Macrophages direct process elongation from adult frog motorneurons in culture

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SUMMARY

Motorneurons and macrophages have been isolated and identified in primary cultures from adult frog (Rana pipiens) spinal cord. Time-lapse video microscopy revealed that during the first two weeks migrating macrophages contact the growth cones of motorneurons. As they continue to migrate, the motorneuron processes elongate in close association with the moving macrophages. Elongating motorneuron processes are thereby brought into contact with other motorneurons and networks are formed. At later stages, the macrophages die but the motorneurons and the networks survive for at least another two weeks. These experiments show that macrophages can promote a directed elongation of motorneuron processes and suggest that they play a similar role during regeneration in vivo.

1. INTRODUCTION

One of the salient features following injury to the peripheral nervous system is that the damaged nerve fibres regenerate and reach their original targets (Letinsky et al. 1976). After damage of the peripheral nerve, the Schwann cells of the nerve tube proliferate and partake in the removal of axons and myelin sheaths in cooperation with macrophages that are recruited to the sites of nerve injury (Nathaniel & Pease 1963; Holtzman & Novikoff 1965; Baichwal et al. 1988). A signal that triggers this Schwann cell proliferation has been shown to originate in the macrophages but the relative roles of Schwann cells and macrophages in the removal of cellular debris and in promoting axon regeneration are not clear. In the central nervous system repair and regeneration following injury are less well understood (Varon & Lundborg 1983). Although macrophages proliferate in the brain during naturally occurring cell death (Perry et al. 1985; Mollgard & Saunders 1986), their recruitment to sites of injury in the central nervous system is sparse (Perry et al. 1987). Nevertheless, a neurotrophic role of brain macrophages has been suggested (Mallat et al. 1989).

In this paper we show that in primary cultures of cells isolated from adult frog spinal cord, macrophages interact with growth cones of identified motorneurons to bring about an elongation of these processes leading to the development of motorneuron networks.

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2. MATERIALS AND METHODS

On the day before the cultures were started, male frogs Rana pipiens, were placed in water containing 50 µg ml⁻¹ gentamycin. The frogs were anaesthetized on ice, the brain rostral to the medulla pithed, and the frog decapitated. In medium at 4 °C, the spinal cord from the obex to T2 was isolated under the dissecting microscope, the meninges removed and the dorsal horn area cut away. The remaining cord was sectioned into 1 mm cubes with a razor blade and washed three times in fresh medium at 4 °C. The pieces of cord were incubated in a mixture of collagenase-dispase (0.1 units ml⁻¹ collagenase, 0.5 units ml⁻¹ dispase (Boehringer-Mannheim) in Leibovitz-15 medium (L-15) (Gibco)) on a rocking table for 1 h at room temperature. The pieces were allowed to settle for 5 min and the supernatant removed. One millilitre of fresh medium was added and the cord gently triturated 20× through a 5 ml pipette. The pieces were allowed to settle and the supernatant collected into a 15 ml Falcon centrifuge tube. Additional 8–10 trituration cycles were done with a 1 ml Gilson-pipette. The supernatants were centrifuged at 100 g for 10 min at 10 °C and the pellet resuspended in medium. The cells were plated at a density of 2-5×10⁵ cells per milliliter onto sterile coverslips (13 mm diameter round or 20 mm x 20 mm) treated with the appropriate substrate. The round coverslips were attached with Sylgard (Dow Corning) to the bottom of 35 mm Falcon petri dishes with a 12 mm hole drilled out (Hawrot & Patterson 1979). The square coverslips were placed into 35 mm culture dishes. The culture dishes were sealed with parafilm and maintained in an incubator at 27 °C.

The spinal cord cells were cultured in L-15 without l-glutamine, with an adjusted osmolality of 240 mOsm; 2 (77%, i-15), at pH 7.2, containing 0.25% Garamycin (Gibco) and 1% foetal calf serum.

