INTERACTIONS BETWEEN MACROPHAGES AND MOUSE CNS NEURONS AFTER NEURITE AMPUTATION VIA LASER MICROBEAM SURGERY IN MONOLAYER CULTURES

A DISSERTATION

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We hereby recommend that the Dissertation prepared under our supervision by Carol R. Gardner entitled Interactions Between Macrophages and Mouse CNS Neurons after Neurite Amputation Via Laser Microbeam Surgery in Monolayer Cultures.

be accepted as fulfilling this part of the requirements for the Degree of Doctor of Philosophy.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

MP	- Macrophage
PG	- Prostaglandin
PGE_2	- Prostaglandin E_2
$6\text{-KetoPGF}_{1\alpha}$	- 6-keto-prostaglandin $F_{1\alpha}$
LT	- Leukotriene
LTB_4	- Leukotriene B_4
LTC_4	- Leukotriene C_4
CNS	- Central nervous system
PNS	- Peripherial nervous system
CSF	- Cerebrospinal fluid
DlSGH	- A balanced salt solution containing various salts, sucrose, glucose and HEPES
MEM	- Earle's minimum essential medium
HS	- Horse serum
FBS	- Fetal bovine serum
MEM 10	- MEM + 10% HS
MEM 10/10	- MEM + 10% HS + 10% FBS
PSF	- Penicillin-streptomycin-fungizone
FdU	- Fluoro-deoxyuridine
mHBSS	- Modified Hank's balanced salt solution
HeNe laser	- Helium-neon laser
ETS	- Electron transport system

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INTRODUCTION AND LITERATURE REVIEW

The brain is normally isolated from the milieu of the rest of the body via the blood-brain barrier which is formed by the endothelium of brain capillaries and to some degree by the end feet of astrocytes (Cuzner 1983, House et al. 1979). Damage to the central nervous system (CNS) often destroys this barrier and allows elements of the immune system to be exposed to brain tissue (Oldstone 1982).

Macrophages (MPs) are one of the immune system components that enter in great numbers after CNS trauma. They have many functions and secretory products, some of which change with the activation state of the MP (Adams and Hamilton 1984, Unanue 1983). The resident tissue MP has some phagocytic activity, shows a moderate response to chemotactic stimuli, and secretes lysozyme, monokines, prostaglandin E_2 (PGE_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto$PGF_{1\alpha}$) and leukotriene C_4 (LTC_4) (Adams and Hamilton 1984, Humes et al. 1980, Humes et al. 1977, Rouzer et al. 1980). Elicited or inflammatory MPs, which are found in areas of non-immune mediated inflammation, are highly phagocytic, react strongly to chemotactic stimuli, and

secrete lysozyme, monokines, a decreased amount of prostaglandins (PGs), neutral proteases, and reactive oxygen (Adams and Hamilton 1984). Leukotriene B_4 (LTB_4) may be secreted at this stage, as well as at higher levels of activation (Bonney and Humes 1984, Goetzl et al. 1983). Primed and activated MPs, which are found in areas of immune mediated inflammation are not only highly phagocytic and react strongly to chemotactic stimuli, but they can also present antigen (Adams and Hamilton 1984, Unanue, 1983). The secretory products are the same as those produced by elicited MPs (there are variations in the amounts of products secreted) with the addition of the secretion of a cytolytic protease (Adams and Hamilton 1984). A main property of activated MPs is their ability to cause cytolysis of target cells (Adams and Hamilton 1984, Olstad et al. 1982, Unanue 1983) (Table 1).

Some macrophages are normally present in the CNS in yet unknown states of activation. They occur in human cerebro-spinal fluid (CSF) at an estimated number of 3000 MPs/ml (Oehmichen 1978) and at a concentration of 186 MPs/g brain tissue (Oehmichen 1983). If compared with an estimated $4X10^4$ monocytes/ml of blood and some $1.2X10^5$ MPs/g body weight (Oehmichen 1983, Oehmichen 1978), one can see that there is a great paucity of these cells in the CNS.

TABLE 1

PROPERTIES OF MACROPHAGES IN DIFFERENT STAGES OF DEVELOPMENT

STAGE	FUNCTIONS				SECRETORY PRODUCTS
	CYTO-TOXICITY	PHAGO-CYTOSIS	CHEMO-TAXIS	ANTIGEN PRESENTATION	
RESIDENT	-	±	±	-	Lysozyme, Monokines, Arachiodinic Acid Metabolites, Complement Components
ELICITED OR INFLAMMATORY	-	++++	+++	-	Same as RESIDENT + Neutral Proteases, Reactive Oxygen
PRIMED	-	++++	+++	+++	Same as ELICITED + Cytolytic Proteases
ACTIVATED	++++	++	+++	+++	Same as PRIMED

(Adapted from Adams and Hamilton 1984)

There is some evidence that microglia in the CNS are similar in function to macrophages. However, a great deal of controversy and debate exists over nomenclature, origin, and function of these cells. "Reactive" microglia, (also referred to as brain macrophages or amoeboid cells by some authors) are believed to be derived from blood monocytes (Aarli 1983, Albrecht and Bleier 1979, Bleier et al. 1982, Hume et al. 1983, Imamoto et al. 1982, Imamoto 1981, Korinkova et al. 1977, Miyake et al. 1984, Murabe and Sano 1983, Oehmichen 1983). These cells are particularly active in fetal and early neonatal life (Bleier et al. 1982, Hume et al. 1983, Imamoto et al. 1982, Imamoto 1981, Korinkova et al. 1977, Miyake et al. 1984, Murabe and Sano 1983). "Resting" or endogenous microglia may also be derived from blood monocytes but are considered by some authors to be terminally differentiated cells and are no longer capable of phagocytosis (Imamoto 1981, Murabe and Sano 1983, Murabe et al. 1981). Other investigators, however, have suggested that resting microglia are not of hematogenous origin but that they actually derive from glioblasts during prenatal development (Fujita et al. 1981).

Monocyte-macrophages have been observed to pass through the blood-brain barrier in small numbers under normal circumstances (Kerns and Nierzwicki 1981, Oehmichen 1978, Oldstone 1982). Lymphocytes are also present in

small numbers in the CNS but neutrophils are never seen under normal conditions (Oehmichen 1983). However, when CNS trauma or autoimmune disorder occurs, large numbers of blood elements enter the CNS and phagocytic elements become very active. Some of these active phagocytes are "reactive" microglia but the vast majority of the phagocytosing cells are of hematogenous origin (Kerns and Nierzwicki 1981, Kitamura et al. 1972, Lampert 1967, Means and Anderson 1983, Oehmichen 1978, Smith and Walker 1967, Stenwig 1972, Walker 1963, Werdelin and McCluskey 1971, Willenborg et al. 1977) and infiltrate in large numbers (Means and Anderson 1983, Oehmichen 1983). One of the functions of these phagocytes is the removal of debris (Beck et al. 1983, Oehmichen 1983). The infiltration of blood elements occurs in waves. First, the neutrophils move into the damaged area accompanied by some MPs to begin cleanup of debris (Oehmichen 1983). This is followed by infiltration by lymphocytes and plasma cells (activated B lymphocytes) (Oehmichen 1983, Walker 1963, Willenborg et al. 1977) and finally by more MPs (Oehmichen 1983). It has been suggested by one author that "resident" mononuclear cells in the CNS may be able to mount a local immune response when damaged by infections or autoimmune disease (Cuzner 1983).

What specifically attracts phagocytes (neutrophils, MPs, or microglia) to the area of a lesion and initiates phagocytosis is unknown. However, damaged tissue (particularly with hemorrhage and ischemia) releases large amounts of arachidonic acid (Chan et al. 1984, Chan et al. 1983, Chan et al. 1982, Floyd and Zaleska 1984, Means and Anderson 1983) which is very easily converted into lipid peroxides and free radical lipids (Braugher and Hall 1982, DeMedio et al. 1983, Demopoulos et al. 1982, Demopoulos et al. 1979, Demopoulos et al. 1977, Means and Anderson 1983, Yoshida et al. 1982). These free radical lipids and lipid peroxides have been shown to be chemotactic and can elicit the oxidative burst in both neutrophils and MPs (Fernández-Repollet et al. 1982, McCord and Roy 1982, McCord et al. 1980, Turner and Tainer 1983, Turner and Lynn 1978), which is characterized by increased production of H_2O_2, production of the superoxide ($O^{\cdot-}_2$) anion, increased oxidation of glucose via the hexose monophosphate shunt, and other reactions (DeChatelet 1978). These reactions allow the phagocyte to destroy ingested particles, mediate cytolysis, and attract more phagocytes to the area (DeChatelet 1978, Keisari et al. 1983, McCord and Roy 1982). Certain polypeptides and proteins have also been shown to be chemotactic (Gallin and Rosenthal 1974, Schiffmann et al. 1975). Bessis (1973) suggested that

concentration gradients of chemotactic signals can be generated from the structural material of cells that had been injured or destroyed (in this case, erythrocytes destroyed by laser irradiation). Phagocytes could then react by moving actively toward the area of highest concentration (Bessis 1973, Bessis and Burte 1965). These signals may result from oxidized arachidonic acid derived from the cell membrane and denatured proteins (Turner and Tainer 1983).

CNS trauma is also accompanied by glial proliferation and scarring (Adrian and Schelper 1981, Guth et al. 1983, Ludwin 1984, Merrill et al. 1984). It is believed that proliferating glia engage in the repair of the blood-brain barrier and the CSF-brain barrier. Some of these glial elements are microglia which are apparently not derived from blood monocytes (Adrian and Schelper 1981, Fujita et al. 1981), but are apparently derived from glioblasts (Fujita et al. 1981). These are believed to actually transform into astrocytes (Fujita et al. 1981). This repair may take 2 to 3 weeks, during which time the CNS is open to immune system components (Berry and Riches 1974). Glial proliferation may be mediated by a lymphocyte derived factor whose secretion may, in turn, be mediated by MPS (Billingsley et al. 1982, Fontana et al. 1982, Fontana et al. 1980a, Fontana et al. 1980b, Merrill

et al. 1984). MPs may be needed for lymphocyte infiltration to occur (Walker 1963). Infiltration and proliferation of lymphocytes is mediated by MP secreted monokines (Unanue 1983). It has been demonstrated that MPS appear to secrete a factor encouraging endothelial migration and proliferation that may allow for repair of cerebral microvessels after trauma (Beck et al. 1983). It is interesting to note that glial proliferation and scarring does not take place in hibernating animals (Guth et al. 1981). Instead the area of spinal cord transection fills with a loose accumulation of MPs and blood vessels, and neurons show a greater tendency to sprout (Guth et al. 1983, Guth et al. 1981). However, the regenerating fibers still do not cross the transected area (Guth et al. 1981).

Between the extremes of healthy cells and cell debris lies a vast realm of different types of cell injuries and different degrees of specific injuries. Nothing is known about MP interaction with injured cells, especially for injuries from which cells normally recover. A specific aim of this study was to determine the effects of the presence of macrophages on the survival and recovery of neurons traumatized <u>in vitro</u> by laser microbeam transection of neurites. Such laser-induced neurite amputation has been shown to mimic neuronal trauma caused by other agents (Gross et al. 1983).

The primary goal of this dissertation was to make a contribution to the clarification of the roles played by macrophages in CNS trauma. To eliminate the overwhelming complexity of *in situ* tissue reactions, studies were conducted in neuronal monolayer cultures where direct interactions between MPs and neurons could be observed in the living state. Also, this model system allowed the removal of most immune system components and cells (heat inactivation of serum added to the medium destroyed complement proteins and treatment of cultures with fluorodeoxyuridine (FdU) essentially removed mitotically active non-neuronal cells). Subsequently a purified population of elicited MPS was added to neuronal cultures. Therefore, the interactions described were obtained from a simplified and comparatively well defined culture system.

The initial guiding hypothesis for this study was based on the assumption that MPs would attack and destroy neurons damaged by laser cell surgery. However, despite the fact that MPs are attracted to process lesions and actively engage in phagocytosis of damaged processes, it has been found that MPs actually enhance the survival of neurons injured by laser cell surgery. Although the mechanism of this surprising result is still unknown, this dissertation presents quantitative evidence for this effect.

METHODS AND MATERIALS

I. Animals Used

BALB/C mice were initially supplied by Charles River, Wilmington, Mass. They were reared and maintained in the departmental animal facility.

II. Neuronal Culture

The culturing of embryonic mouse spinal cord neurons was carried out following the method of Ransom, et al. (1977), with an added enzymatic dissociation step (Gross and Lucas, 1982). Briefly, spinal cords were removed from 13-15 day mouse embryos and minced in a petri dish. One ml of DlSGH (a balanced salt solution containing various salts, sucrose, glucose and HEPES) was added to the minced cords which were tranferred to a 15ml centrifuge tube. One additional ml of DlSGH was added to the dish as a rinse which was added to the same centrifuge tube before centrifugation for 2 min at 200xg. The supernatant was discarded and 3ml 0.25% trypsin with 0.5gm/ml DNAase was added to the cords. The cords were then resuspended and placed into a 60mm tissue culture dish. The dish containing the cords was placed in a 37^{o}C incubator for 15 to 30 minutes. The spinal cord fragments were then pipetted

into a centrifuge tube containing 6ml of Earle's minimum essential medium (MEM) with 10% horse serum (HS) and 10% fetal bovine serum (FBS) (MEM 10/10). The dish was then rinsed with an additional 1ml of MEM 10/10, the rinse was added to the centrifuge tube and centrifuged for 1 minute at 200xg. The cord pellet was transferred to another tube containing 1.5ml MEM 10/10 and triturated at least 10 times. Fragments were allowed to settle to the bottom of the tube and supernatants (containing the suspended CNS cells) were pooled. The previous step was repeated using a narrowed pipette. Pooled cells were counted by utilizing a hemocytometer. The pool was then diluted to yeild $1X10^6$ cells/ml and the suspension was seeded on coverslips coated with poly-lysine (mw 400,000). These coverslips were previously prepared by flaming briefly with a propane torch to increase wettability of the glass surface and hence, enhance the adhesion of cells (Hightower et al. 1983), and were subsequently glued to the culture plate with Eukitt (a xylene based mounting medium; O. Kindler GmbH, Freiburg, Germany). Cultures established to contain an internal control area consisted of neurons seeded on opposite sides of the coverslip separated by a silicon rubber gasket placed prior to seeding (Gardner et al. 1984) (Fig. 1).

