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TRANSFORMATION OF LEECH MICROGLIAL CELL MORPHOLOGY AND PROPERTIES FOLLOWING CO-CULTURE WITH INJURED CENTRAL NERVOUS SYSTEM TISSUE

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Summary

When the leech central nervous system (CNS) is injured, microglial cells migrate to the site of the lesion. It is possible that the injured CNS releases diffusible substances that alter the properties of microglial cells; to investigate this, microglial cells were cultured in the presence of injured or uninjured CNS tissue. Grown on Concanavalin A (Con-A), 75 % of microglial cells are rounded in shape and are avoided by growing neurites. However, when chains of leech ganglia with damaged connectives were cultured on Con-A next to microglial cells, many of the microglial cells changed their morphology. The number of rounded cells present decreased to 48 %, 4 % became spindle-shaped and 48 % had an intermediate form. In addition, the presence of crushed ganglionic chains allowed more growth of neurites across microglial cells than occurred under control conditions, although round-shaped microglia were still avoided by growing neurites. Similar changes in microglial cells were produced in cells plated on Con-A in the presence of conditioned medium from crushed ganglionic chains. Hence, a diffusible substance from injured CNS tissue caused the morphology of the microglial cells plated on Con-A to become more like that of microglia plated on laminin, on which only 22 % of the cells are rounded while the remainder are spindle-shaped and are readily crossed by neurites. Changes in morphology were not observed when microglial cells were cultured with frozen and crushed ganglionic chains or with uncrushed chains. These experiments demonstrate that substances released from damaged leech CNS cause microglial cells plated on Con-A to change their morphology and the way in which they interact with growing neurites.

Key words: axon sprouting, nerve regeneration, glia, trophic factor, leech, *Hirudo medicinalis.*

Introduction

In the leech, regeneration after injury depends on extrinsic factors (see von Bernhardi and Muller, 1995) that can enhance or inhibit axon growth. Such factors include extracellular matrix (ECM) molecules, cell adhesion molecules and growth factors in the environment. Extrinsic factors can be provided by non-neuronal cells, such as microglial cells (Morgese et al., 1983; McGlade-McCulloh et al., 1989). Leech microglia, which are macrophage-like cells resident in the central nervous system (CNS), resemble vertebrate microglia in their morphology (Coggeshall and Fawcett, 1964; Elliott and Muller, 1981). They are elongated and are evenly distributed along the connectives and in the ganglia. In culture, microglial cells show different properties depending on the nature of the surface on which they are plated (Masuda-Nakagawa et al., 1994). On a substratum rich in laminin, microglia are spindle-shaped, motile and are contacted by growing neurites (Masuda-Nakagawa et al., 1994). On Concanavalin A (Con-A), microglia are rounded, immobile and are avoided by neurites, which collapse after contacting the microglia (Chiquet and Nicholls, 1987).

After injury to the leech CNS, microglial cells rapidly accumulate at the lesion site, where they play a role in repair. Microglial cells have been show to phagocytose debris (Ling, 1981; Perry and Gordon, 1988), to synthesise laminin and to deposit extracellular matrix components (Masuda-Nakagawa et al., 1994). This accumulation of microglia in the leech resembles the invasion of haemocytes observed at sites of injury in the cockroach CNS (Treherne et al., 1984; Smith et al., 1987). Isolated mammalian microglia undergo transformations in response to cytokines and other factors (Suzumura et al., 1991) and are themselves are able to secrete soluble growth factors (Frei et al., 1987; Banati et al., 1993; Elkabes et al., 1996). In the present experiments, microglial cells were plated on Con-A and were exposed to injured ganglion chains or to medium conditioned by injured CNS. The morphological responses of the microglial cells were

examined together with their interactions with growing neurites.