One osmole contains one mole of osmotically active particles.
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Figure 1. (a–c) Cell identification. Motorneurons in culture were identified by their fluorescence following in vivo retrograde labelling with the fluorescent dye Dil. Two motorneurons, 13 d in culture, under phase optics (a) and fluorescence illumination (b). Macrophages were identified by their ability to phagocytize Zymosan granules, (c) A macrophage in culture for 4 d; Zymosan granules are seen as dots within the vacuoles. The second cell (arrowhead) in (c) is a motorneuron. (c–d) Development of macrophage networks. (d) For the first 2 d macrophages had a ‘fried-egg’ appearance and were not in contact with one another (arrowheads); interspersed between the macrophages were motorneurons with short processes (arrows) and unidentified cells (asterisk); (e) migrating macrophages formed confluent cell clusters and extended processes over each other as well as over the substrate on the dish (arrowheads); two motorneurons (arrows) were located on the macrophages and extended processes (5 d in culture). (f–h) Motorneurons and their networks remain after the disappearance of macrophages. (f) Initially motorneurons (black arrows) and their processes (white arrows) were associated with macrophages (10 d); (g) with time, and loss of the macrophages, the motorneuron networks became increasingly evident (note the presence of only three macrophages); (h) by 16 d, and after the disappearance of macrophages, virtually pure motorneuron networks remained. Bar = 50 μm. Denervated muscle extract substrate.

Muscle extracts were prepared from cutaneous pectoris muscles. For denervated muscle extracts the brachial nerve was cut 3 d before preparation of the extract. Extracts were made by using a modification of the procedure described by Dohrmann et al. (1986). After dissection of the muscle from its attachments, it was frozen at −70 °C. The tissue was then thawed and homogenized in four volumes of 10 mM Tris-HCl, pH 7.4, containing (in millimoles per litre): 1 EDTA; 2 N-ethylmaleimide and 2 phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 23000 g for 1 h at 4 °C. The supernatant was dialysed overnight at 4 °C against 0.1 M PBS or 0.15 M Sorenson buffer, sterile-filtered and stored.
at -20 °C. The glass coverslips in the culture dishes were incubated with the denervated muscle extract for 1 h, washed twice with distilled water and then with medium.

Cultures were viewed on an inverted microscope at magnifications of 25 x and 40 x. Two systems were used for collecting time-lapse images. The first was a Panasonic TV camera connected to a JVC professional video cassette recorder. One frame was recorded every 20 s. The second system was an image analysis system (Image 1/AT, Universal Imaging Corporation, Media, Pennsylvania). Images were collected with a silicon intensified target camera (Model 66, Dage-MTI Inc., Michigan City, Indiana). Each image consisted of an average of 128 frames. The averaged image was sharpened with a simple convolution kernel, a background image subtracted to correct for uneven illumination, and the result stored on a laser disk recorder (Panasonic TQ 2028 F). One processed image was recorded every 20 s.

Macrophages were identified by their morphology (Giulian & Baker 1986; Jordan & Thomas 1988) and their ability to phagocytose trypan blue (Bloom & Fawcett 1968) and Zymosan (Giulian & Baker 1986). Cultures were incubated with trypan blue (Amimed) (0.4% solution diluted 5-fold with medium) for 5-15 min, washed with medium and then examined. Other cultures were incubated with Zymosan (35 x 10^6 particles per dish) (Sigma) for 40 min to 3 h at 27 °C in L-15 containing 10% foetal calf serum. The cultures were then washed several times with medium and the cells examined for the presence of phagocytized Zymosan. Counts were made of all labelled macrophages in each culture.

Motorneurons were retrogradely labelled in situ, with the lipophilic dye Dil (1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate, Molecular Probes Inc., Eugene, Oregon) (Honig & Hume 1986; Kuffler 1990). Briefly, crystals of Dil dissolved in 100% ethanol were placed on the proximal cut-end of the brachial nerve where it exits from the spinal cord (Kuffler 1990). Examination of sections of brachial cord under fluorescence illumination one to six weeks after dye application, showed a high yield of intensely labelled cell bodies and their processes in the ventral horn. These labelled cells were considered to be motorneurons. Upon dissociation a high yield of viable and intensely labelled cell bodies was recovered.