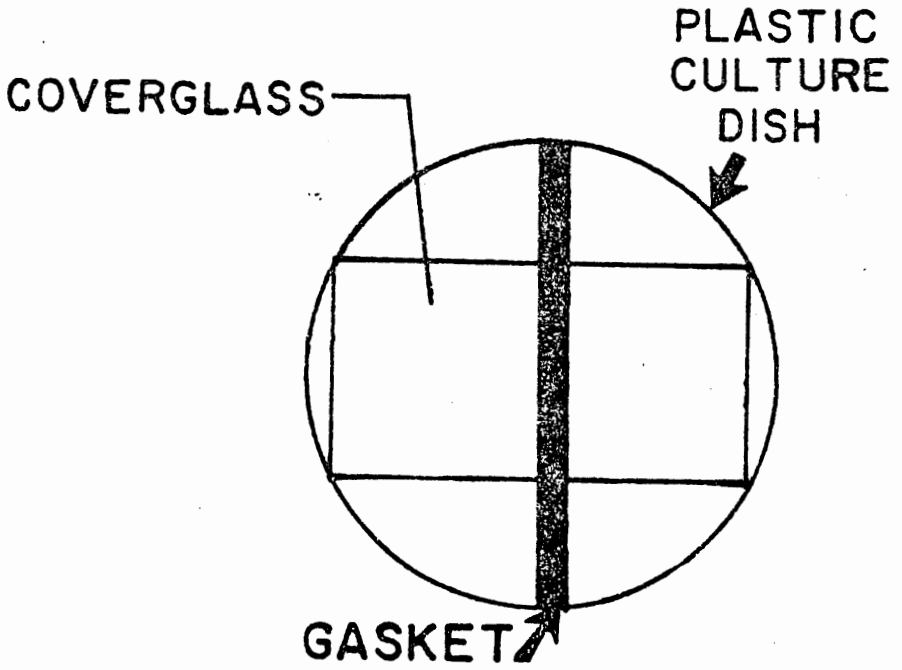


Figure 1. Culture system developed to allow studies of neuronal survival after laser cell surgery in the presence and absence of macrophages under the same culture conditions (internal control). Neurons were seeded to either side of the gasket. When cultures were approximately 3 weeks old, macrophages were seeded on one side of the culture only.

After allowing 24 hours for the adhesion of cells, 3ml of MEM 10/10 was added to the dishes. At 7 days post seedng, dishes were treated with fluoro-deoxyuridine (FdU) for 72 hours to retard the growth of mitotically active cells in the "carpet". Cultures were fed every 3 days with MEM + 10% HS (MEM 10) that was equilibrated to the proper pH and temperature prior to use.

III. Macrophage Culture

The procedure utilized for macrophage culturing was modification of one described previously (Garvey et al. 1977). Male mice were injected intraperitoneally with 3ml sterile paraffin oil (Saybolt viscosity 350) 3 days prior to culture date. The mice were killed by CO_2 asphyxiation. Macrophages were harvested via peritoneal lavage with modified Hank's balanced salt solution (mHBSS). HBSS was modified by addition of 1ml FBS/100ml and 10U/ml heparin. Harvested cells were centrifuged for 10 minutes at 300xg at 0-2^{o}C The supernatant was discarded and cells were resuspended in 4ml mHBSS and run on a Ficoll-Paque gradient for 30 minutes at 400xg at 20^{o}C. (Density 1.077, used specifically for the isolation of mononuclear cells). The resulting interface layer containing the cells was harvested, washed 2 times by centrifuging for 10 minutes at 300xg in 3 volumes mHBSS and suspended in MEM

10/10 including 1ml/100ml penicillin-streptomycin-fungizone (PSF) (Gibco). The medium was equilibrated to proper pH and temperature prior to use. Cells were counted utilizing a hemocytometer and $2X10^6$ cells were seeded per established 3-4 week old neuronal culture (60mm dishes) or $1X10^6$ cells on one side of cultures with internal control. After a 2 hour incubation period to allow for macrophage settling and adhesion, half the medium was removed and fresh medium was added. The medium added depended upon the study being undertaken. The cultures were kept in a 37^oC, 10% CO_2, moist incubator and were not used until cultures were at least 2 days old. Cells were fed every 3 days with MEM 10/10 + PSF (MP reaction time and internal control studies) or with MEM 10 (external control studies). External control studies were carried out by utilizing neuronal cultures of a particular culture date paired with neuron-macrophage cocultures of the same neuronal culture date.

IV. Identification Methods

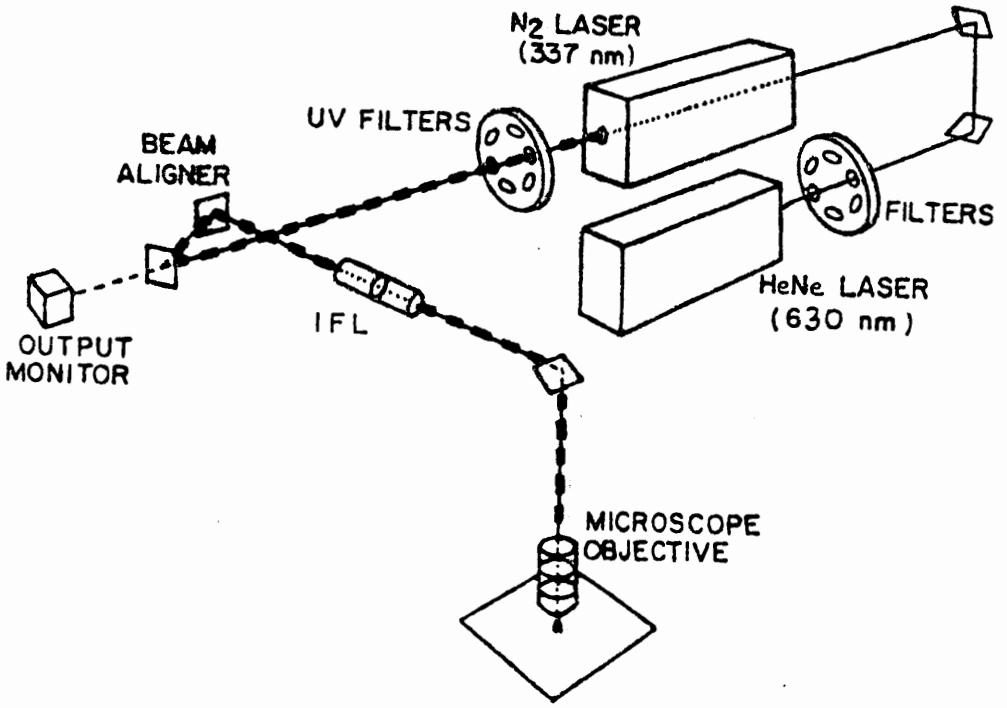
Live macrophages can be identified by general morphology, but cytological staining is more accurate. Wright's stain for blood elements was used to determine nuclear shape and the presence or absence of granules in cell cytoplasm (Humason 1972). This stain was used in

combination with a non-specific esterase stain to improve identification (Gardner et al. 1984). The non-specific esterase stain indicates functional maturity of MPs and is used as an identifying characteristic (refer to Fig. 5).

The non-specific esterase was the first staining method employed. The method used was a modification of that contained in Sigma Technical Bulletin Number 90. First the culture medium ws removed from the culture dish and the dish rinsed with DlSGH or Hank's balanced salt solution. After fixing the culture with 3% glutaraldehyde in Millonig's buffer (Ph 7.4) for at least 5 minutes, the fixative was then removed by soaking in distilled water for 10 minutes. The stain was prepared by combining 7.5mg Fast Blue RR in 15 ml Tris(hydroxymethyl)aminomethane-maleate buffer (pH 7.6, 20mm) warmed to 37^{o}C. To this, 0.6ml of an α-napthyl acetate solution (prepared by dissolving 10mg α-napthyl acetate in 1 ml ethylene glycol monomethyl ether) was added while stirring for 15-20 seconds. The coverslips were placed in a columbia jar and the stain was poured over them. Staining proceeded at 37^{o}C for 15 minutes in the dark. This was followed by a 3 minute rinse in distilled water. The coverslips were then counterstained with Wright's stain for 2 to 4 minutes and mounted in a water soluble mountant.

V. Laser Cell Surgery

The laser microbeam system was constructed by BTG Biotechnik, Munich, G.F.R. and consisted of a 630nm continuous mode helium-neon (HeNe) laser aligned with a pulsed nitrogen work laser. The lasers were coupled to a Leitz Orthoplan microscope (Fig. 2) (Higgins et al. 1980). The N_2 work laser emited UV radiation at 337.1nm in 12ns pulses. Irradiation of cells was carried out utilizing a 32X phase quartz glycerine immersion Zeiss Ultrafluar objective producing a minimum laser focus diameter of 2.2 µm. The objective was placed in a plastic sleeve to which a quarts coverglass was attached (Fig. 3). This sleeve served to protect the objective from heat stresses as quartz optics are very sensitive to temperature variations and can actually be damaged at temperatures above 35°C. As the sleeve was directly lowered into the culture medium, the objective in the sleeve and the cover plate were irradiated with a germicidal UV light 15 minutes prior to surgery to minimize the possibility of culture contamination. Since the initiation of this practice, fewer than 5% of cultures used for surgery have become contaminated. Following irradiation, the sleeve, and, hence, the objective, were attached to the microscope, lowered into the medium and focused on the cell layer. The desired process was targeted with the HeNe laser at a



(Adapted from Higgins et al. 1980)

Figure 2. Optical arrangement of the laser microbeam system used. The diagram shows essential components, beam alignment and coupling to the light microscope. The laser - microscope coupling was achieved by an adjustable quartz interface lens (IFL) which allowed movement of the laser focus along the vertical axis without changing the image plane.

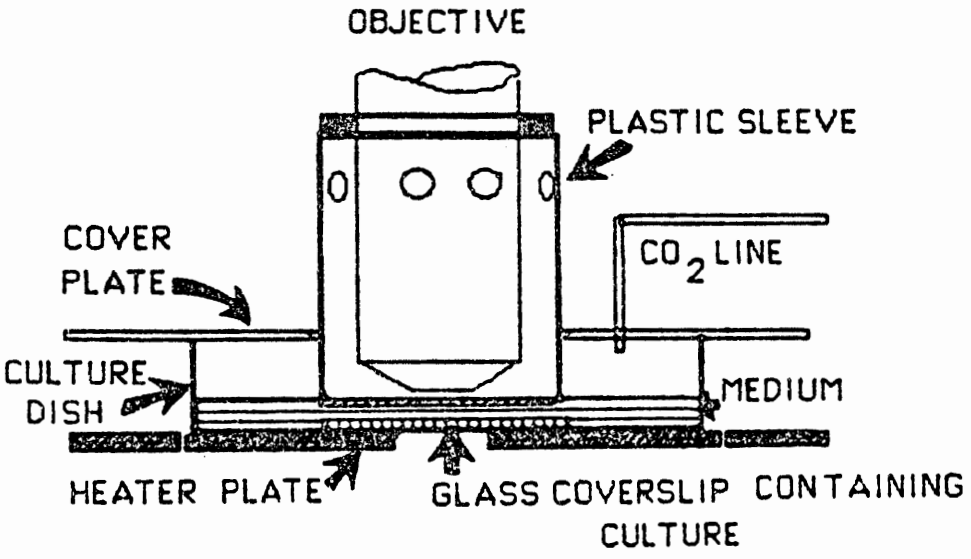


Figure 3. A quartz phase contrast objective is placed in a plastic sleeve to protect the objective from saline and from heat damage. The sleeve has a matched quartz coverglass attached to allow for passage of UV radiation. It allows the immersion of an objective into culture media without damage to the objective and without optical distortion. (Thickness of coverglass: 350µm)

given distance from the cell body and the process was then either transected or damaged. Cells were irradiated at a frequency of 20Hz, and an energy density between 2.4 and 3.6$\mu J/\mu m^2$. Cumulative energy densities varied with the length of time of irradiation. Firing continued until the desired effect on the process was achieved. The effect on the process was either a complete transection or a pinching of the neurite as described in Gross et al. (1983). The pinching was reversible if lasing was halted at a time before transection occurred. Surgery was carried out on a heater plate that kept the culture temperature between 38° and 40°C. A stream of moist air with 10% CO_2 was passed through a small opening in a chamber cover to maintain pH.

VI. Data Collection and Analysis

An area in a culture was selected containing a sufficient number and appropriately positioned MPs near neurons chosen as targets for surgery. The area was usually photographed for future analysis.

A. Time Course of Macrophage Response

These studies were carried out to determine the time of initial MP response to a lesion and also the distance of the responding MPs. Photos were taken immediately prior to and after trauma and every 2 minutes or other

suitable interval consistent with MP activity and movement. The time of initial MP movement toward the lesion was taken as the initial response time and the initial distance of the MP from the lesion was taken from measurements off the photographs.

B. Long-Term Survival Studies

1. Determination of Cell Death

The determination of the cell survival obviously depends on a good functional definition of cell death. For the experiments in this dissertation, cell death had to be determined from light microscopic observations. In neuronal cultures without MPs, over 80% of the cells that had died were frequently identifiable only as debris or they floated off the substrate and could not be relocated. On those occasions where cells were relocated and their viability was in doubt, the presence of Brownian motion within the soma was indicative of cell death. However, in the absence of Brownian motion, at least 2 of the following criteria had to be met for a cell to be considered dead: 1) somal outline not clearly visible; 2) no clear nuclear outline; and 3) severe granularity of the soma. These criteria of cell death were confirmed by MP removal of dead cells. As described in the results section, MPs do not destroy injured cells that have

the capacity to recover but will attack those which apparently have died. In practically all cases, dying or dead cells could be reliably identified by the criteria listed above before they were removed by MPs. Consequently, dye exclusion tests were not needed to determine validity of the visual observations.

2. Internal Control Studies

The surgery of neurons was carried out as described in Section V. In cultures with internal control, 10 cells were damaged by process transection on the control side and an equal number on the MP seeded side (lesion distance was 100μm from the cell body). The gasket separating the two sides was removed 1 to 3 minutes prior to placement of the plate on the microscope to allow for medium continuity (Gardner et al. 1984). To facilitate relocation of injured cells, the glass substrate was marked with laser craters below the glass surface. These craters were produced with high energy ($6\mu J/\mu m^2$) single shots and did not disturb the culture. Photos were taken both before and immediately after trauma. The culture was subjected to a full medium change with MEM 10/10 + PSF before it was returned to the incubator. Photos were taken at 24 and 48 hours post trauma and were

analyzed with special attention to the survival or damaged neurons utilizing the Student's t test as the main statistical procedure.

3. External Control Studies

For experimental plates without internal controls, MP-neuronal cocultures were paired, when possible, with normal neuronal cultures of the same culture date. Six cells per plate were subjected to laser irradiation and 6 additional cells were chosen as controls to judge general culture health. Such an experiment lasted approximately 1 hour because it took time to choose neurons with appropriate parameters (neurons with well defined morphology and neurites, MPs in vicinity, lesion distance measurements). This choice, therefore, represented a compromise between minimizing culture stress and the need for a suitable sample size. This procedure also allowed a direct comparison of data with other investigators in our laboratory. Surgery was carried out and the glass substrate marked as described above. The cultures were subjected to a half medium change with MEM 10 24 hours after trauma. Again, photos were taken at 24 and 48 hours post trauma. Results were analyzed with the Student's t test.