Materials and methods

Cell culture

Leeches (*Hirudo medicinalis*) were obtained from Ricarimpex (Audenge, France). Neurones were isolated as previously described (Dietzel et al., 1986). In brief, after enzymatic digestion with 2 mg ml^{-1} collagenase/dispase in Leibowitz 15 medium (L-15; Gibco), single Retzius and Anterior Pagoda (AP) neurones were removed from the ganglia by suction with a fire-polished glass micropipette. Alternatively, ganglionic chains were washed with sterile medium, and clumps of cells were prepared by disrupting the ganglia with fine forceps. Both the isolated cells and the cell clumps were plated in Lab Tek dishes (Nunc, Inc.) coated with Con-A or laminin-rich extracellular matrix extract (ECM; as described by Masuda-Nakagawa et al., 1988) and covered with L-15 supplemented with 6 mg ml^{-1} glucose, 2 mmol l^{-1} glutamine and 0.1 mg ml^{-1} Gentamycin for 1–5 days at 20 °C. Cells were then fixed for 30 min in 4 % paraformaldehyde in phosphate buffer, pH 7.4, and washed four times for 10 min each with phosphate-buffered saline (PBS). Cell nuclei were labelled using Hoechst 33258 dye, at a concentration of 10 mg ml^{-1} in PBS for 10 min, after which the cells were covered with 50 % PBS/50 % glycerol. The only cellular elements in the desheathed ganglion are the neuronal cell bodies, the large glial cells of the neuropile and the microglia (Coggeshall and Fawcett, 1964). By using cells from desheathed ganglia, we were able to obtain populations of microglial cells to plate them with the cultured neurones (Kai-Kai and Pentreath, 1981). That these cells are true microglia has been shown in numerous experiments (Masuda-Nakagawa et al., 1990, 1993, 1994; Morgese et al., 1983; McGlade-McCulloh et al., 1989). They have been shown to migrate after injury, to stain characteristically and to synthesize laminin, and they display the distinctive morphology of microglia in other animals. In addition, these cells were described as ‘microglia’ in the leech by del Rio Hortega (see Masuda-Nakagawa et al., 1993), who first coined the term microglia.

Co-culture of microglial cells with injured ganglionic chains or conditioned media

Chains of ganglia were dissected from leeches under sterile conditions. For the co-culture experiments, all connectives were crushed at several places with forceps (Dumont no. 5). To prepare conditioned medium, ganglia with crushed connectives were cultured in supplemented L-15 medium (100 µl per ganglionic chain) for 48 h. Conditioned medium was filtered through a membrane (0.2 µm pore diameter) with a low affinity for proteins before being added to the microglial cultures. Crushed connectives or conditioned medium were added to the microglial cell cultures either at the time when cells were plated or 2 days later. In control experiments, microglial cells were co-cultured with uncrushed or frozen crushed chains of ganglia. Conditioned media were prepared as above using either uncrushed or crushed and frozen ganglionic chains. Preparations were viewed by phase contrast and differential interference contrast microscopy and photographed under an Olympus microscope with a 40× objective. Microglial cell morphology was categorised as (i) rounded (broad lamellipodia completely surrounding the cell body), (ii) intermediate (microglial cells with a defined long axis, but still with lamellipodia partially surrounding the cell body) or (iii) spindle-shaped (elongated cells with two or more thick processes arising from a cell body devoid of lamellipodia).

Assay for the interaction of growing neurites with microglia

To test for interactions between neurones and microglia, Retzius cells, AP cells and other neurones were plated on 2-day-old microglial cell cultures. Cultures were observed by phase contrast microscopy with a Leitz inverted microscope and kept until neurites sprouted (2–5 days). Cultures were fixed and stained with Hoechst dye as previously described. Three types of neurite–microglial interactions were arbitrarily defined: (i) neurites that avoided a microglial cell, collapsed or grew around the microglia without contacting it; (ii) neurites that contacted a microglial cell, touched it or grew a short distance over or under it (less than one-third of the cell diameter); and (iii) neurites that crossed a microglial cell and grew over or under it.

Statistical analysis

The results were analysed with contingency tables and calculated χ^2 values. Differences were analysed using a Mann–Whitney *U*-test. A Kolmogorov–Smirnov test was used to compare the distribution of microglial cell morphologies under the different experimental conditions. Spearman rank order correlation was used to evaluate the dependence of morphology on the number of cells plated.

