Journal of Cell Science 103, 571-579 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

Manuscript no.: JCS7720

Axonal growth on astrocytes is not inhibited by oligodendrocytes

JAMES W. FAWCETT*, N. FERSHT, L. HOUSDEN

Physiological Laboratory, Downing Street, Cambridge CB2 3EG, England

M. SCHACHNER and P. PESHEVA

Department of Neurobiology, ETH-Hönggerberg, HPM, CH 8093 Zürich, Switzerland

*Author for correspondence

Summary

Axon growth *in vitro* may be inhibited by contact with oligodendrocytes, but most axons grow readily on the surface of astrocyte monolayers. Since both cell types are in close contact with one another in the damaged nervous system, we have examined the growth of axons on cultures which contain both astrocytes and oligodendrocytes. Cultures derived from neonatal rat forebrain develop with a monolayer of large flat astrocytes attached to the culture dish, and with many smaller cells of the oligodendrocyte lineage on their surface. Dorsal root ganglia placed on these cultures grow axons readily, the overall extent of growth being unaffected by the presence or absence of oligodendrocytes, many of which express galactocerebroside and the inhibitory molecule janusin. A previous set of experiments had shown that growth of these axons is inhibited by oligodendrocytes by themselves. Scanning electron microscopy coupled with silver-intensified immunostaining reveals that the axons grow on the surface of the astrocytic layer, underneath the oligodendrocytes, and are therefore in contact with both cell types as they grow. The presence of astrocytes therefore alters the results of axonal contact with oligodendrocytes.

Key words: axon growth, axon regeneration, astrocyte, oligodendrocyte, growth cone.

Introduction

Axons fail to regenerate in the mature mammalian central nervous system (CNS) in part because the CNS environment is inhospitable to axon growth. The environment surrounding damaged CNS axons is made up primarily of astrocytes interspersed with a variable quantity of oligodendrocytes; either or both of these two cell types must therefore be responsible for blocking axon growth (Carlstedt, 1985; Liuzzi and Lasek, 1987; Reier and Houle, 1988; Patterson, 1988). There is evidence from in vitro experiments that both astrocytes and oligodendrocytes can be inhibitory to axon growth. Oligodendrocytes have inhibitory effects on axon growth cones, and possess two types of inhibitory molecule on their surface, IN35 and J1 160/180 (janusin) (Schwab, 1990; Fawcett et al. 1989b; Pesheva et al. 1989a; Pesheva et al. 1992; Morganti et al. 1990). Astrocyte tissues are penetrated with difficulty by axons, although astrocyte monolayers are a surface on which many types of axons grow readily (Fawcett et al. 1989a; Fawcett and Housden, 1990). From in vivo experiments there is also evidence which implicates both astrocytes and oligodendrocytes in the prevention of axon regeneration.

In regions of damaged brain, oligodendrocytes are always found accompanied by astrocytes. Tissue culture studies of axon-glial interactions, however, have focused on purified populations of the two cell types. Axon growth cone behaviour can be radically changed by contact with different environments and different cell types (Tosney and Landmesser, 1985; Mattson et al. 1988; Cox et al. 1990; Keynes et al. 1990; Argiro et al. 1984; Kater and Guthrie, 1990; Johnson et al. 1991). We have therefore examined the growth of postnatal dorsal root ganglion axons, which we have previously shown to be inhibited by oligodendrocytes (Fawcett et al. 1989b), in mixed cultures of astrocytes and oligodendrocytes to see whether their interactions with one cell type are altered by the presence of the other cell type. We have done this by growing axons on cultures derived from newborn rat forebrain, which contain both astrocytes and oligodendrocytes; the type 1 astrocytes forming a monolayer on the culture surface, and the cells of the oligodendrocyte lineage growing on top of them. We examined cultures with light and scanning electron microscopy to show overall axon growth characteristics, and also the precise anatomical interactions between the various cell types. A preliminary report of this work has been published (Fawcett et al. 1987).

