palette transformation where pixels are assigned to individual colors over a range of 250 pixel values. A range of individual colors were then assigned for counting following a transformation of all of these assigned values to a single color (i.e. green); 2) the image is broken into three primary colors (red/green/blue) each represented by a unique file, the darkest values of each color (those associated with the HRP labeled macrophages) are chosen and assigned a single pixel value to be transformed to a single color. Prior to counting in both methods, the histological data (HRP labeled macrophages,

Fig. 2A) is binarized, and the assigned/transformed color to be counted becomes green, the rest of the image white (Fig. 2B), and the green regions counted to provide total pixels. To do this all regions not to be counted assume a "0" value, which is black, Fig. 2C. Visual analysis of the transformed images suggested that the most accurate representation of the macrophages was achieved with the second method of color/graphic transformation. This was later reconfirmed by comparison of the computer counts with actual visual counts of macrophages within 10 histological sections.

This methodology has generated a manuscript in progress entitled "Immunocytochemical Evaluation of Macrophage Infestation following Acute Spinal Cord Injury" to be submitted to the *Journal of Neurotrauma*. Our studies indicate macrophage accumulation to be 3 fold more intense than previously reported[25]. Secondly, we are in the final stages of preparing data on the effect of the applied voltage gradients (ca. 0.4-0.5 mV/mm) on this special cell population. This is as well intended for submission in an article "The Effect of an Applied Electric Field on Macrophage Localization following Acute Spinal Cord Injury" to be submitted to the *Journal of Neurotrauma* in January 1996. (These data are still being evaluated during the preparation of the "results" section of the manuscript. Completed manuscripts or published reprints will be provided in next year's annual report.)

Table 2 shows the total number of animals in each group of this second study (experimental and control) used to determine the effect of an applied voltage gradient on macrophage accumulation in such 3 week old severe contusion injuries in the rat model. Indwelling active and sham stimulators are identical to those used in the study discussed on page 9.

Table 2

Total animals	Mortality	Experimental	Control	Time at Sacrifice
45	10	13	22	21±3 days