Results

Changes in microglial cell morphology after exposure to injured ganglionic chains

Earlier experiments by Masuda-Nakagawa et al. (1994) have shown that microglial cells in culture adopt distinctive shapes when plated on the plant lectin Concanavalin A (Con-A) and on laminin. To provide a basis for assessing changes in morphology, quantitative estimates of the various forms of the cells on the two substrata were made by scoring the morphology of 8000 microglial cells from 21 separate experiments. On Con-A, 75 % of microglial cells were rounded and immobile (Fig. 1A; Table 1), 25 % were intermediate in form, and spindle-shaped cells were absent. In contrast, the proportions of cells showing these morphologies on laminin-rich substrata were as follows: 64 % of the cells were intermediate in form, 14 % were spindle-shaped and mobile, and only 22 % were rounded (Fig. 1B; Table 1).

When microglial cells plated on Con-A were exposed to

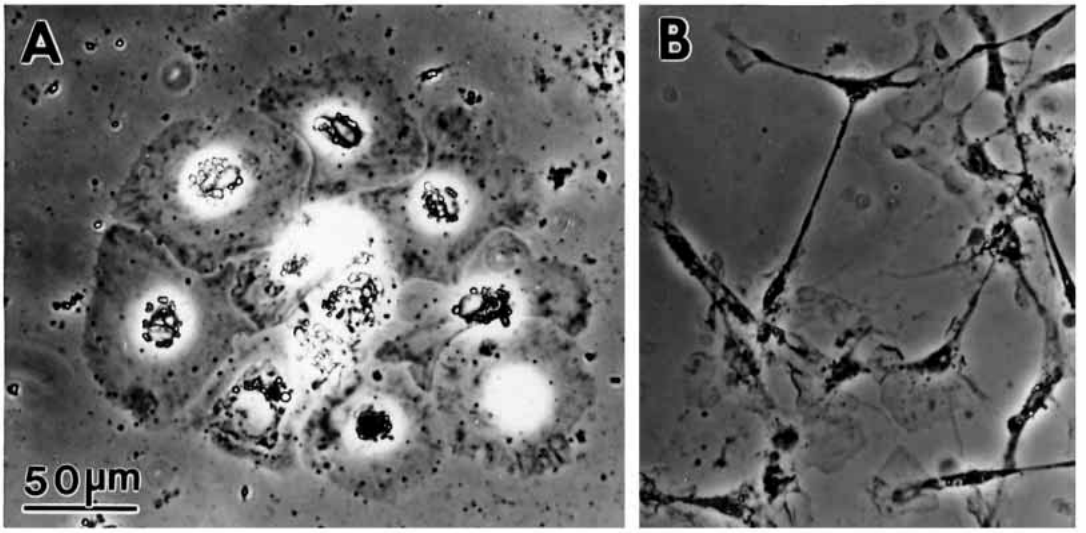

Fig. 1. Microglial cells had different shapes on different substrata. Distribution of cell morphologies and phase contrast micrographs of microglial cells plated on Con-A and on a laminin-rich substratum for 2.5 days. (A) On Con-A, most cells were rounded and showed abundant lamellipodia. (B) On a laminin-rich extracellular matrix extract, microglial cells were elongated and lamellipodia were virtually absent. These results confirm those reported by Masuda-Nakagawa et al. (1994).