3. RESULTS

At the time of plating, one population of dissociated cells had an intense uniform fluorescence of their cell body plasma membrane. These cells were similar in size and labelling to motorneurons in vivo; in this paper they are referred to as motorneurons. Two days after plating, motorneurons made up approximately 65% of the total cell population. In addition to the labelled cells, there was a variety of unlabelled unidentified cell types. Figure 1 a, b shows, with both phase optics and fluorescence illumination respectively, a labelled motorneuron in culture for 13 d. A Zymosan labelled (with the Zymosan in the vacuoles of the macrophage) macrophage is shown in figure 1 c.

The labelled macrophages assumed a variety of shapes. Within a few hours of plating, they took on a round 'fried egg' appearance with diameters of 30-100 μm. The centres of the cells had many vacuoles surrounding a dense nucleus. The cell cytoplasm, peripheral to the central core, appeared granular and contained vacuoles (figure 1 d). These cells resemble activated macrophages described by Giulian & Baker (1986).

By day 3 in culture the macrophages took on a fan-like shape, became motile and migrated around the dish. With time, macrophages became increasingly vacuolated and crenated. Based on their morphology, macrophages could be identified in the absence of uptake markers.

Measurements were made in three cultures of the total numbers of macrophages with time after plating on denervated muscle extract. Between days 1 and 4 the number of macrophages increased approximately 40-fold. After day 4, the numbers decreased until they had virtually disappeared by day 14-16 (figure 2). Similar changes in macrophage number were seen when the cells were plated on other substrates (including innervated muscle extract, mouse tumour-laminin and polylysine, data not shown). Despite the disappearance of the macrophages, other cells including motorneurons survived for more than four weeks.

Between 2-3 d when the number of macrophages rapidly increased, the macrophages began to migrate with their fan-like edges leading the direction of migration. When migrating macrophages contacted one another, they separated but maintained contact by extending processes between each other over distances of more than 200 μm (figure 1 e, f). With time-lapse microscopy such interactions were seen in 42 out of 59 (71%) contacts between macrophages. As the macrophages migrated and contacted each other, networks developed. Macrophages also formed confluent cell clusters (figure 1 e).

During the period of macrophage network formation and clustering, identified motorneurons extended short processes that became incorporated into both the macrophage networks and their confluent cell clusters. Motorneuron cell bodies were also seen located on the macrophages with processes extended over the surface of the macrophages as well as onto the substrate of the dish (figure 1 e, f). These observations show that macrophages provide a substrate that is permissive for motorneuron adhesion and process elongation.

In time-lapse microscopy, motorneuron processes were seen to elongate in association with migrating macrophages. In all cases (n = 28) in which a macrophage contacted the growth cone of a motorneuron process and continued its migration, the motorneuron process elongated in association with the

![Figure 2. Number of macrophages from three cultures plotted against time when plated on denervated muscle extract.](http://rsbp.royalsocietypublishing.org)
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Figure 3. Elongation of motorneuron processes by a macrophage recorded with time-lapse microscopy. (a) Two Dil-labelled motorneurons under fluorescence illumination; (b) same field as (a) with phase illumination. Macrophage (arrowhead) in contact with the two fluorescently labelled motorneurons shown in (a); the macrophage is also in contact with the process of a third motorneuron whose cell body is out of the field at the top of the frame. (c–d) With migration of the macrophage (in direction of arrow) the processes of the motorneurons elongated. Time between (a) and (d) is 3 h 10 min. Denervated muscle extract substrate. Bar = 40 μm.

macrophage by as much as 200 μm (figure 3b–d). Migrating macrophages always led the direction of elongation with the motorneuron growth cones in apparent contact with the trailing edge of the macrophage.