RESULTS

I. Macrophage-Neuronal Coculture

Fluoro-deoxyuridine (FdU) treatment of primary spinal cord cultures eliminated macrophage-like cells from these cultures (Fig. 4). Most of these endogenous macrophages may have been derived from blood monocytes released during the culture procedure. It is also possible that some of these cells were blood monocyte derived resident brain macrophages or glioblast derived phagocytic microglia. The data indicated that these endogenous MPs stain positively for non-specific esterase. This could indicate that they were indeed derived from the blood monocyte pool and were simply obtained from unavoidable contamination of the culture (Hansson et al. 1980). No cells positively identifiable as MPs were found in FdU treated spinal cord cultures when these were stained with Wright's stain or for non-specific esterase.

The exogenous MPs that were isolated via peritoneal lavage had a viability of better than 95% at the time of seeding, as established by trypan blue exclusion. These cells formed a heterogeneous population composed of cells 5μm to 25μm in diameter. Virtually 100% of the cells that

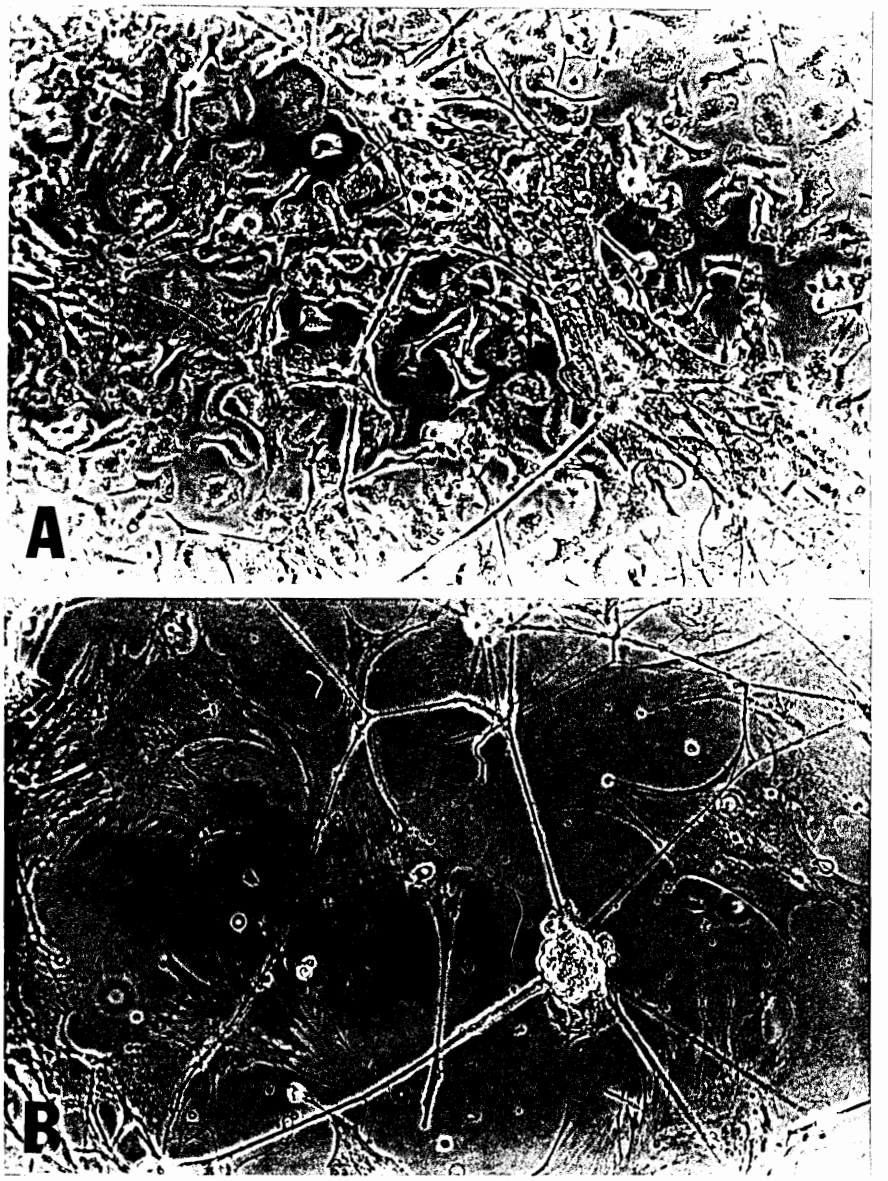


Figure 4. Fluoro-deoxyuridine effects on neuronal cultures. Primary neuronal cultures were treated with FdU 7 days after seeding to control growth of rapidly dividing cells. Cultures A and B were both seeded at the same time and were 3 weeks old at the time of photography.

A. Culture not treated with FdU on day 7; revealing the presence of macrophage-like cells.

B. Culture treated with FdU on day 7; macrophage-like cells are absent.

adhered to the glass, stained positive for non-specific esterase (Fig. 5) and showed the characteristic nucleus of a monocyte via Wright's stain. Non-adherent cells present at the time of isolation were predominantly erythrocytes, with very few, if any, lymphocytes.

Exogenous MPs and neurons were found to coexist in culture for the duration of these experiments (up to 3 weeks). MPs do not interfere with the health of neuronal cultures. On the contrary, they appear to perform a function similar to the debris removal performed in vivo. This function, unfortunately, can not be easily quantified in our system. However, it was consistently observed that cocultures had less floating debris and no recognizable dead cells. Also, neurons in cocultures had cleaner looking processes (most neurites in regular cultures had what appeared to be debris on them). By 3 weeks time, some of the MPs took on the appearance of giant foreign body cells. Such cells are formed by the fusion of MPs (Mims 1964). There were no observations taken after 3 weeks, because it appeared that MP function had begun to decline and the cultures were discarded.

There was essentially no difference observed in the behavior of MPs cocultured with neurons and those in purified MP cultures. MPs in purified cultures reacted to laser damaged cells with chemotactic and phagocytic

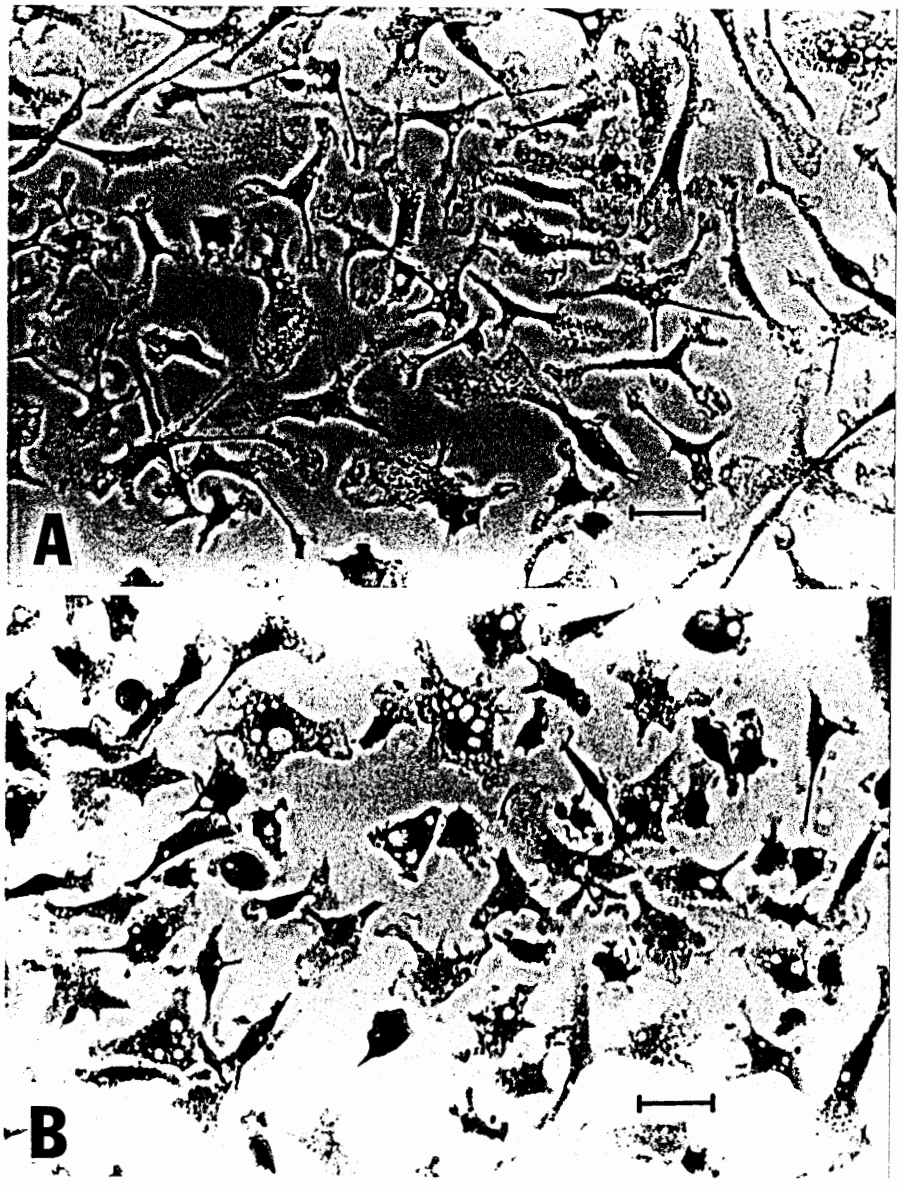


Figure 5. Stained macrophages on coverslips in 48 hour purified macrophage cultures.

A. Esterase stain of macrophages. Note all cells stain positively for non-specific esterase. This indicates functional maturity and is an identifying characteristic of macrophages.

B. Combined esterase and Wright's stain. Note the darkly staining non-lobulated nuclei characteristic of monocyte-derived macrophages. The lack of any cells with lobulated nuclei suggests that no neutrophils were present. Scale indicates 25μm. The greater number of vacuoles present in these cells represent a selection artifact.

responses in a similar, if not identical manner, to that observed in MP-neuronal cocultures. Likewise, MPs in cocultures were morphologically identical with those in purified MP cultures (refer to Fig. 5 and 6).

Several cocultures were stained with a combined non-specific esterase and Wright's stain to determine if these stains could be used to identify MPs specifically in these cultures. It was found that both neurons and MPs stain with this procedure, leaving cells in the carpet essentially unstained (Fig. 6 and 7). This carpet is composed of fibroblasts and various kinds of glia.

Fig. 6 clearly shows that MPs in coculture with neurons remain functionally mature and retain the characteristic morphology of MPs. They retain the ability to actively respond to damaged cells and initiate phagocytosis as do MPs in purified cultures.

II. Macrophage Response to Neurite Lesions Produced by Laser Cell Surgery

A. Responses to Neurite Transection

Elicited macrophages responded to neurite transections by extending pseudopodia and migrating to the site of the lesion (Fig. 8). Upon arrival at the lesion most MPs made contact with the cut ends of the proximal or distal neurite segments. Occasionally, single MPs were observed to bridge the lesion site and contact both

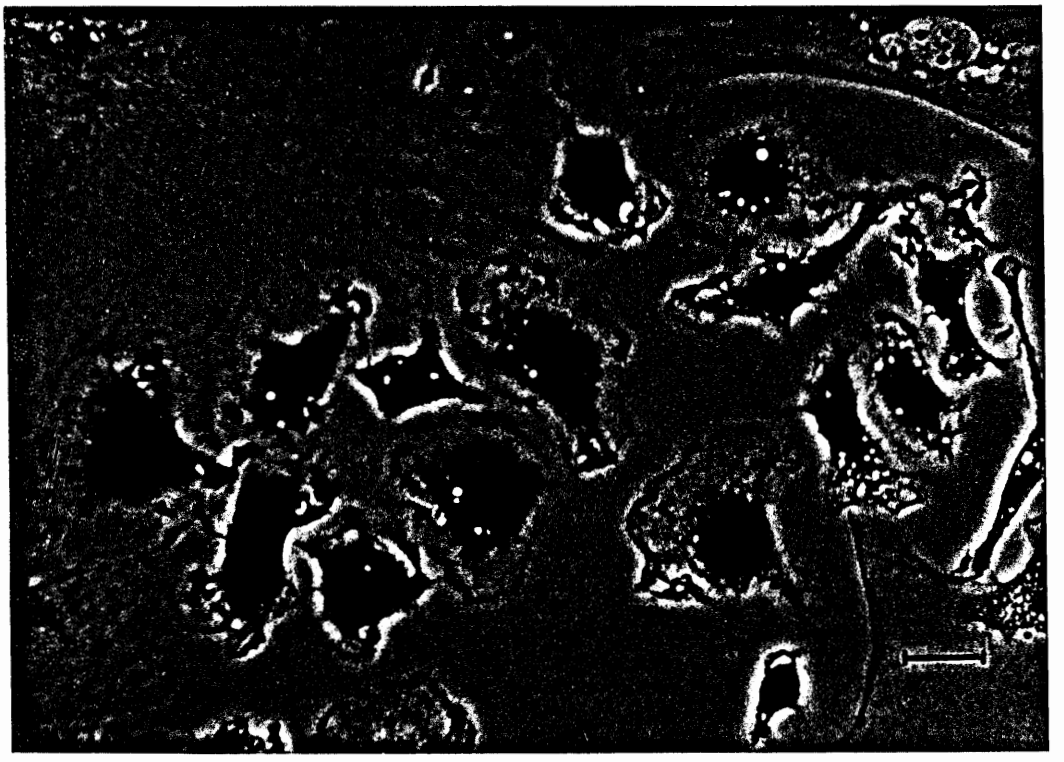


Figure 6. A combined non-specific esterase and Wright's stain of a 4 day macrophage-neuronal coculture shows intense staining of flattened macrophages with little or no staining of the non-neuronal "carpet" cells. (Neurons are not visible in this photograph. Scale indicates 15µm.