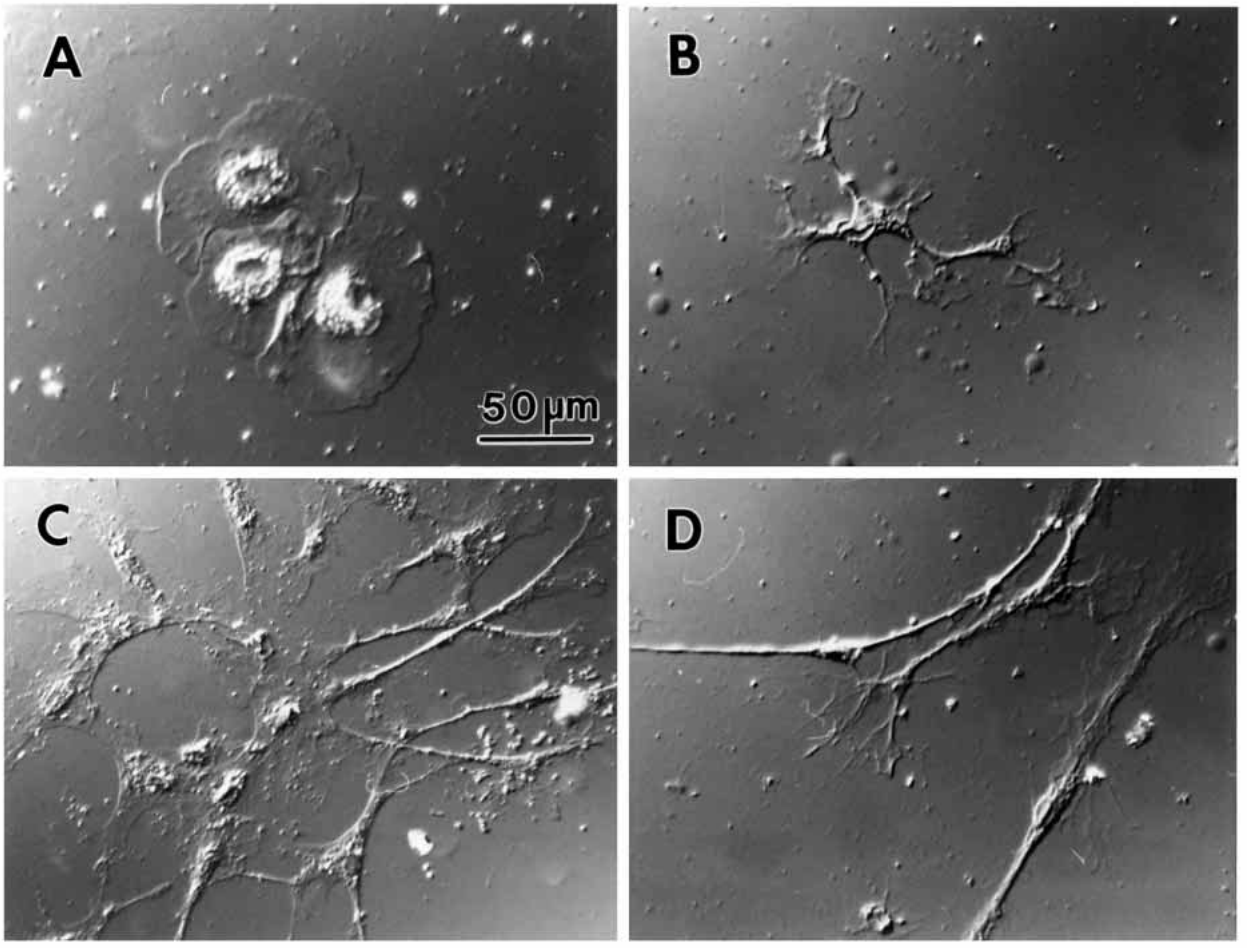

Fig. 2. The shapes of microglial cells were influenced by co-culture for 2 days with injured chains of ganglia. Microglial cells were plated on Con-A in the presence or absence of injured nerve cords for 2 days. On Con-A, 75% of microglia were rounded (A). In the presence of injured nerve cords, over 50% of the cells plated on Con-A became intermediate (B,C) or spindle-shaped (D). The results in Figs 1 and 2 were representative of those obtained from an analysis of 8000 microglial cells in 21 experiments.

chains of ganglia with crushed connectives, elongated microglia became more abundant and presented longer processes (Fig. 2; Table 1). Only 48 %, compared with 75 %, remained rounded. The changes in the numbers of rounded, intermediate and spindle morphologies on Con-A were highly significant in the presence of crushed ganglionic chains ($P<0.001$). Microglial morphology also changed when cells were exposed to conditioned medium prepared from crushed CNS. Significantly more (36 % compared with 25 %) of the microglial cells became intermediate in shape ($P<0.001$). The changes were, however, less marked than those for cells exposed directly to crushed CNS, and spindle-shaped cells were absent. Tests were made to investigate whether the morphological changes in microglia exposed to crushed connectives were the result of changes in the shape of each individual cell or whether they were due to selection of a particular population since it seemed possible that intermediate- or spindle-shaped microglial cells might survive better than rounded ones in the presence of injured ganglia. Individual microglial cells plated on Con-A were observed for several days in culture. After exposure to injured CNS, cells that were originally rounded became elongated; 1 day after removal of the injured ganglionic chain from the culture dish, the cells began to round up again.