Methods and methods

Mixed glial cultures

Two-day-old rat pups were anaesthetized with ether, their brains

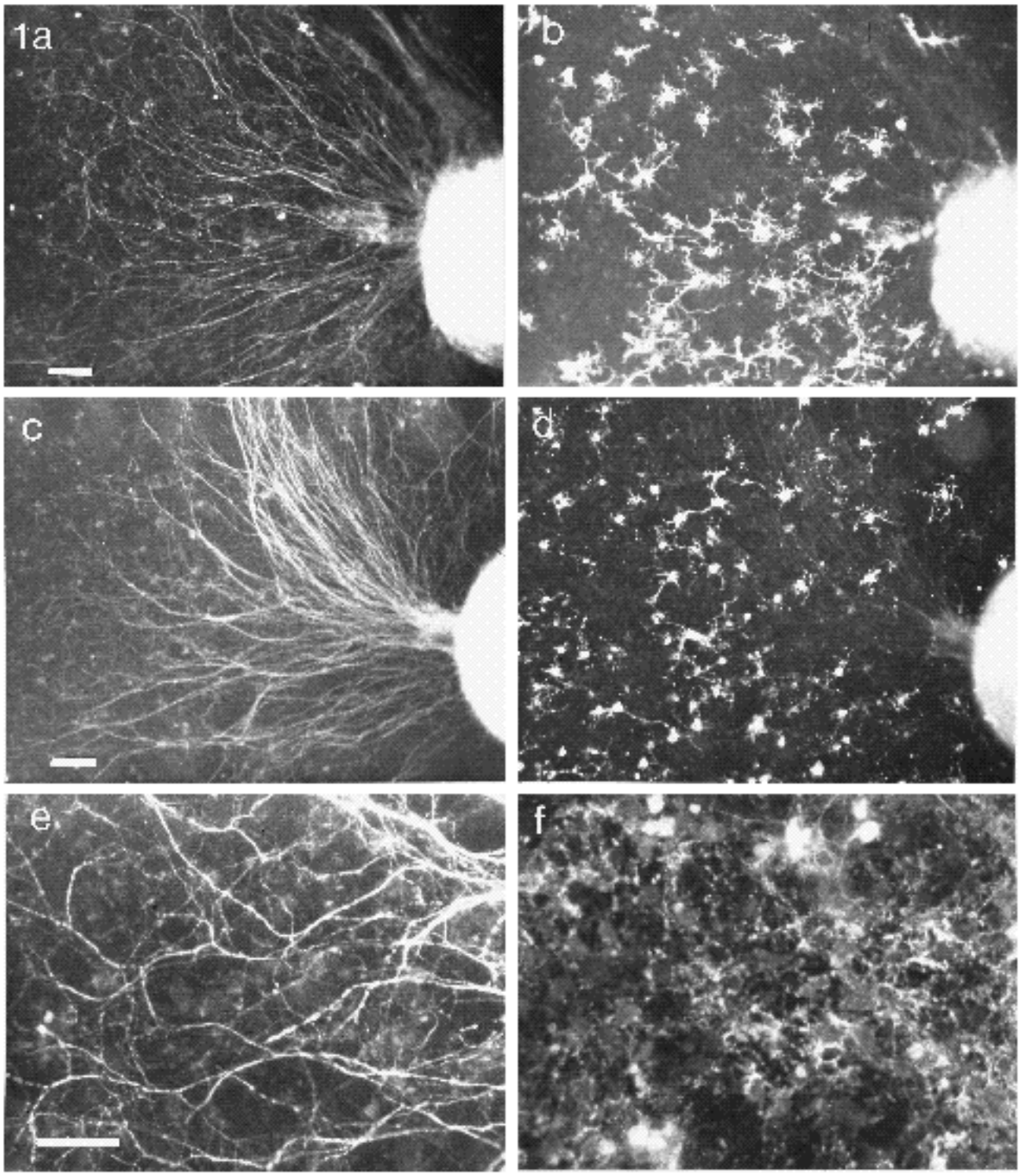

Fig. 1. Axons, stained with anti-L1 growing from dorsal root ganglia over the surface of mixed glial cultures (a,c,e). Oligodendrocytes in the same cultures are stained with anti-galactocerebroside (b,d) or anti-janusin (f). Many instances can be seen in which axons have passed directly through the images of oligodendrocytes without stopping or deviating. Anti-galactocerebroside stains oligodendrocyte cell bodies and processes, while janusin is mostly present on the periphery of the cells. A larger number of cells stain for janusin than for galactocerebroside, so the janusin-stained culture is almost completely covered in stained oligodendrocyte processes. Bar, 100 μm.