When a migrating macrophage and an elongating motorneuron process contacted another macrophage or motorneuron, connections formed (figure 1f). The macrophage, initially in contact with the motorneuron process, could then migrate away from the newly established contact point leaving the other cells in contact. In this way, networks of motorneurons formed (figure 1g). With time and the loss of macrophages, the motorneuron networks became increasingly evident. By day 16, when most of the macrophages had disappeared, motorneuron networks still remained and survived for at least an additional two weeks (figure 1h).

When migrating macrophages contacted motorneurons at sites other than the growth cone, no elongation was seen (32 of 32 contacts). These results show that the motorneuron growth cones have sites for macrophage–motorneuron interactions.

4. DISCUSSION

We have shown that adult frog motorneurons identified by retrograde labelling (Kuffler 1990), survive and extend processes in culture for at least four weeks. We have also identified a second cell type in these cultures as macrophages. It cannot be ruled out that the 'macrophages' are phagocytic microglia; however, because of their uptake of specific markers we refer to them as macrophages. The origin of the macrophages in the cultures is not known. They may arise from resident macrophages, microglia, blood-borne monocytes or other precursor cells in the spinal cord at the time of dissociation.

The sites at which these macrophage–motorneuron interactions occur are restricted to the growth cones. Contact by macrophages with motorneurons at sites other than the growth cones, do not cause process elongation. Several mechanisms can be proposed by which macrophages elongate motorneuron processes. First, it is known that elongation requires lipid for membrane biosynthesis. A direct source of such lipids for the cultured motorneurons is the debris of dissociated axon membranes and myelin remaining in the culture medium after plating. However, macrophages are known to phagocytize axons and myelin and secrete apolipoprotein-E. This secretion of apolipoprotein-E by macrophages in close association with motorneuron growth cones would provide them with a high concentration of a readily usable form of lipid. Further, apolipoprotein-E receptors have been shown to be present on the tips of growing neuronal processes (Ignatius et al. 1987; Boyles et al. 1989). These two observations taken together have given rise to the proposal that macrophages may facilitate the uptake of lipid by the tips of the neuronal processes by secretion of apolipoprotein-E (for review, see Mahley (1988)). In addition, Ignatius et al. (1987) have suggested that macrophages play a role in redistribution of lipid during axon regeneration.

An alternative mechanism for elongation may result from the ability of macrophages to secrete extracellular matrix proteins and cell adhesion molecules such as fibronectin, chondroitin sulfate proteoglycans, a gela-
tin-binding protein as well as a variety of growth and synergistic factors (Giulian & Baker 1983; Nathan 1987).

Pure motorneurons have not yet been cultured in the absence of macrophages. Our observations do not rule out that motorneuron axons in vivo regenerate in the absence of macrophages; nor do they exclude other mechanisms that might be involved in promoting or directing axonal regeneration, such as release of diffusible tropic and trophic factors by denervated axonal targets (Kuffler 1989) or the synthesis of adhesion molecules by cells of the denervated nerve tube. The present results do (i) show that in culture macrophages direct the elongation of motorneuron processes and (ii) provide support for the hypothesis that macrophages promote axonal outgrowth in vivo.

We are grateful to Dr J. G. Nicholls for his constructive advice and encouragement throughout the course of this work and for making facilities available to S.D.E. during his sabbatical leave. Thanks are due to Dr W. Adams for valuable assistance with the time-lapse recording experiments and suggestions on the manuscript; Dr K. Muller for advice on the manuscript and Dr M. Westerfield for introducing the photographic assistance, and Joes Wittker and Rosanne Baettig for providing the mouse tumor-laminin, Dr W. Halfter for Boehringer Ingelheim Fonds fellowship to B.S.

Weiss Cell Research Grant DDAL03 90-G-0189 to D.K., by a Swiss National Science Foundation Grant 3.516.86 and application of the dye Dil to us; Dr M. Paulson for kindly advice and encouragement throughout the course of this study; Dr K. Muller for advice and encouragement throughout the course of this study; Dr M. Paulson for kindly

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