Control experiments were performed with crushed and frozen or with uncrushed CNS material. Such cultures showed no significant changes in morphology ($P>0.05$) compared with cells plated on Con-A (Table 1). In other control experiments, microglia were plated on Con-A and cultured for different periods (1–5 days). Such cells also showed no progressive change in morphology. Similarly, increasing the density of microglial cells in culture had no effect on cell shape. On laminin-rich substrata, where cells are already elongated, damaged ganglionic chains induced no changes in microglial morphology.

Interactions of microglia with growing neurites after exposure to injured ganglionic chains

When microglial cells are cultured on Con-A, neurites growing from neuronal cells in co-culture avoid them, grow around them or collapse (see Masuda-Nakagawa et al., 1994). In contrast, microglial cells plated onto laminin-rich ECM do not repel neurite outgrowth: neurites readily cross spindle- and intermediate-shaped microglia. After microglial cells plated on Con-A had been exposed to injured CNS material, cells that remained rounded were still avoided by neurites (Figs 3, 4). There was, however, a significant difference in the response of

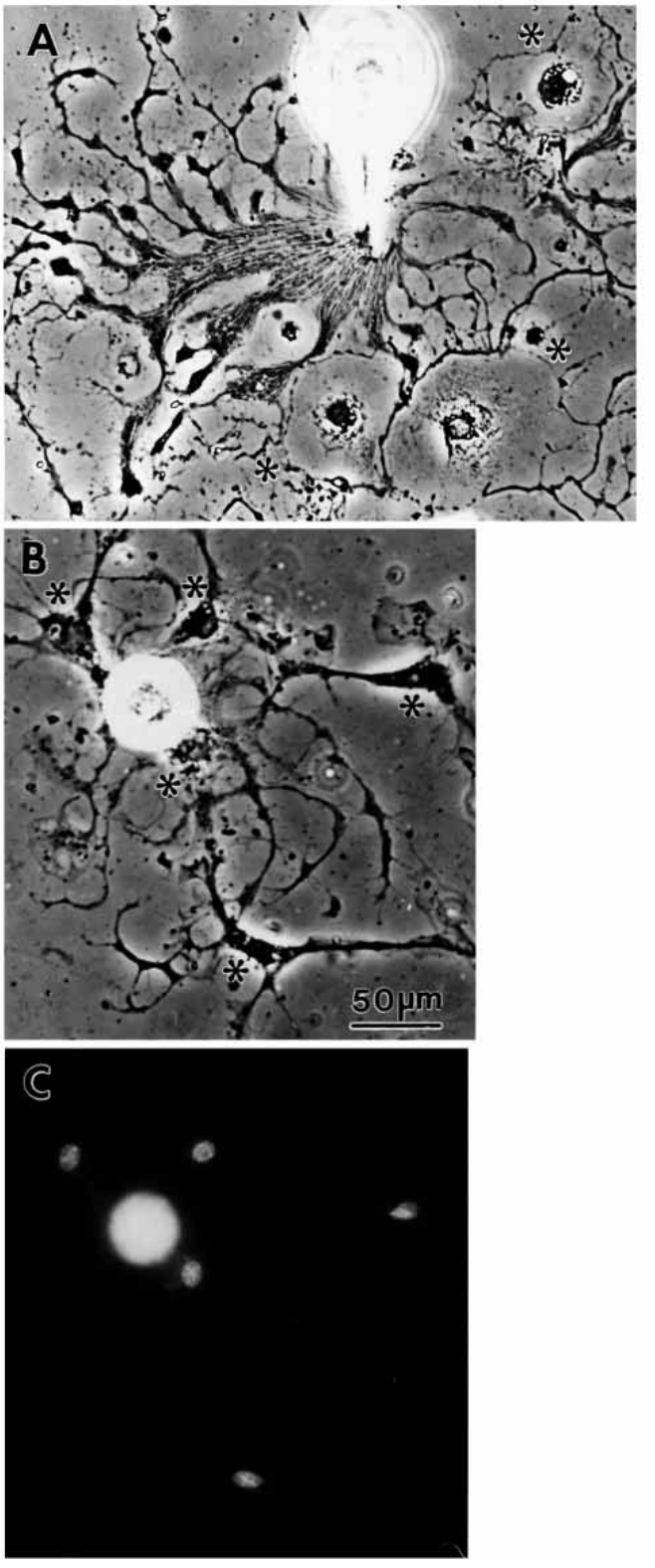

Fig. 3. Altered interactions between microglial cells and neurites in the presence of injured central nervous sytem (CNS) tissue. Phase-contrast micrographs of neurones and microglial cells plated on Con-A for 3 days. (A) Neurites avoid crossing the rounded microglial cells and grew around the edge of the lamellipodia. Asterisks show lamellipodia that are not crossed by neurites. (B) When cells are co-cultured with injured leech CNS, intermediate-shaped cells (asterisks) are crossed by growing neurites. (C) A fluorescent image of the cells shown in B showing five microglial cell nuclei stained with Hoechst dye 33258 visible near the neurone. The processes of these microglial cells were thicker, shorter and darker than those of neurones. Microglial and neuronal interactions such as those in this figure were analysed in 17 experiments in which 998 individual neurones were cultured with microglial cells.

Table 1. *Effects of crushed central nervous system tissue on microglial cells*

Culture conditions	Shape (% of total)		
	Rounded	Intermediate	Spindle
Control: Con-A substratum	75±1.3	25±1.5	0.05±0.04