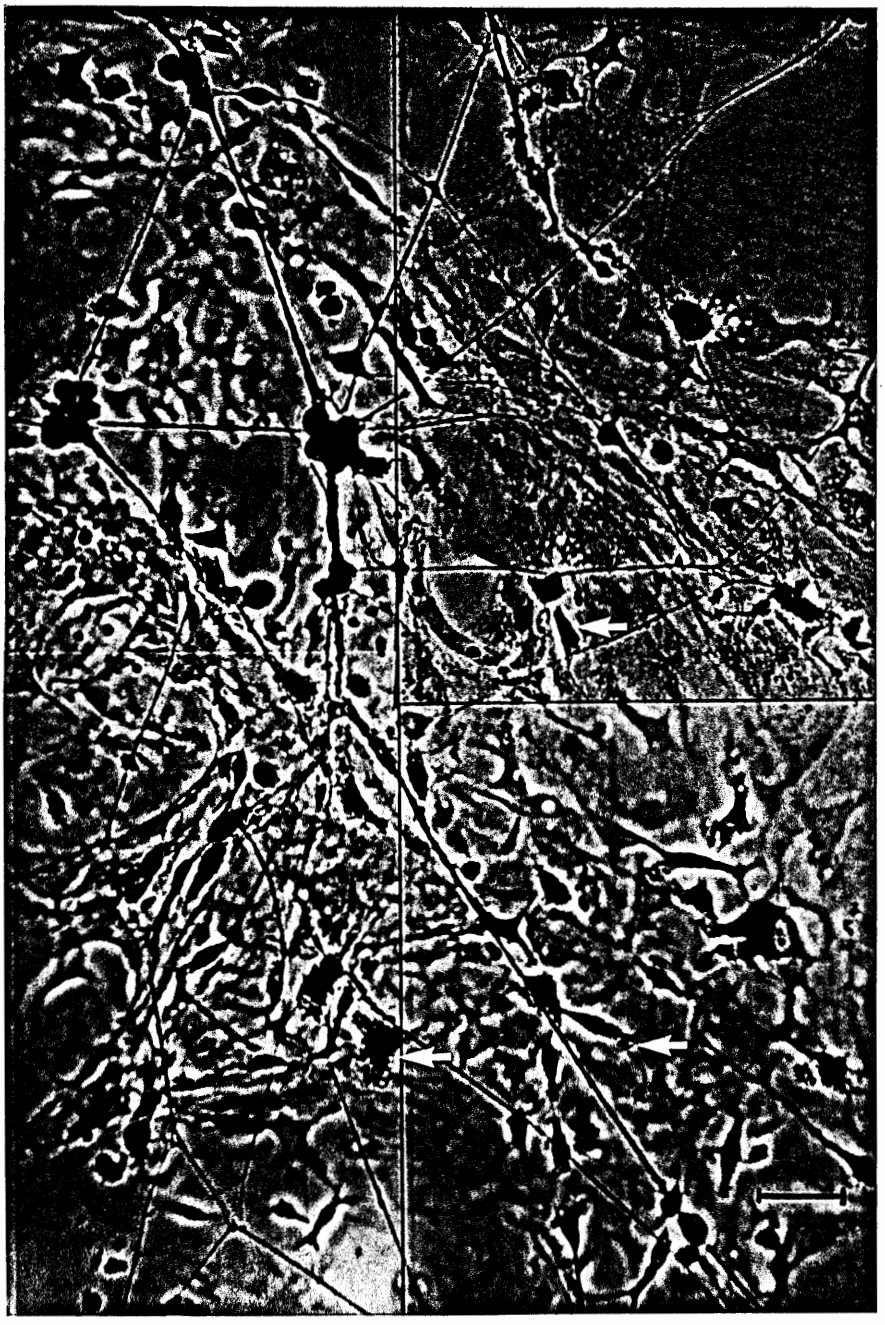


Figure 7. A combined non-specific esterase and Wright's stain of a 4 day macrophage-neuronal coculture demonstrating that only neurons and macrophages stain strongly with this procedure. Arrows indicate representative macrophages. Most neurons are in small clusters interconnected by relatively straight multifiber fascicles. For cell surgery, clustered neurons were avoided. Scale = 55μm.

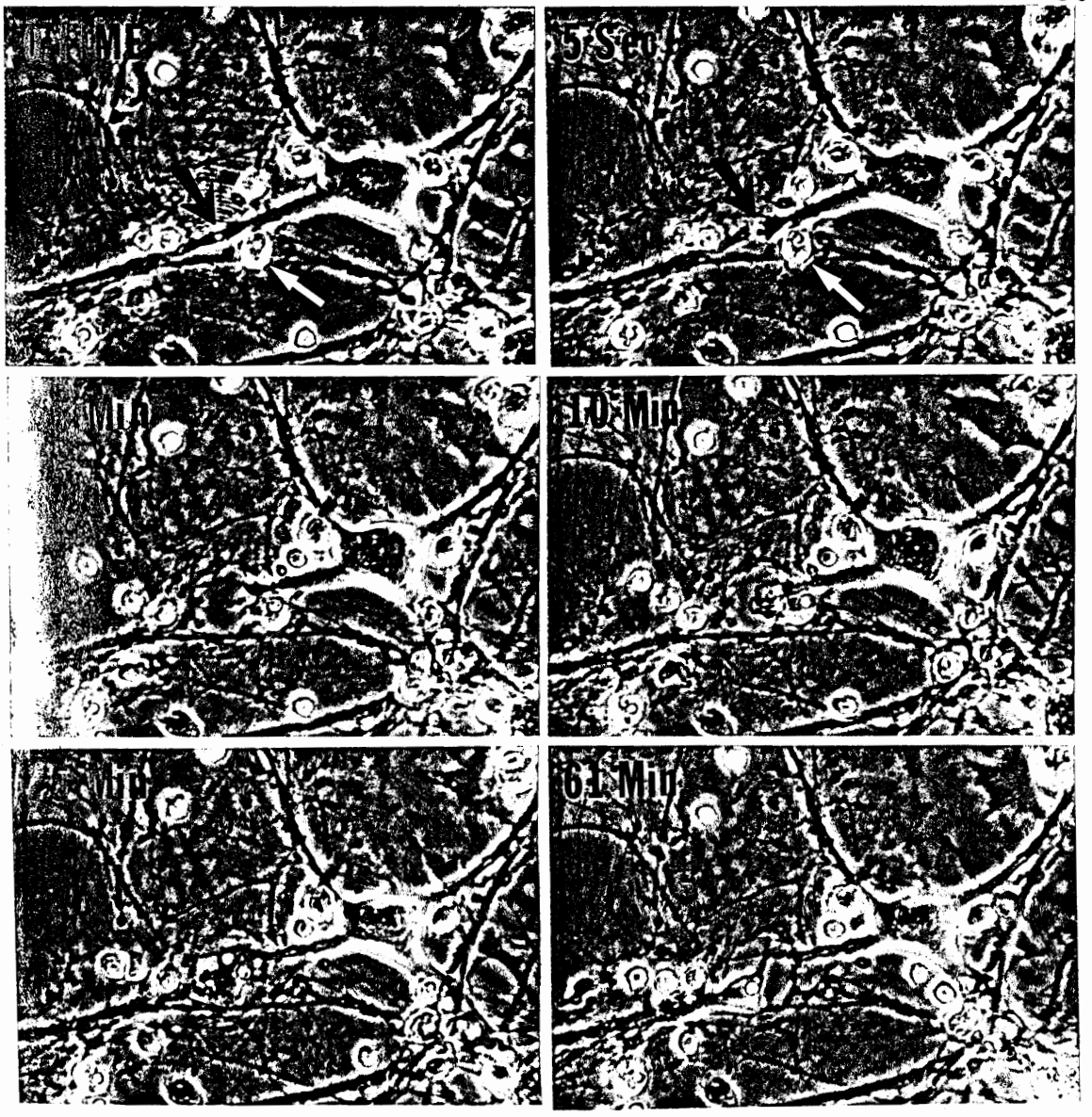


Figure 8. Macrophage response to a process transection. Lesion site is indicated with a black arrow, the responding macrophage is indicated with a white arrow. At T=0, the lesion site is targeted by the HeNe laser. Five seconds after lasing (frequency-4Hz, energy density-3μJ/μm^2, 74 shots), a transection is clearly seen. The MP had begun to respond to the lesion before the 7.5 min. mark and is seen here and at the 10 min. mark attacking the proximal segment of the transected process. By 25 min., the MP had bridged the gap between the proximal and distal segments and by 61 min., the MP had released the proximal segment (which may be resealed) and appears attached to the degenerating distal segment.

neurite segments simultaneously. In the case of contact with only one neurite segment, no obvious preference for proximal or distal sites was observed.

Once in contact, a variety of different responses occurred. Some MPs initiated active phagocytosis discernable through the appearance of vacuoles in the MPs and the concomitant shortening of the contacted neurite. However, when this response was observed, the neurite shortening never exceeded 5-10μm. Thereafter, the MPs seemed to become refractory and did not appear to be capable of further phagocytic activity during the usual 1 hour observation period. It also was evident that such cells could not respond to other chemotactic stimuli. In addition, other MPs initiated abortive approaches, being attracted to but never reaching the lesion. Some MPs never responded at all regardless of their proximity to the lesion. MPs also reacted to the degenerating process and occasionally to the neuronal soma in cases of severe damage. In the case of MP response to the soma, the soma frequently took on a granular appearance and in some instances began to extrude cytoplasm into the surrounding area. This sort of somal reaction took place when lesions were placed too close to the cell body (under 50μm away). The response of MPs to a lesion indicated that transection

of a process released some substance(s) that are chemotactic for MPs. The suspected nature of this substance will be elaborated upon in the Discussion.

B. Responses of Macrophages to Neurite Pinching

At low energy densities, the laser causes gradual damage to the neurite. After many shots, the cytoplasm of the process contracts in the laser focus producing a clearly discernable pinching. It has been determined by Gross et al. (1983) that such pinching is associated with a loss of microtubules and some reduction of microfilaments in the laser focus. If irradiation of the process is halted during the development of process pinching, it is usually reversible, i.e. the original process diameter can be attained in about 5-10 minutes after lasing (Gross et al. 1983). However, the pinching is associated with a temporary, partial depolarization as measured by intracellular electrodes in the soma (Lucas, personal communication). This indicates that at least a small change in permeability is associated with this phenomenon.

It is of importance to determine if the minor, reversible lesions described above release chemotactic substances. Therefore, experiments were conducted to ascertain MP responses to process pinching. From 10 observations, it was clear that MPs responded to pinching

processes. This is demonstrated in Fig. 9 where a pinching of the process is clearly visible. With time, 2 MPs were attracted to this site but did not initiate phagocytosis. MP 1 was apparently not stimulated sufficiently to migrate onto the process and indeed retracted by 46 minutes post-lesioning. However, MP 2 received enough stimulation to initiate migration to the lesion site. In its migration, MP 2 displaced and appeared to stretch the process. This stretching could enhance the release of chemotactic molecules. The MPs were no longer chemotacticlly stimulated when the neurite presumably resealed. This type of response may indicate that a small molecule might be the chemotactic factor. This factor appeared to be released not only after a complete transection but also when there was minimal injury to the process, as with pinching.

C. Macrophage Response Time to a Lesion

The initial response time is defined as the time from the completion of the lesion to the first indication of MP movement. This depends on the distance of the MP from the lesion, the rate of production, and diffusion of the chemotactic substance, interaction of the substance with the MP and the initiation of MP movement. As shown in Figures 10 and 11, the initial response distance can be

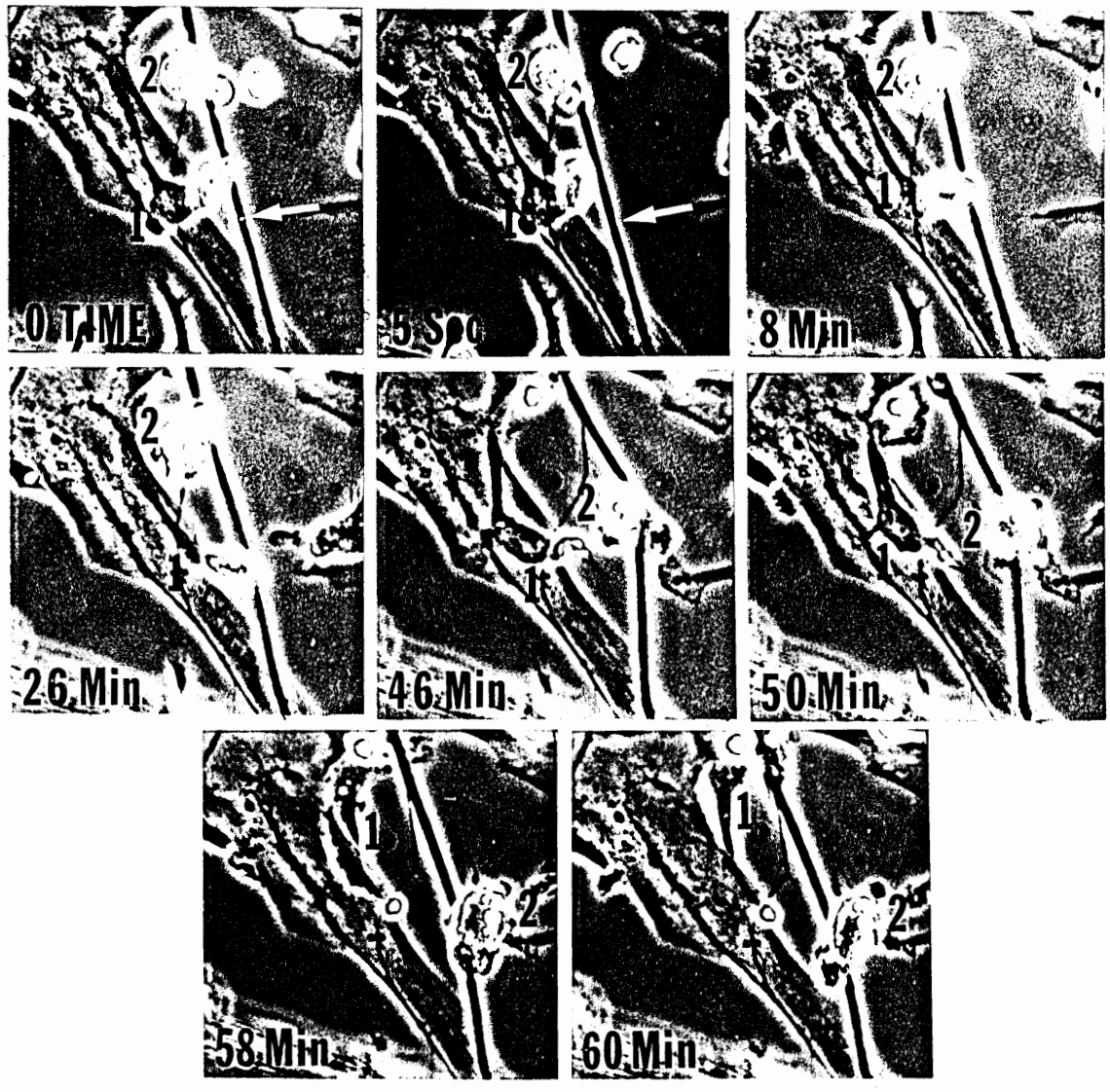


Figure 9. Macrophage response to a pinching process. Laser impact site is indicated by the white arrow. Two macrophages had responded to the pinching. Macrophage 1 extended a pseudopod directly onto the lesion site by 8 minutes post surgery and then retracted only to migrate onto the process at the top of the photo by 1 hour. Macrophage 2 began to react to the lesion by 26 minutes and actually migrated down the process to directly cover the lesion site by 1 hour, where it remained. It is interesting to note that during migration, macrophage 2 displaced the neuronal process. A possible stretching of the membrane could enhance the release of chemotactic molecules from the injured neurite.