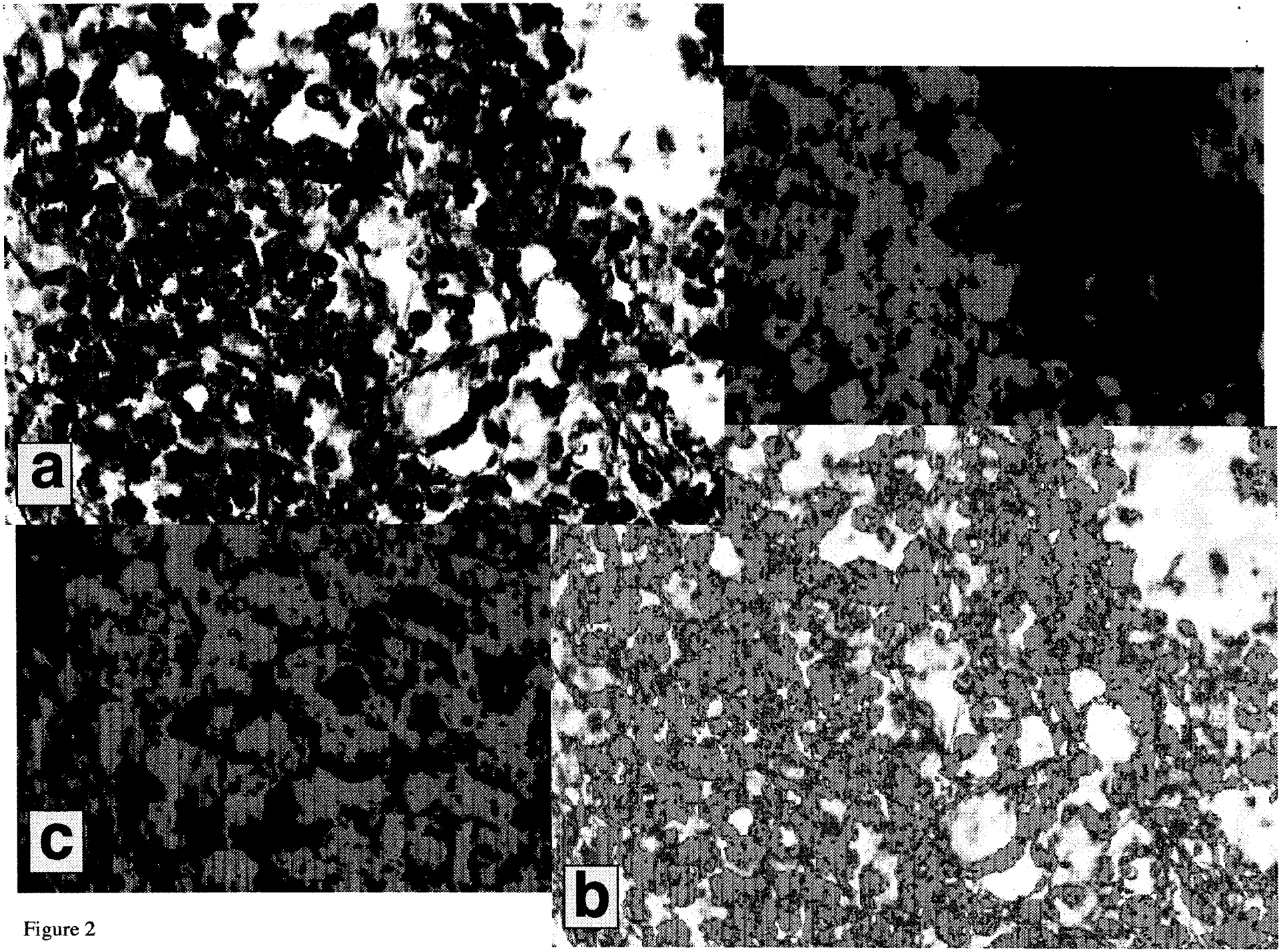


Figure 2

We have hypothesized that the improved outcome in functional recovery mediated by the applied voltage gradient (see original USAMRMC proposal, 03/30/95; see also citations 13 and 14) may be mediated by reducing the severity of the so-called secondary damage to CNS parenchyma as explained above. Macrophage invasion of the CNS lesion is hypothesized to be affected by the applied voltage since these cells are known to clearly migrate within an applied voltage in *in vitro* tests[29] Another indicator of secondary damage is the degree of *cavitation* produced at the level of scar, as well as the *extent of gliosis* itself. In the first year of this award, we have developed a technique to evaluate the extent of cavitation within severe spinal cord injuries using 10 rats. To do this properly, it is required to delineate cavities within the parenchyma (micro and macrocysts) from the increase *in capillary formation within the lesion* (well known to occur, in response to the