Crushed CNS: Con-A substratum	48±1.5	48±1.7	4±0.9
Uncrushed or frozen CNS: Con-A substratum	73±1.7	26±1.8	1±0.2
ECM-laminin substratum	22±2.3	64±6.9	14±3.7

Results were obtained from 20–47 cultures containing 1000–7000 microglial cells for each condition.

Values are means ± S.E.M.

Con-A, Concanavalin A; CNS, central nervous system; ECM, extracellular matrix.

neurites to intermediate-shaped microglia. In the presence of crushed connectives, elongated microglial cells were crossed by neurites twice as often as were elongated cells in controls (P<0.005). Fig. 4 shows neurites growing from a single identified neurone; where these neurites encountered a microglial cell of intermediate shape, they crossed it (to the left of the arrowhead), while other neurites avoided the two round-shaped microglial cells (asterisks). Conditioned medium from injured leech CNS produced similar effects. No change in behaviour of growing neurites was observed when microglial cells plated on ECM were exposed to damaged ganglionic chains.

Discussion

Microglial cells are known to present characteristic morphologies on different substrata (Masuda-Nakagawa et al., 1994). Microglial cells plated on Con-A and exposed to injured chains of ganglia changed their morphology from rounded to more elongated shapes. This change appeared to depend on a diffusible factor since the morphological changes occurred when medium conditioned with injured chains of ganglia was used. The cells resumed a rounded shape after injured ganglionic chains had been removed from the culture system. This result is in accord with the observation that the transformation of mammalian microglial cells in response to cytokines and other soluble factors is reversible (Suzumura et al., 1991).

Rounded microglial cells were avoided by neurites, but intermediate-shaped cells were readily crossed by them, indicating that, as a result of their exposure to injured chains of ganglia, the inhibitory effect of the microglial cells on neurite growth had changed. Laminin stimulates neurite outgrowth *in vitro* (Bixby and Harris, 1991) and acts as a permissive substratum for growing leech axons (Chiquet et al., 1988; Masuda-Nakagawa et al., 1988). Unlike those plated on Con-A, microglial cells plated on laminin were elongated and readily crossed by growing neurites unless injured tissue was present.