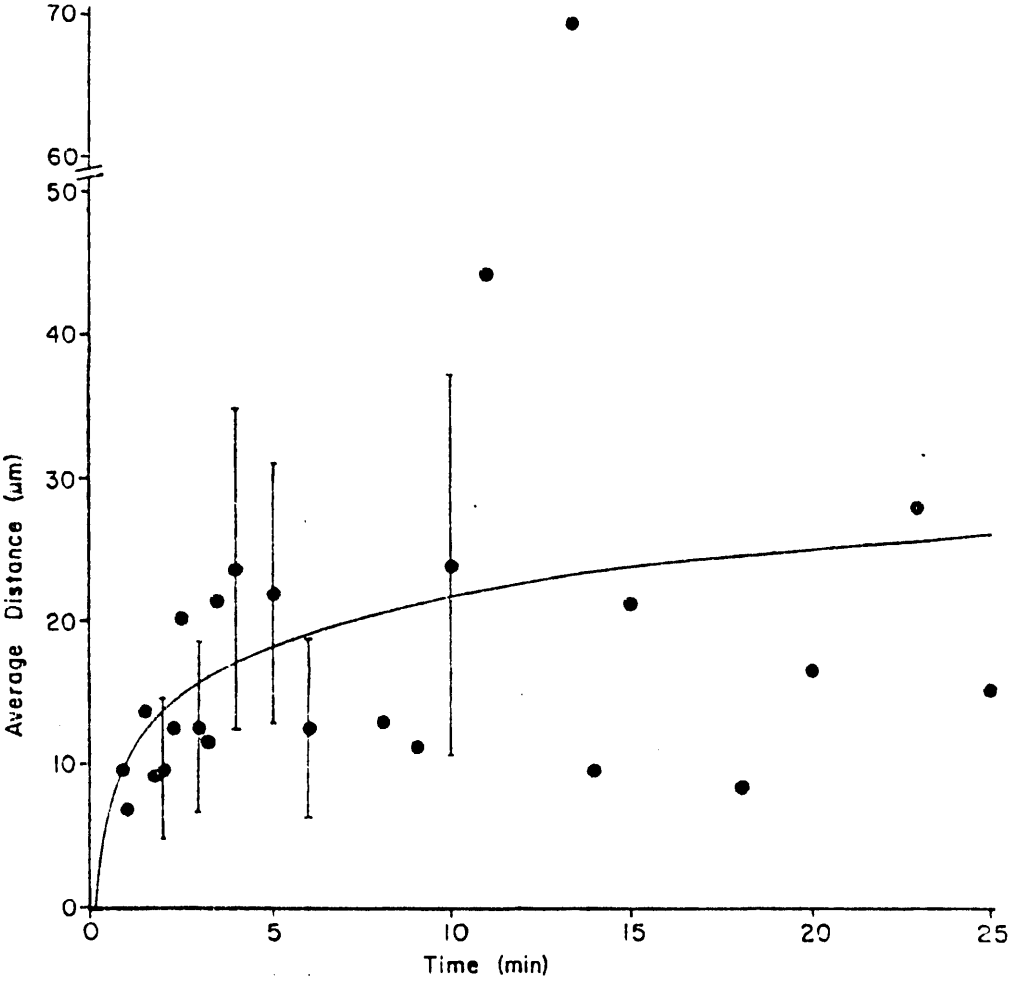


Figure 10. Macrophage response to a process lesion. The average distance is the distance of the MP from the lesion. Time is defined as the amount of time post-lasing that it took the MP to initiate a migratory response. Only those MP that actually reached a lesion site were included in this graph. Also, single observation points were excluded and the values plotted are averages of 2 to 15 observations. The graph depicts the distances from the laser lesion at which directed MP movement could be determined as a function of time after lasing. There was a wide range of response distances for any given time point; however, there is enough evidence to suggest a logarithmic relationship between the distance of a MP from a lesion and it's initial time of response. Error bars indicate standard deviations (N=4 to 15 observations). Points without error bars represent less than 4 observation points per average.

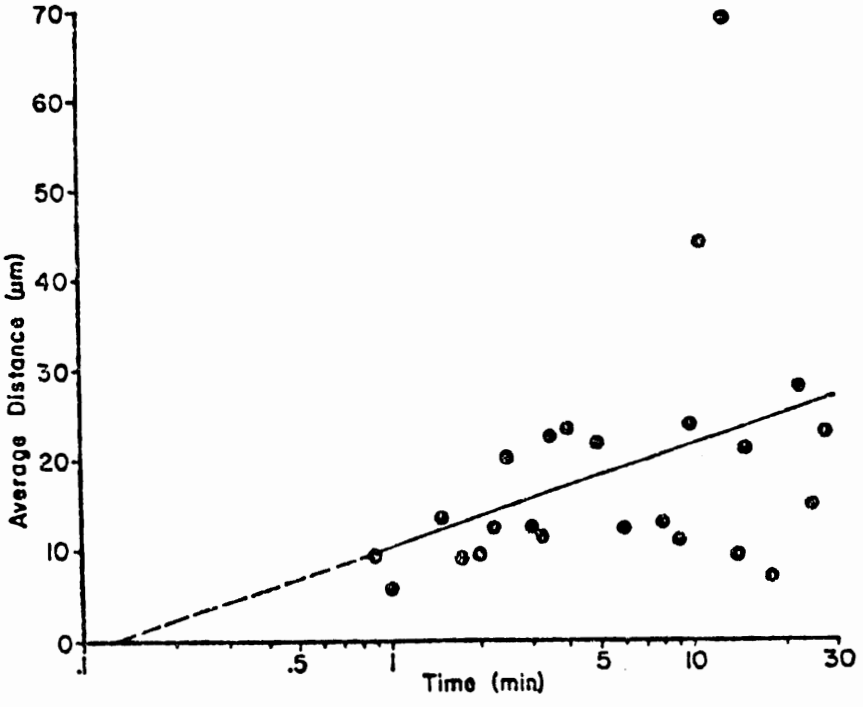


Figure 11. Macrophage response to a process lesion: Time-Distance Study. The initial response distance can be approximated by a logarithmic function of initial response time. A MP needs a certain amount of time to initiate a response to a chemotactic stimulus. The extrapolated intercept with the abscissa suggests that a minimum distance from the lesion, a MP will take about 7 seconds to show a visible response. The correlation coefficient for these data is 0.34 which indicates that only a weak linear correlation exists. The presence of convection currents in the medium was probably the main cause for the large scatter of the data.

represented as a logarithmic function of initial response time. The scatter of the data past the 10 minute time interval indicates a breakdown of the distance-time relationship. This is probably due to convection currents in the culture medium which modify concentration gradients, as well as the possibility of MPs being recruited by other MPs that had already responded. However, below this time period, a reasonable correspondence can be attained. It can be determined from these figures that a MP 10μm from a lesion would respond to that lesion in approximately 1 minute and a MP 20μm away would respond in approximately 8 minutes. This is further demonstrated by the semi-log plot of Fig. 11, which also reveals that a logarithmic relationship between initial response time and distance from the lesion is a reasonable approximation. Furthermore, the extrapolated intercept of Fig. 11 suggests that a MP located virtually on top of a lesion needs at least 7 seconds to respond to that lesion. A maximum response distance of 70μm was observed for a MP that reached the site of a transection lesion. A diffusion coefficient for the chemotactic substance could not be calculated due to convection currents set up in the medium by the heater plate and the CO_2 stream.

III. Neuronal Survival in the Presence of Macrophages

In view of the observation that MPs attack and phagocytose transected processes on neurons, it was hypothesized that MPs would continue phagocytosis of the proximal segment of transected neurites. It was believed that this would prevent resealing of processes and hence, lead to the death of these neurons. Experiments were carried out to test this hypothesis. Surprisingly, MPs were found to actually enhance the survival of neurons which had neurites transected via laser cell surgery.

The survival of neurons was assessed after neurite amputation trauma in the presence and absence of exogenous macrophages to determine if MPs affect survival. Functional criteria for the determination of cell death were established as follows. The presence of Brownian motion within the soma was, by itself, indicative of cell death as it indicated a dissolution of the cytoskeleton. In the absence of Brownian motion, at least two of the following criteria had to be met: 1) somal outline not clearly visible; 2) no clear nuclear outline; and 3) severe granularity of the soma. However, the task of determining cell death was simplified by the fact that at least 80% of dead neurons were identifiable only as debris or had lost adhesion to the carpet and floated off.

Furthermore, in neuron-MP cocultures, cells that died were phagocytosed by MPs and never relocated.

In MP-neuronal cocultures, there seemed to be no correlation between the survival of injured neurons and MP-proximal neurite contact, based on 24 hour observation. However, this interaction may have been missed as the protocols for these experiments did not allow for prolonged observation periods after surgery. It was important to minimize the stress placed on the cultures to give injured neurons the opportunity to recover.

A. Internal Control Experiments

The purpose of this study was to determine whether or not MPs had an effect on the survival of neurons while under identical culture conditions as neurons without MPs. MPs were seeded on one half of the neuronal culture separated by a silicon rubber gasket. This gasket was removed only 1 to 3 minutes prior to positioning the dish under the microscope, so the medium was continuous for only that short period of time. Following surgery, the culture was subjected to a full medium change with penicillin-streptomycin-fungizone (PSF) to prevent contamination of the culture (refer to Methods section VI).

Eleven experiments were carried out at lesion distance of 100μm from the soma. Each experiment

represented a total of 20 cells per dish, or 10 neurite transections per side. It was found that there was no significant difference in the survival of traumatized neurons regardless of the presence or absence of macrophages (Fig. 12). Neuronal survival under these conditions was 61% when MP conditioned medium only was present and 65% when MPs were present. This indicated that MPs did not interfere with the recovery of neurons with transected neurites.

The mean age of the neuronal cultures used was 24 $\pm$ 6.7 days and were surgically manipulated an average of 5.8 $\pm$ 4.4 days after macrophage addition. Morphological parameters of neurons selected for surgery were similar for cells on either side of the plate (with MPs or with MP conditioned medium only). A random sampling of 5 of the 11 experiments indicated an average process diameter of 2.6 $\pm$ 0.9µm (N=49 cells) and an average somal radius of 9.4 $\pm$ 2.4µm (N=43 cells) for neurons with MP conditioned medium. On the side of the dish containing MPs, neurons had an average process diameter of 2.4 $\pm$ 1.0µm (N=50 cells) and an average somal radius of 9.1 $\pm$ 2.2µm. For the formulae that were used in these calculations please refer to Table 2.

The survival depicted in Fig. 12 was consistently higher than the survival determined by other researchers

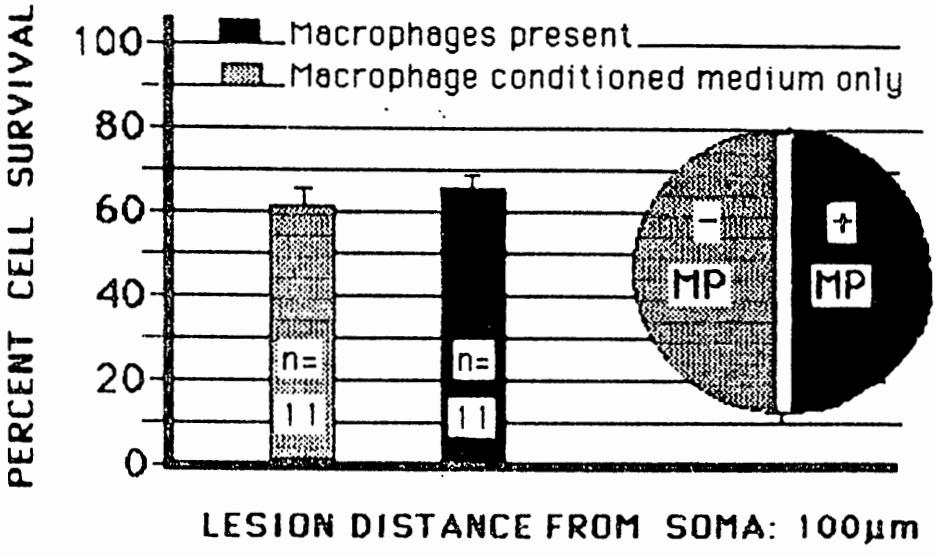


Figure 12. Neuronal survival after process transection in the presence and absence of macrophages. Macrophages (+MP) were seeded only on one side of a neuronal culture. Medium continuity was established by removing the gasket 3-5 minutes prior to surgery. There is no significant difference in the survival of neurons in the presence or absence of MPs as long as MP conditioned medium was present. (Note: These experiments were conducted in the presence of penicillin-streptomycinfungizone (Gibco).) This treatment reduces the survival of injured neurons to 30% (Lucas et al. 1985).) Lesion distance: 100µm; Process diameters: MP- = 2.6 $\pm$ 0.9µm; MP+ = 2.4 $\pm$ 1.0µm. Error bars indicate standard error of the mean.

in our laboratory working without MPs or without MP conditioned medium. Therefore, it was necessary to directly compare neuronal survival in the presence and absence of MPs in separate culture dishes without the complicating conditions of MP conditioned medium.

B. External Control Experiments

Experiments were carried out with paired neuronal cultures, one containing macrophages, the other in normal (i.e. non-conditioned) medium and without MPs (refer to Methods section VI B3). The data demonstrated a highly significant increase in survival of traumatized neurons in the presence of MPs. This unexpected effect was quantitatively analyzed at lesion distances of both 100 and 50μm from the soma. Mean neuronal culture age for the 100μm and 50 m lesion distances were 27.4 $\pm$ 3.6 days and 33.1 $\pm$ 8.4 days respectively. Cultures with macrophages added were surgically manipulated at an average MP culture age of 5.3 $\pm$ 3.6 days for the former and 10.3 $\pm$ 5.3 days for the latter study. No culture age related differences were noted in the survival of injured neurons. Similarly, there were no culture age related differences in macrophage behavior within the stated age ranges. MP activity did appear to decrease in cocultures over 3 weeks of age so these cultures were never used in the above studies.

The data at a lesion distance of 100μm were obtained from 10 experiments from which MPs were absent and 11 experiments in which MPs were present (Fig. 13). Each experiment represented 6 neurite transections per culture plate. Despite the low number of transections per experiment (chosen to minimize culture stress under the microscope (see Methods section VI B3)), the standard errors of the mean are small and reveal a high consistency that the presence of MPs had a positive effect on cell recovery from process amputation.

The results obtained at a 50μm lesion distance are even more surprising. At this distance, almost 70% of process amputations lead to cell death in normal medium. In the presence of MPs this was reduced to 35% (i.e. 65% cell survival in Fig. 13).