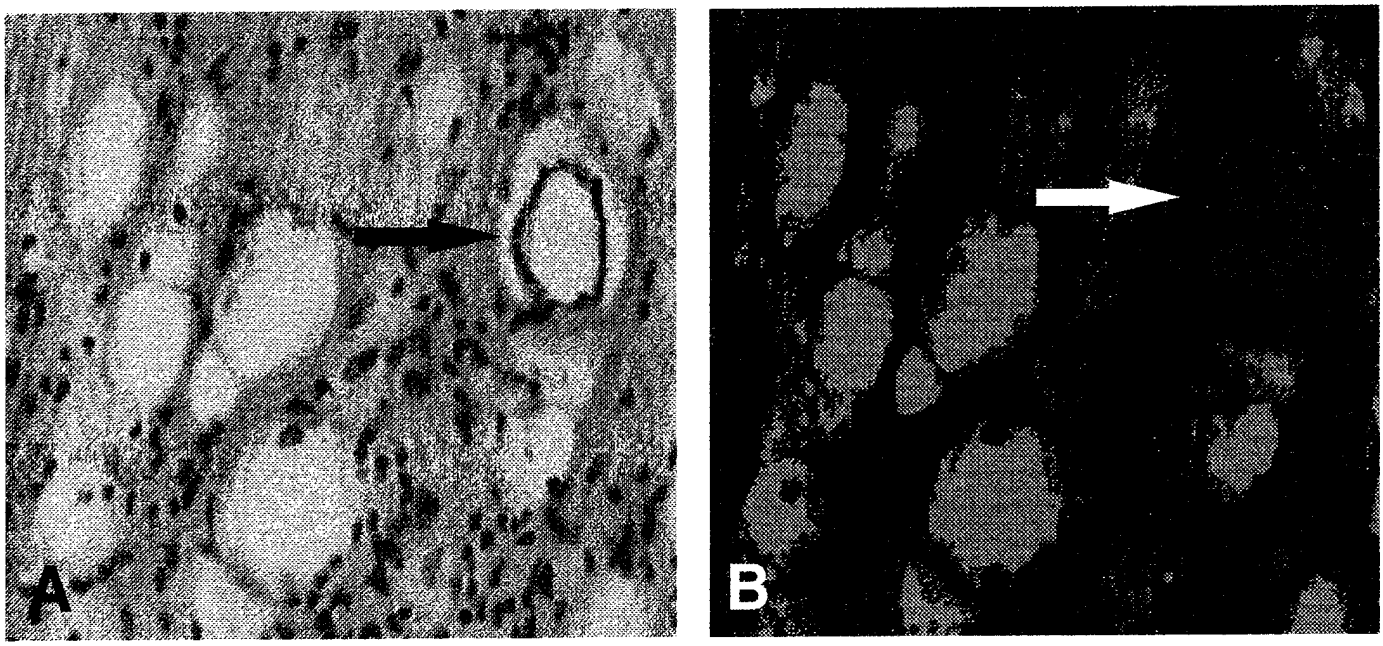


Figure 3

A high magnification digitized photomicrograph showing numerous cavities within the region of a spinal cord injury. The large arrow indicates a capillary (note the ring of perfused ink contained within it) surrounded by cysts of a similar size and shape. In B this capillary is blocked out (pink fill) and would be excluded from computer assisted counting of the remaining cysts (green fill). The captured micrographs are managed by a software program (IP Lab spectrum) which color contrasts each image, then colorizes the cavities and cysts, which are numbered and their total unit area calculated.

angiogenesis factors produced by macrophages infesting the area of insult[23, 24]) We accomplish this by immunocytochemically marking capillaries with factor VIII antibody during histological preparation of the spinal cord tissue containing the lesion, *and as well by* perfusion of the animal with ink during sacrifice (by intracardiac perfusion/fixation). This latter technique labels the lumens of even the smallest caliber capillaries with crystals of ink when seen at high magnification. The remaining cystic cavities are then scored and counted (Fig. 3), as well as the *total unit area of cavitation* which can also be determined from these images. Presently we are beginning experiments to determine if cavitation is reduced by the applied voltage (0.4-0.5 mV/mm) by comparing equal groups of sham and current treated animals (30 animals total).

III. Increased magnitude of OFS field will yield Greater levels of Functional Recovery in Canine Paraplegics.

We have previously used implantable stimulators that switch the polarity of the field (250 μA total current, 15 minute duty cycle) in seminal tests of efficacy in clinical cases of canine paraplegia[22]. We wished to improve this design by incorporating changes that will allow 1) *a three fold increase in the magnitude of the field imposed,* and 2) the voltage gradient to be imposed over a *greater length of the spinal cord*. This design has been accomplished during the first year of this award.

Total current delivered through individual platinum/iridium electrodes (coiled to increase surface area) cannot increase over the level now used without producing unwanted electrode product contamination of adjacent tissues and subsequent necrosis. To effectively increase the magnitude of the field safely, we should deliver current through an electrode array, each electrode delivering the same amount of total current as previously used. Therefore, the current density at each electrode will not be any greater while six electrodes (three anode, three cathode) would deliver three times the total current and three times the applied field at the spinal cord. The circuit to accomplish this is now in implementation.