Following injury to the leech CNS, microglial cells accumulate at the site of injury *in vivo*. Laminin-specific antibody labelling appears in advance of regenerating fibres, and since microglial cells accumulate before the laminin, it is possible that they are the source of the laminin (Masuda-Nakagawa et al., 1993). *In vitro*, anti-laminin antibodies label the surface of leech microglia (Masuda-Nakagawa et al., 1994; M. D. Neely and R. von Bernhardi, unpublished observations), and *in situ* hybridisation has shown that microglial cells express leech laminin RNA (Luebke et al., 1993).

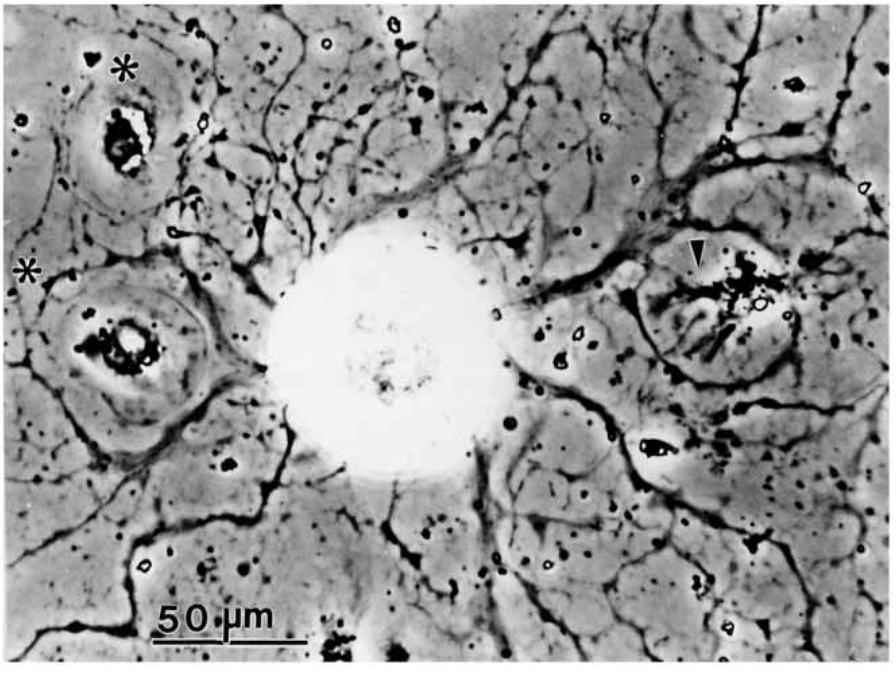

Fig. 4. Single neurones interact with microglial cells in a different manner depending on their shape. Phase-contrast micrographs of a neurone and microglia plated on Con-A for 2.5 days and co-cultured with injured leech ganglionic chains. Neurites were broad, curved and paler than microglial processes. An intermediate-shaped cell (arrowhead, right) with thick processes was contacted and crossed by growing neurites (see region close to arrowhead). Two rounded microglia that were devoid of processes were not crossed by neurites (asterisks, left). A clear space was apparent between the neurite and the lower of the two rounded microglial cells. Interactions of this sort in which neurites of a single neurone approached both rounded and elongated microglial cells were seen on nine occasions.

Microglial cells are implicated in a number of repair processes: they guide regenerating nerve fibres (Fernández and Fernández, 1974; McGlade-McCulloh et al., 1989; Wallace et al., 1977) and ensheath axon bundles, they take the place of the giant connective glial cell when it is destroyed by injection of proteases (Elliot and Muller, 1981, 1983a,b), they produce surface molecules that influence neurite outgrowth (Masuda-Nakagawa et al., 1993, 1994; Elkabes et al., 1996) and they phagocytose cell debris (Ling, 1981; Perry and Gordon, 1988). Our results indicate that the different roles and properties displayed by microglial cells may depend on how they are activated by signals coming from a lesion. One can speculate that microglial cells acquire growth-promoting properties after damage and switch these off after completion of sprouting and regeneration when they are no longer needed. Leech neurones and microglial cells offer considerable advantages for demonstrating these phenomena, but the isolation and purification of the molecules secreted by the damaged tissue remain key problems in analysing the cellular and molecular mechanisms by which microglia are activated.

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