Note that the viabilities in Figs. 12 and 13 can not be compared quantitatively, since the data were gathered utilizing different surgical protocols. Internal control studies were carried out in MEM 10/10, in which the presence of extra serum may have affected the system. Also, cultures used in these studies were subjected to a full medium change following surgery, procedure which may have further stressed the culture, and perhaps most significantly, PSF was routinely added to the medium both before and after surgery. PSF is known to reduce the

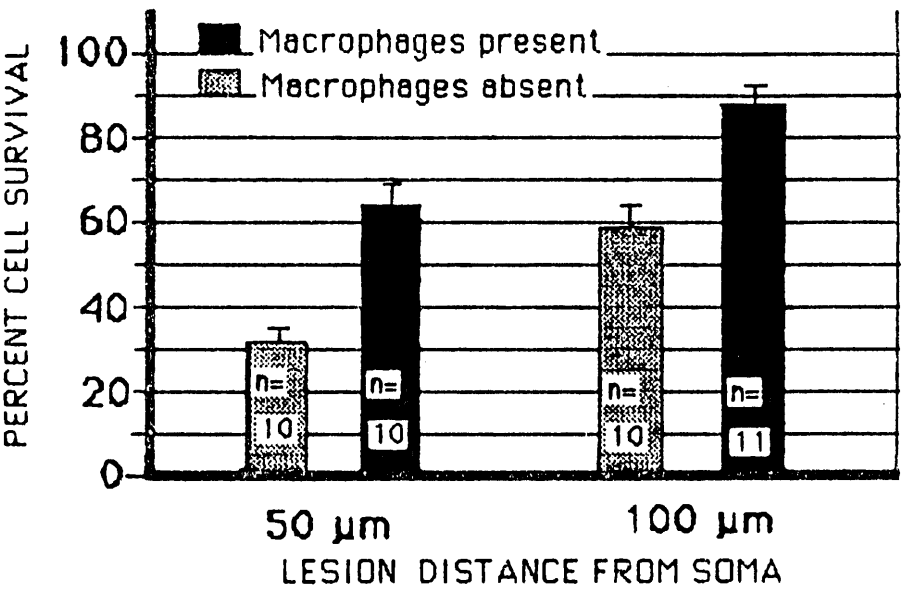


Figure 13. Neuronal survival after process transection at lesion distances of 50µm and 100µm from the soma. Experiments were conducted in the presence and absence of MPs in separate culture dishes but usually matched cultures (same culture date and procedures). At both 50µm and 100µm lesion distances, there was a highly significant ($P \leq 0.01$) increase in the survival of injured neurons in the culture dishes containing MPs. Error bars indicate standard error of the mean.

viability of injured neurons in culture (Lucas et al. 1985), hence, its presence may have artificially lowered the survival of injured neurons. The protocol was changed for the external control experiments to allow for direct comparison of data gathered in these studies with those collected by other researchers in our laboratory.

As stated in the Methods section, the Student's t test was the main statistical procedure used to analyze the data. The use of this test was justified as, in most instances, the data were normally distributed about the mean (Appendix Fig. 1). In those cases where the data were not normally distributed, they approximated half of a Gaussian curve as the mean neuronal survival increased.

C. Morphological Parameters of Neurons Chosen for Process Amputation

Morphological parameters of surgically manipulated neurons (data from External Control Experiments only) can be found in Table 2. Note that cells chosen for laser cell surgery had a fairly consistent somal radius and process diameter. Therefore, the increase in the survival of injured neurons in the presence of MPs was not due to the selection of neurons with quantitatively different morphological characteristics.

The morphological parameters of neurons chosen for injury are important in trying to determine the mechanisms

TABLE 2

MORPHOLOGICAL PARAMETERS OF NEURONS INJURED BY LASER CELL SURGERY

Parameter	Lesion Distance (μm)			
	100		50	
Macrophages	-	+	-	+
Number of Neurons	60	64	58	60
Average Process Diameter (μm)	3.2 ± 0.1	2.9 ± 0.1	3.2 ± 0.1	3.4 ± 0.1
Average Somal Radius (μm)	9.1 ± 0.3	9.8 ± 0.3	9.2 ± 0.2	9.9 ± 0.3
Somal Volume (μm^3)	2016 ± 307	2337 ± 329	2223 ± 464	2402 ± 308
Somal Surface Area (μm^2)	843 ± 71	953 ± 70	826 ± 41	971 ± 69
Volume of Cut Process (μm^3)	874 ± 66	725 ± 39	442 ± 38	497 ± 36
Surface Area of Cut Process (μm^2)	1001 ± 40	925 ± 26	506 ± 19	534 ± 20

All reported values are means of the individually calculated values for each cell. For descriptions of calculation methods, refer to the following page.

Calculation Footnotes for Table 2

Average process diameter was calculated by taking the diameters of the process at the soma, lesion point and half way between the two and averaging them together.

Average somal radius- neurons appear as ellipses, so the long and short axes were measured. An ellipse that is rotated on its long axis will define a prolate spheroid. The volume of a prolate spheroid is given by $4/3\pi ab^2$, where a is the radius of the long axis and b is the radius of the short axis. Therefore, if the volume of a prolate spheroid is set equal to an ideal cell with a spherical volume ($4/3\pi r^3$), the average somal radius can be calculated with the equation $\sqrt[3]{ab^2}$.

The volume of the soma was calculated by utilizing the formula for the volume of a sphere ($4/3\pi r^3$) divided by 2 (i.e. $2/3\pi r^3$), where r was the average somal radius. The soma of a neuron has the appearance of half of a sphere when viewed by electron microscopy (Lucas, personal communication).

Somal surface area was calculated with the equation $3\pi r^2$, where again r was the average somal radius. The equation for somal surface area was derived from the equation for the surface area of half of a sphere ($2\pi r^2$) plus the area of the equatorial plane (πr^2).

The volume of the cut process was calculated by multiplying the lesion distance by πr^2, where r was the average radius of the process.

Surface area of the cut process was calculated by multiplying the lesion distance by πd, where d was the average process diameter.

of cell survival. Ca^{2+} fluxes are believed to be the primary cause of the initiation of cell death. Ca^{2+} may enter a lesion from the extracellular medium or be released from a number of intracellular pools upon injury. Large quantities of free intracellular Ca^{2+} have the effect of causing dissolution of the cytoskeleton and activation of phospholipase A_2 (which causes changes in membrane phospholipids). These conditions may cause death of the cell. A given neuron, however, may be able to handle these fluxes either by Ca^{2+} pumping or sequestration of Ca^{2+} within intracellular pools (such as mitochondria), thus preventing the changes described above and allowing recovery from the injury. This ability to stabilize damaging changes in intracellular Ca^{2+} concentrations depends greatly on cell geometry since the latter determines the number of Ca^{2+} pumps and the cytoplasmic resistance to injury currents. However, the calculation of these important Ca^{2+} fluxes is beyond the scope of this dissertation.

DISCUSSION

Macrophages are known to exist in the CNS but in much smaller numbers than in the rest of the body (Oehmichen 1978). MPs are believed to be able to penetrate the basal lamina of the blood-brain barrier and thus gain access to the CNS (Kerns and Nierzwicki 1981). It is not known in what state of activation these resident CNS MPs are but it has been shown that they stain positively for esterase and negatively for peroxidase which indicates that they are mature and blood monocyte derived (Albrecht and Bleier 1979). Resident brain MPs have been termed microglia by some authors. There is great confusion in the literature as to what kind of cell the term microglia should be applied. Some investigators have attempted to establish rules for naming and identifying microglia. According to Fujita et al. (1981), the term microglia should only be used when referring to resting microglia. These are cells that are present in adult brain and include those that can be shown to be directly derived from resting microglia. Brain MPs and transformed monocytes should not be referred to as microglia, because they are derived from circulating blood monocytes at the time of injury. The microglia are derived from glioblasts late in the differentiation of the

CNS and can be identified on the ultrastructural level. Microglia react to CNS damage with cytoplasmic swelling and DNA synthesis (reactive microglia). Following this, these cells differentiate into fibrous astrocytes. This view differs considerably from that of Aarli (1983) who states that brain phagocytes are microglia and/or belong to the monocyte-macrophage series of cells. Hume et al. (1983) have presented evidence that MPs differentiate into the microglia of the retina but retain a macrophage specific marker. These investigators also claim to be able to locate and stain microglia in the CNS by utilizing the same marker. As one can see, there undoubtedly will be debate on this subject for some time to come.

In this dissertation, peritoneal MPs have been shown to coexist with neuronal tissue in vitro without causing any overt damage. However, when MPs are cocultured with other cell types (such as tumor lines), cytolysis has been shown to occur (Olstad et al. 1982).

Utilizing peritoneal MPs elicited with paraffin oil in these studies is justified because: 1) they are easily harvested in large numbers (Garvey et al. 1977); 2) antiserum against exudate MPs and histological stains specific for MPs have been shown to be reactive with MPs present in brain tissue in in situ work (Albrecht and Bleier 1979, Murabe and Sano 1983); and 3) MPs elicited

with paraffin oil are more reactive and show a more vigorous response to compounds that elicit the oxidative burst than those considered "resident" MPs or those elicited by other sterile inflammatory stimuli (Keisari et al. 1983). This may be more representative of activity producted in vivo, since many types of CNS trauma, such as cerebrovascular accidents and closed head injuries, are sterile inflammatory stimuli.

As reported in the Results, exogenous MPs have the ability to remove debris from a neuronal culture. This activity has been documented in primary cultures of rat or mouse brain where the presence of these cells was believed to be due to contamination of the culture with blood monocytes and not due to any microglial elements (Hansson et al. 1980). Contamination with blood monocytes may be the origin of the macrophage-like cells present in spinal cord cultures that have not been treated with FdU. As demonstrated in this dissertation, no cells positively identifiable as MPs were present in neuronal cultures treated with FdU. This was the case for cultures stained for non-specific esterase and/or with Wright's stain.

In neuronal-MP cocultures, neurons and MPs stain with almost identical intensity with a combined non-specific esterase and Wright's stain. Apparently, neurons contain high levels of esterase activity (some of which is

probably acetylcholine esterase). Because Wright's stain has some of the same components as Nissl stain, the Nissl substance is probably being highlighted. MPs and neurons also stain similarly with a combined Bodian-Nissl stain (Gardner et al. 1984). Both neurons and MPs are metallophilic cells and stain with the metals present in the Bodian. In view of the apparent similar chemical and biochemical compositions of neurons and MPs, it would be difficult to stain any of these cell types preferentially using histochemical staining methods. Preferential staining would have to be carried out utilizing immunohistochemical techniques (specific marker antibodies).

When the CNS is damaged either by ischemia, concussion, compression, freeze lesion, surgical disruption, or high power laser irradiation, MPs infiltrate in large numbers along with neutrophils (Beck et al. 1983, Lampert et al. 1966, Means and Anderson 1983, Oehmichen 1978). In view of the fact that MPs respond *in vivo* to CNS trauma caused by laser irradiation (Lampert et al. 1966), it should be no surprise that in the *in vitro* culture and laser cell surgery system used in the present study, transection or damage of a neuronal process attracted MPs to the damaged process and soma (see also Gardner and Gross 1983).

The attraction of MPs to a lesion site was approximated by a logarithmic relationship between the distance of a MP from a lesion and it's initial time of response. However, these response data contained a large amount of scatter, some of which was probably due to a number of factors, such as 1) recruitment of additional MPs by MPs that had already responded to the lesion, 2) convection currents setup in the medium by the heater plate, 3) movement of the medium casued by the CO_2 stream, and 4) movement of the objective during scanning of the culture. Therefore, any further analysis of these data (as in determination of diffusion coefficients for chemotactic factors) would not be justified.

It is unknown what attracts the phagocytes (neutrophils and MPs) to damage sites but it has been shown that lipid peroxides are primary chemotactic agents (Hertz and Cloarec 1984, Means and Anderson 1983, Payan et al. 1984, Piper 1983, Rouveix et al. 1983, Samuelsson 1983), collect in the CNS after injury (Bazan et al. 1983, Moskowitz et al. 1984, Sun et al. 1983, Willmore and Rubin 1984, Wolfe and Pappius 1983), and are a main cause of nervous tissue damage (Beyer-Mears et al. 1984, Bhattacharyya et al. 1984, Demopoulos et al. 1980, Demopoulos et al. 1979, Demopoulos et al. 1977, Faden 1983, Petito and Pulsinelli 1984, Semchenko et al. 1983, Siejo et al. 1980).

A hypothesis can be proposed linking laser damage at 337nm with lipid peroxidation and hence with a plausible chemotactic factor, one conceivably small enough to be released in cases of process pinching as well as transection. If a chemotactic factor is being produced it could stimulate the migration of and phagocytosis by MPs (Fig. 14).

Two molecules that are prevalent in the cell and have absorption maxima near 337nm are NADH and NADPH (absorption maxima-340nm (Lehninger 1975)). Other substances which are not present in high concentrations, such as serotonin, also absorb near this wavelength. A two-photon absorption for NADH has been demonstrated by a number of investigators (Berns and Salet 1972, Passarella et al. 1982, Rounds et al. 1966). NAPH and NADPH are concentrated in the mitochondria, which are disrupted following laser irradiation (Gross et al. 1983). The disruption of mitochondria by laser microbeam irradiation in vitro resembles (on an ultrastructural level) that caused by impact injury of spinal cord (Clendenon and Allen 1979) and that of isolated mitochondria in the presence of reduced glutathione or Fe^{2+} (Slater 1972). NADPH and NADH in a higher energy state could transfer energy to electron transport system (ETS) proteins and cause run-away free radical reactions which in turn could

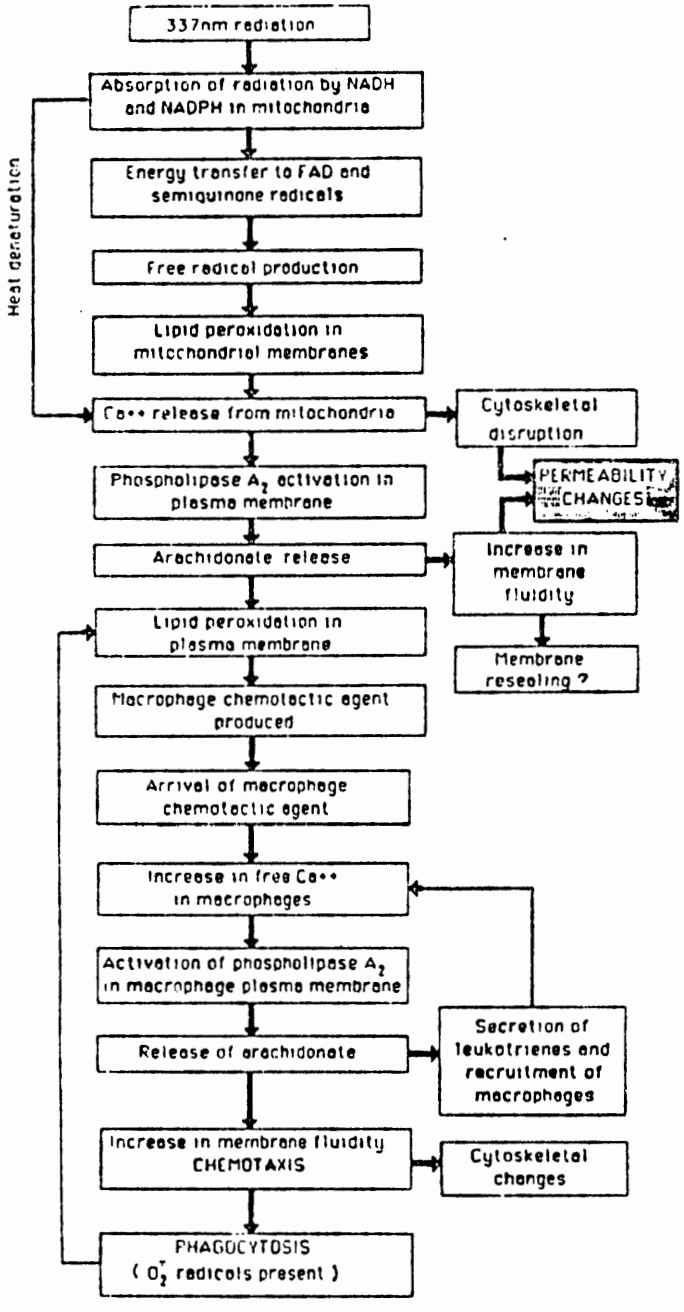


Figure 14. Flow chart of putative events leading to process transection and macrophage chemotaxis. Permeability changes can lead to process transection as this allows for a massive influx of extracellular calcium which will cause collapse of the cytoskeleton.

produce lipid free radicals and cause lipid peroxidation or cause lipid free radical production directly. NADPH can induce lipid peroxidation in a number of cases (Bhattacharyya et al. 1983, McCay et al. 1976, Slater 1972). Auto-oxidation of ETS components may occur as well (Demopoulos et al. 1980).