To allow three pairs of electrodes to deliver a threefold increase in imposed voltage (see above) the following changes have been made:

The oscillating field stimulator we have designed is capable of delivering a constant current (50-200 μA) that reverses polarity after virtually any time period desired. The circuit and fabrication has been described in detail[13]. The new circuit uses in-line current regulators rather than an op-amp to control the current. Six pairs of LM134 three terminal constant current sources will be used immediately ahead of six electrodes. The LM134 is also a current rectifier and so mirror image pairs of LM134's are used before each electrode to allow bilateral current flow. The oscillation frequency is controlled by the CD4060 binary ripple counter which sends a control pulse to a quad (2 NO, 2 NC) SPST analog switch acting as a bipolar inverter. This switch will essentially reverse the supply voltage leads to the arrays of LM134's, resulting in reversal in the polarity of current flow through the electrodes. Pairs of LM134's are available as dual surface mount packages. The CD4060 and the analog switch are also available in surface mount, so a complete circuit would require 8 surface mount IC's, a capacitor and 2 resistors to set frequency, and 12 current set resistors in a SIP package. This circuit has been tested to be functional, and is no larger in size than previous units used clinically in studies of canine paraplegia. *We are presently ahead of the research schedule suggested in our proposal of March 1993. During Year 1 we have already tested the new OFS units to be functional and have implanted naturally injured canines with these units in blinded study.*

At the present time, 13 study animals have been recruited into this clinical trial (neurologically complete, disc herniation induced trauma and meeting the other inclusion criteria as described in the original proposal). We have taken complete neurological records on these dogs (presurgical, 6-8 week and 6-8 month recheck) including urodynamic evaluation (cystometry and urethral pressure profile), electrodiagnostic (i.e. somatosensory evoked potential study, spinal evoked potential study), and a large battery of other neurological measures as described in the original proposal. These have all been videotaped and/or physiological records captured to computer, however we have not moved these to spreadsheet or evaluated this data in any way. This protocol has been used to maintain blinding and to eliminate any bias during the *continuing* clinical neurological evaluations of study animals.

CONCLUSIONS:

During year one of USAMRMC support we have learned:

1) Weak DC (0.4 - 0.5 mV/mm) voltage gradients, imposed across dorsal hemisections of the adult guinea pig spinal cord, induce nerve fiber regeneration. Fine (<1 μm) branching axonal processes project to the plane of transection, around it, and through the dense glial scar that forms at the region of injury. In sham-treated controls, less than 20% of the animals display dye injected axonal terminals even within the caudal most boundary of the glial scar.

2) Infestation of the 3 week old rat spinal cord lesion by macrophages is severe. The lesion is actually defined by these cells in the rodent in subacute compression or piercing injuries. This adds strength to an emerging picture that the inflammatory processes following CNS injury is hyperbolic and leads to further destruction of CNS parenchyma. An additional study designed to quantify the effects of applied DC fields on macrophage infestation, is nearing completion and this data is in the final stages of preparation for publication. A consequence of auto-propagated inflammatory damage in CNS is extensive cavitation of the parenchyma near the site of CNS damage. We have developed a means to quantitate this cavitation (free of inclusion of micro-capillary formation within the lesion) and we are now testing the effect of the applied voltage on such cavitation.

3) Clinical trials of OFS stimulators of spinal injures in naturally occurring paraplegia is ahead of the schedule proposed in the workplan of the original proposal (see also discussion on "Budget" below).

BUDGET

During the first year of this award, there were more expenditures in both salary and supply and equipment funds than originally proposed. This resulted from an increased capability to begin specific areas of the workplan *during* year one, that had been originally budgeted to begin in year two. This happened because of unanticipated advancements that were made after the proposal was reviewed favorably by the USAMRMC and the actual initiation of the award nearly 10 months later. In the original proposal, full time clinical activity was not budgeted until year two - however this was begun during year one since design of the clinical OFS stimulator had been completed. We do not anticipate any overall change to the 3 year work plan or budget based on this early start on some of these individual studies.

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