Lipid peroxidation may have the effect of severely damaging mitochondrial membranes and causing Ca^{2+} release (Vercesi 1984). Increase in intracellular Ca^{2+} has been shown to cause activation of phospholipase A_2 (Ninnemann 1984a, Yawo and Kuno 1983) and consequent release of arachidonic acid (DeMedio et al. 1983, Means and Anderson 1983, Oldstone 1982, Pappius and Wolfe 1983, Yawo and Kuno 1983), a major component of neuronal membrane phospholipids (Bhattacherjee and Eakins 1984). Arachidonic acid is readily oxidized by enzymatic and non-enzymatic pathways into lipid peroxides and other free radical products (Demopoulos et al. 1979, Demopoulos et al. 1977, Wolfe 1981, Yoshida et al. 1982). [See Appendix Fig. 2 and 3 for reactions.]

This fatty acid release has been described by some authors as a prelude to membrane resealing after transection (Yawo and Kuno 1983). However, if lipid peroxidation occurs, further damage can occur to the membrane

and the peroxides themselves are chemotactic (Payan et al. 1984, Turner and Tainer 1983, Turner and Lynn 1978). This will attract neighboring phagocytes (Fernández-Repollet et al. 1982, Means and Anderson 1983, Turner and Lynn 1978) (Fig. 14). The chemotactic factor produced may be LTB_4, which is a very potent chemotactic factor for neutrophils (Goetzl et al. 1983) but there is debate on whether or not it is chemotactic for MPs (Payan et al. 1984, Samuelsson 1983). It has been demonstrated that some arachidonate metabolites are chemotactic for MPs (Rouveix et al. 1983) and stimulate phagocytosis (Fernández-Repollet et al. 1982). This hypothesis would be difficult to test as the very reactions that are believed to function in membrane resealing are basically the same as those required for MP movement (Hirata and Axelrod 1980, Hirata et al. 1979) and the generation of the oxidative burst (Bromberg and Pick 1983, Janco and English 1983). To inhibit arachidonic acid release in one system would prevent the activation of the other.

In my original hypothesis, in which MPs were proposed to hinder the survival of neurons, it seemed plausible that when the MPs attacked and began phagocytosis, the superoxides and peroxides produced by the MPs themselves would have the ability to potentiate free radical lipid peroxidation of the membrane. This would have effectively

prevented resealing and recovery of the neuron. However, in view of the fact that MPs have been shown to enhance neuronal survival after injury, this mechanism of potentiating lipid peroxidation appears not to be operating. MPs do appear to play a significant role in CNS trauma but not as killer cells.

In traumatized CNS tissue, large numbers of phagocytes are present (Kerns and Nierzwicki 1981, Kitamura et al. 1972, Means and Anderson 1983, Oehmichen 1978, Walker 1963). Some of these clearly function in the removal of dead and dying cells and debris (Beck et al. 1983, Oehmichen 1983). As stated in the results, this appears to be the case in my model system as well. MPs do clean up a culture, removing dead cells and engulfing debris. However, I have also shown that the presence of MPs significantly enhance the survival of neurons with transected neurites at approximately 30% at both the 50 and 100μm lesion distances (Fig. 13). This highly significant enhancement of neuronal survival came as a great surprise, as it was shown in previous experiments that MPs actively attack and phagocytize at transection sites. In MP-neuronal cocultures, there appeared to be no correlation between cell survival and MP-proximal neurite contact. This impression is based primarily on 24 hour observations. However, it is possible that such contact

was made earlier and missed by the experimental protocol. It should also be recalled that in the Internal Control Studies, where one side received only MP conditioned medium, direct contact with MPs was not possible yet enhancement of neuronal survival was observed (refer to Fig. 12). This could be of medical importance if the mechanism of this enhancement is elucidated.

It is possible that MPs are in some way mediating the resealing of transected neurites. MPs are known to be highly secretory cells secreting a wide range of arachidonic acid metabolites and other substances (Adams and Hamilton 1984, Adams et al. 1983, Beck et al. 1983, Koyama et al. 1982, Moskowitz et al. 1984, Ninnemann 1984b, Rogers et al. 1984, Scott et al. 1982, Takemura and Werb 1984, Unanue 1983). In view of this fact and the results of experiments carried out in plates with MPs only on one side but with continuous medium during surgery (Fig. 12), a hypothesis can be made that MPs mediate neuronal recovery via a secreted "factor".

Some of these MP secreted substances may affect and perhaps enhance the ability of neurons to reseal. It is known that some of the leukotrienes can modulate nerve excitability in both the CNS and PNS (Gazelius et al. 1984) and one, LTB_4, can act as a calcium ionophore for some cells (Serhan et al. 1983). However, excessive

calcium loading by neurons leads to cell death (Simon et al. 1984). This factor, therefore, is not believed to be leukotriene because, as stated above, some LTs actually may lead to cell death. The LTs are also relatively unstable. Prostaglandins can not be ruled out as MPs do produce significant quantities of PGE_2 and 6-Keto $PGF_{1\alpha}$ and the PGs are more stable. MPs also produce a large number of proteins and the probability exists that the "survival factor" may indeed be a protein. At this time, the mechanism of this enhanced survival remains to be elucidated but these studies are currently being carried out. Initial data from experiments utilizing co-culture conditioned medium show only control levels of survival (33% of injured neurons at the 50μm lesion distance). If a soluble factor is involved in enhanced neuronal survival it is possible that a critical concentration of the substance is needed for the effect. It must also be remembered, that MPs produce a wide variety of substances, some of which are very labile. It is conceivable that the "factor" may have a short halflife or could be easily oxidized by the simple act of pipetting the medium. If no soluble factor is found, other mechanisms such as a lowered Ca^{2+} level in the medium, must be considered. MPs require Ca^{2+} for many of their activities and may remove

enough to effectively reduce the Ca^{2+} concentration in the medium. Low Ca^{2+} has the effect of maintaining the cytoskeletal structure of cells and could conceivably enhance the survival of neurons. Another possible influence on neuronal survival may be osmolarity changes caused by MPs within the culture. However, the addition of $2X10^6$ MPs to a neuronal culture does not create a measurable change in osmolarity. As a consequence osmolarity may be excluded as a pertinent parameter.

In summary, exogenous macrophages have been shown to coexist with neurons and glia in primary mouse spinal cord cultures without causing any overt damage. However, when neurites of neurons are injured by laser cell surgery, MPs are attracted to the lesion by the release of a chemotactic substance which may be an arachidonic acid metabolite. MPs have been shown to actively engage in phagocytosis of proximal and distal segments of transected neurites. Despite these observations, which would lead to the conclusion that MPs would decrease the chances for survival of injured neurons, a significant enhancement of survival was found. This enhancement of neuronal survival may be to a soluble "factor" produced by MPs, may be caused by a MP-induced lowering of medium Ca^{2+} concentrations, or by some other mechanism.

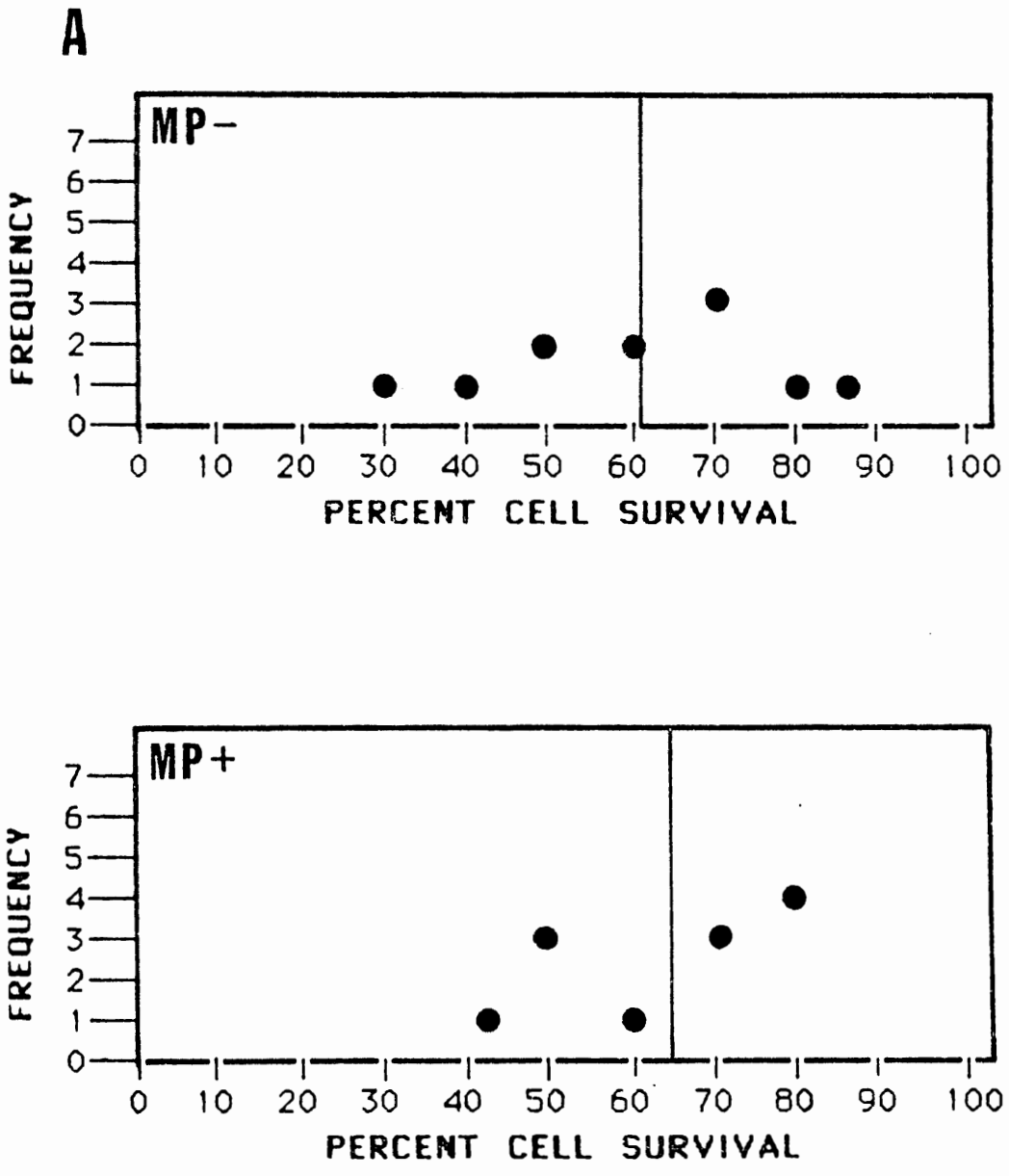
APPENDIX

Appendix Figure 1. Plots of Normally Distributed Data- Long-Term Survival Studies. For the Student's t test to be used as a main statistical procedure, data must be normally distributed about the mean. Much of the data collected from the long-term survival does fit this parameter. On the following graphs, frequency is defined as the number of observations at a given percentage. The bars indicate the means of the data.

A. Internal Control Studies- MP- data have the appearance of the beginnings of a Gaussian curve while the MP+ data do not. However, additional observations would probably lead to the development of a Gaussian curve.

B. External Control Studies- 50μm lesion distance. Both sets of data appear to be normally distributed about the mean.

C. External Control Studies- 100μm lesion distance. MP- data are normally distributed. MP+ data have the appearance of half of a Gaussian curve. As there can be no observations greater than 100% neuronal survival, the other half of the curve does not exist.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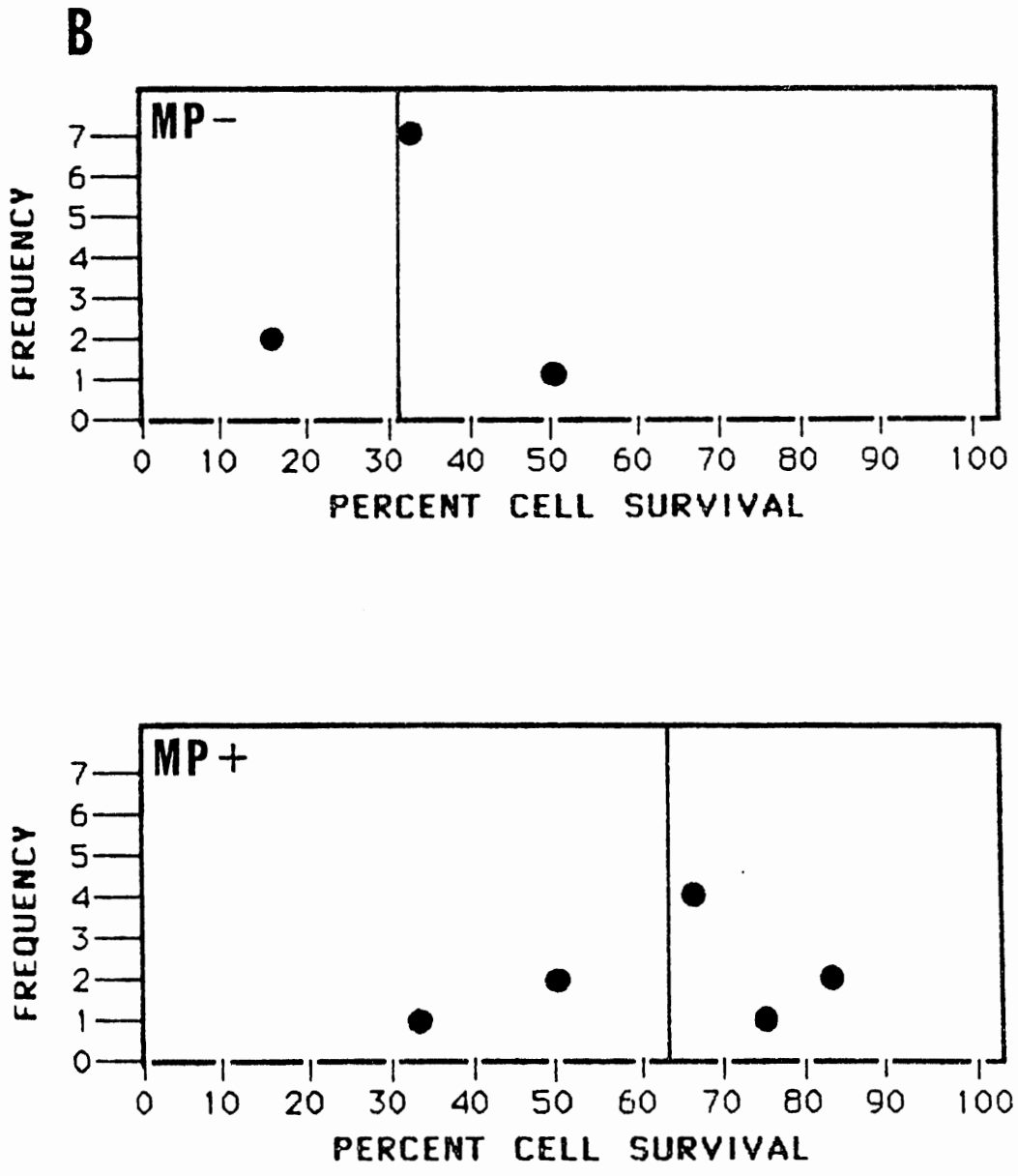
PERCENT CELL SURVIVAL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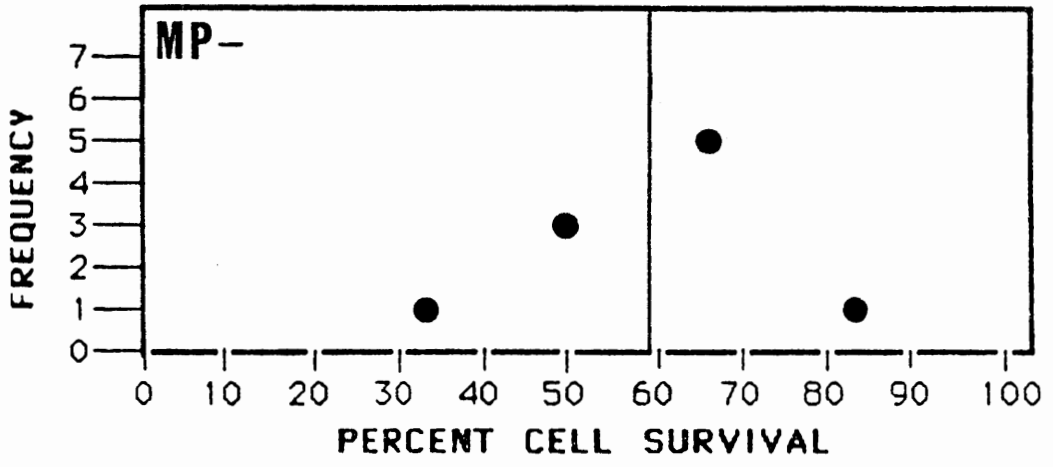
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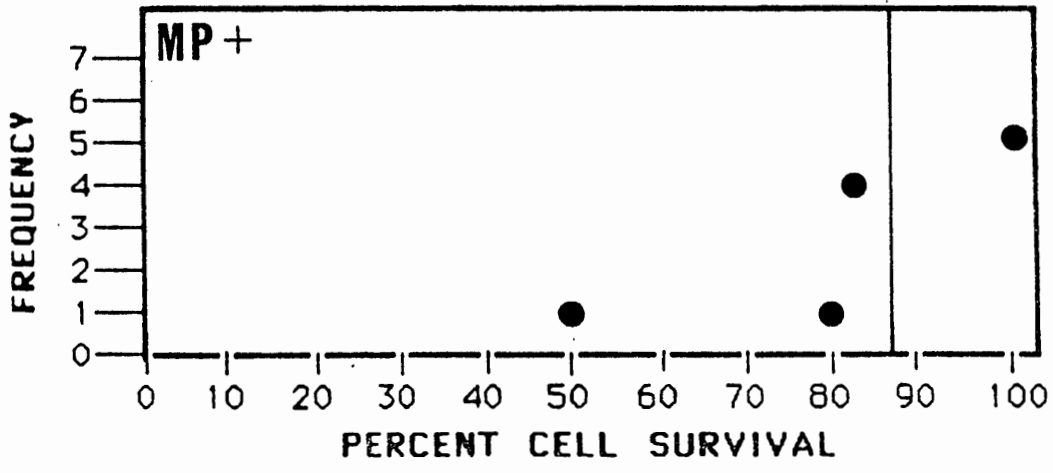
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Appendix Figure 2. Pathways of Lipid Free Radical Damage

Eq. 1: The initial step in the initiation and formation of metastable intermediary products. Part of a fatty acid chain containing a pair of double bonds can react with X·, a free radical, which is usually molecular oxygen or the hydroxyl radical.

Eq. 2 and Eq. 3: A radical center forms adjacent to carbon that is near to carbons with double bonds. The free radical has abstracted a hydrogen and is no longer a radical. There will be immediate configurational changes caused by massive electron shifts.

Eq. 4: Oxygen adds via free radical reactions to cause peroxide formation.

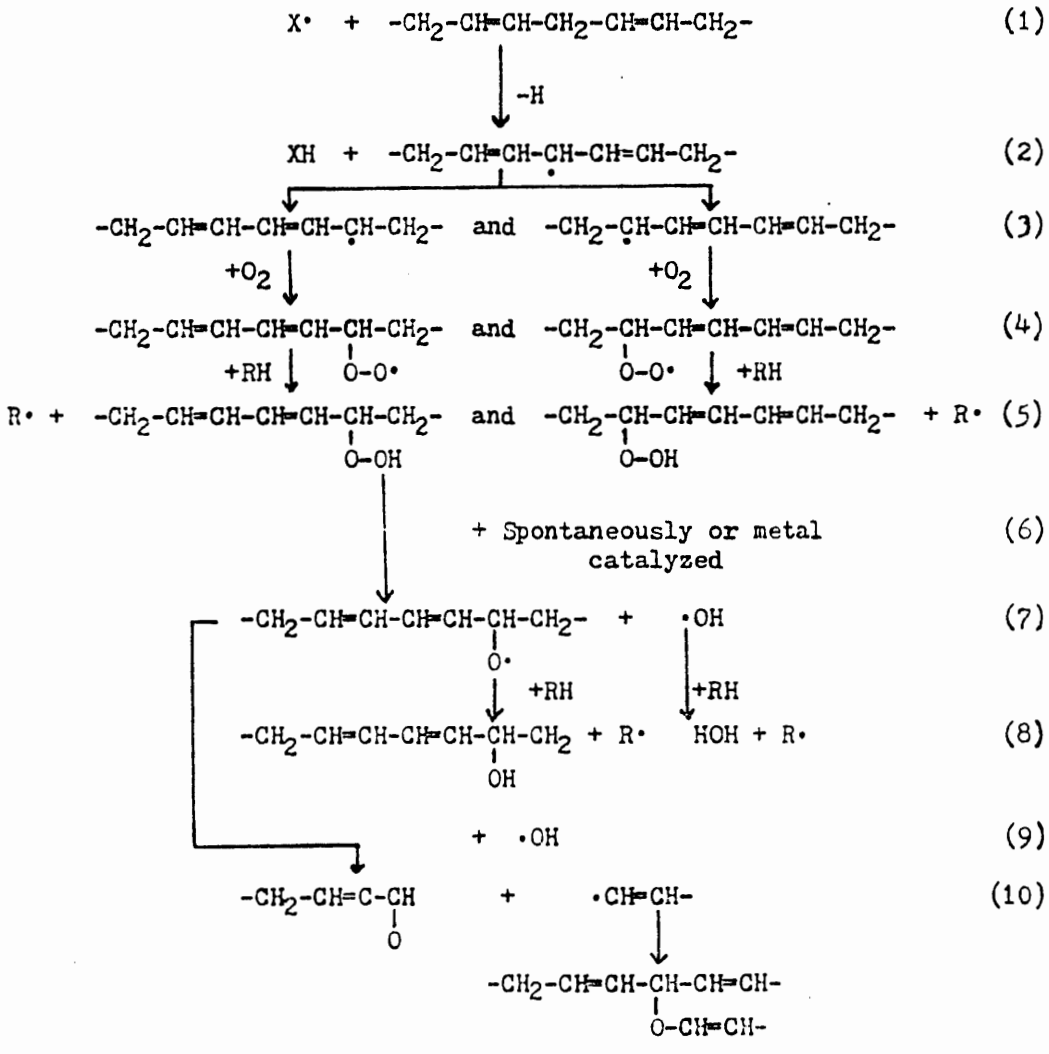
Eq. 5: Peroxy radicals can abstract hydrogen from other nearby molecules forming an unstable hydroperoxide.

Eq. 6 and Eq. 7: A hydroperoxide can break up spontaneously to form hydroxyl radicals and oxygen centered radicals on the lipid (alkoxy radicals).

Eq. 8: Alkoxy and hydroxy radicals can be terminated by abstracting a hydrogen from other molecules but these molecules in turn become free radicals.

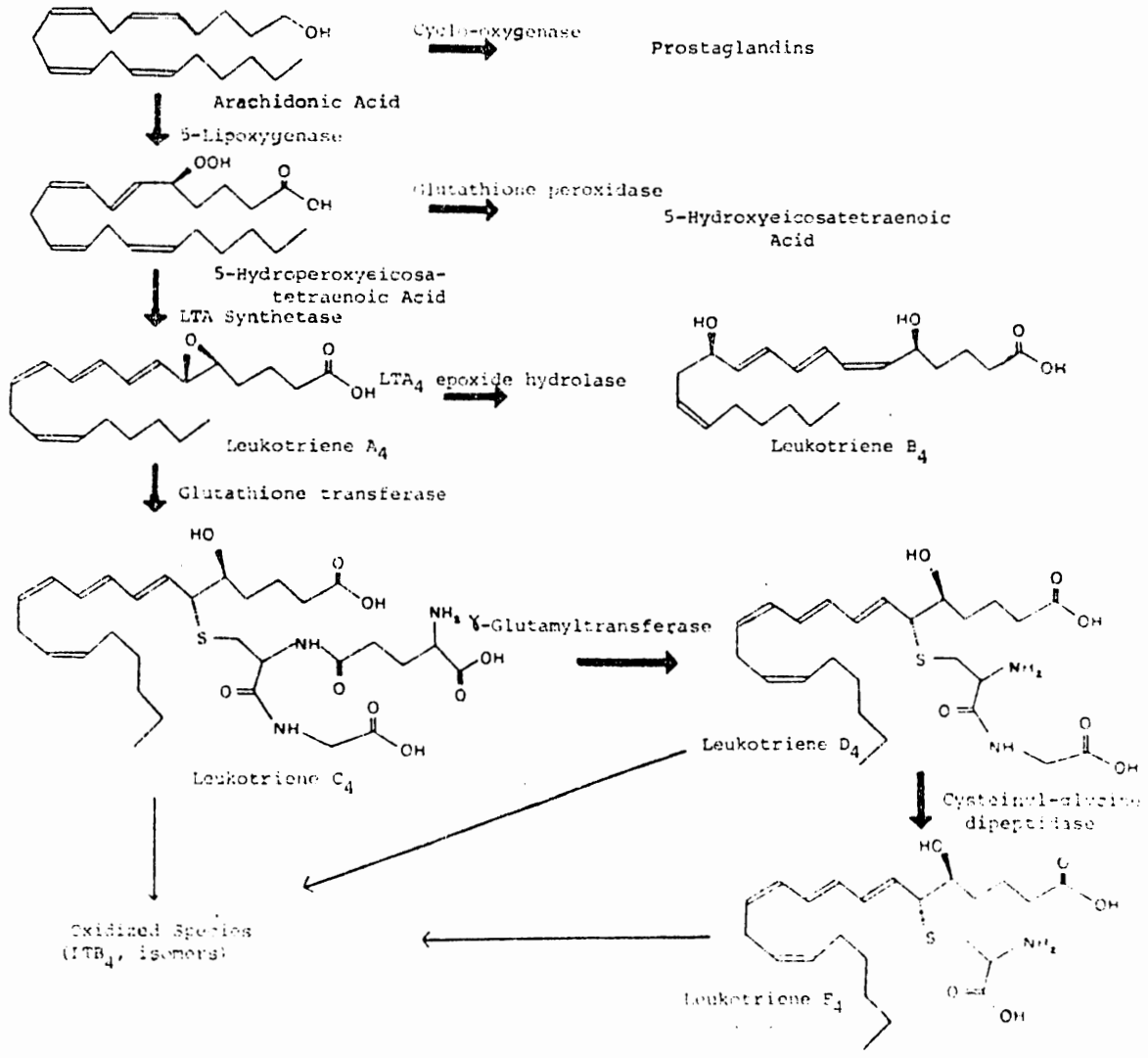
Eq. 9: An alkoxy radical can be oxidized and fragmented if free radical attacks continue on other carbons that are adjacent to the double bonds. This results in the formation of bis-hydroperoxides, fragmentation of the fatty acid and formation of aldehydes and alkyl radical fragments.

Eq. 10: Alkyl radicals can react with other radicals and terminate in an oxygen-linked bridge.



(Adapted from Demopoulos et al. 1979)

Appendix Figure 2. Pathways of Lipid Free Radical Damage



(Adapted from Taylor and Morris 1983)

Appendix Figure 3. Arachidonic acid cascade via lipoxygenase pathway leading to formation of leukotrienes. The leukotrienes have been found to be potent chemotactic agents (Turner and Tainer 1983). They are also implicated in allergy reactions, inflammatory responses, and may also act as neuromodulators.

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