removed into Hanks' balanced salt solution (HBSS), demembranated and then chopped. The tissue was incubated with 0.1% porcine trypsin (Sigma T0134) for 20 minutes, then DNase (20 μg/ml, Sigma D5025) added, the tissue spun down and resuspended in triturating solution (300 mg BSA, 50 mg soyabean trypsin inhibitor (Sigma T9003), 1 mg DNase (Sigma D5025) per

100 ml PBS) and triturated with flamed glass Pasteur pipettes. Medium (10%FCS in DMEM) was added, the suspension left for 15 minutes for undissociated tissue to settle out, and then the cells were plated onto collagen coated 22 mm glass coverslips or aclar squares in 35 mm wells, 1 brain to 10 wells. Some cultures were made on aclar sheets inserted into 25 cm^2 culture flasks. After 7 to 10 days these cultures were shaken overnight to deplete them of oligodendrocytes (McCarthy and de Vellis, 1980), then the astrocyte-coated aclar sheets were removed, cut into squares, and used for axonal growth assays.

Dorsal root ganglia (DRGs)

DRGs were dissected from 2-day-old rat pups into HBSS. Glial cultures on coverslips were placed into 35 mm dishes, and NGF-containing (Sigma N6009, 10 ng/ml) medium was added until it just covered the surface of the culture. DRGs were placed on the glial cultures, held in place by surface tension overnight, then more medium was added on the following day.

Immunohistochemistry

Cultures for fluorescence microscopy: cultures were washed with phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde for 20 minutes, then blocked with 5% goat serum in PBS for 30 minutes. Primary antibodies, anti-janusin monoclonal or polyclonal (Pesheva et al. 1989b; Pesheva et al. 1992), anti-galactocerebroside monoclonal (Ranscht et al. 1982), anti-L1 polyclonal or monoclonal (Lemmon et al. 1989; Rathjen et al. 1987), were diluted in 1% goat serum in PBS, and applied for 2 hours, followed by fluoresceine-labelled goat anti-rabbit and biotinylated goat anti-mouse (Caltag) for 30 minutes and tetramethylrhodamine-labelled streptavidin for 30 minutes.

Cultures for scanning electron microscopy

Cultures on aclar were washed in PBS, fixed in 1% paraformaldehyde for 10 minutes, blocked with 5% goat serum in PBS for 30 minutes, then incubated with primary antibody (as above) for 2 hours, followed by biotinylated anti-mouse and anti-rabbit secondaries (Caltag) for 30 minutes. This was followed by goat anti-biotin tagged with 1 nm gold particles, after which the cultures were post-fixed for 2 hours in 2% glutaraldehyde in PBS. The gold stain was silver-intensified using a kit from Biocell, the end-point being when silver staining of axons and/or oligodendrocytes was visible under light microscopy (see Fig. 2). Cultures were then critical point dried, and carbon coated. Some cultures which were not immunostained were fixed in 2% glutaraldehyde, critical point dried and gold coated. The specimens were examined in a Jeol J35 microscope, surface structure being observed using the secondary electron detector, and silver staining using the backscattered electron detector (Taylor et al. 1984).

Analysis of axon growth

The length of outgrowth of axons from dorsal root ganglia after 2 days growth was quantified by measuring the length of the 5 longest axons and averaging them together. The density of galactocerebroside- or janusin-labelled oligodendrocytes in the immediate vicinity of the axons growing from each DRG was estimated by counting the number of cells in a 500 μm × 500 μm square placed over the longest axons.

Results

Growth of mixed glial cultures

During the first days of culture large numbers of neuronal

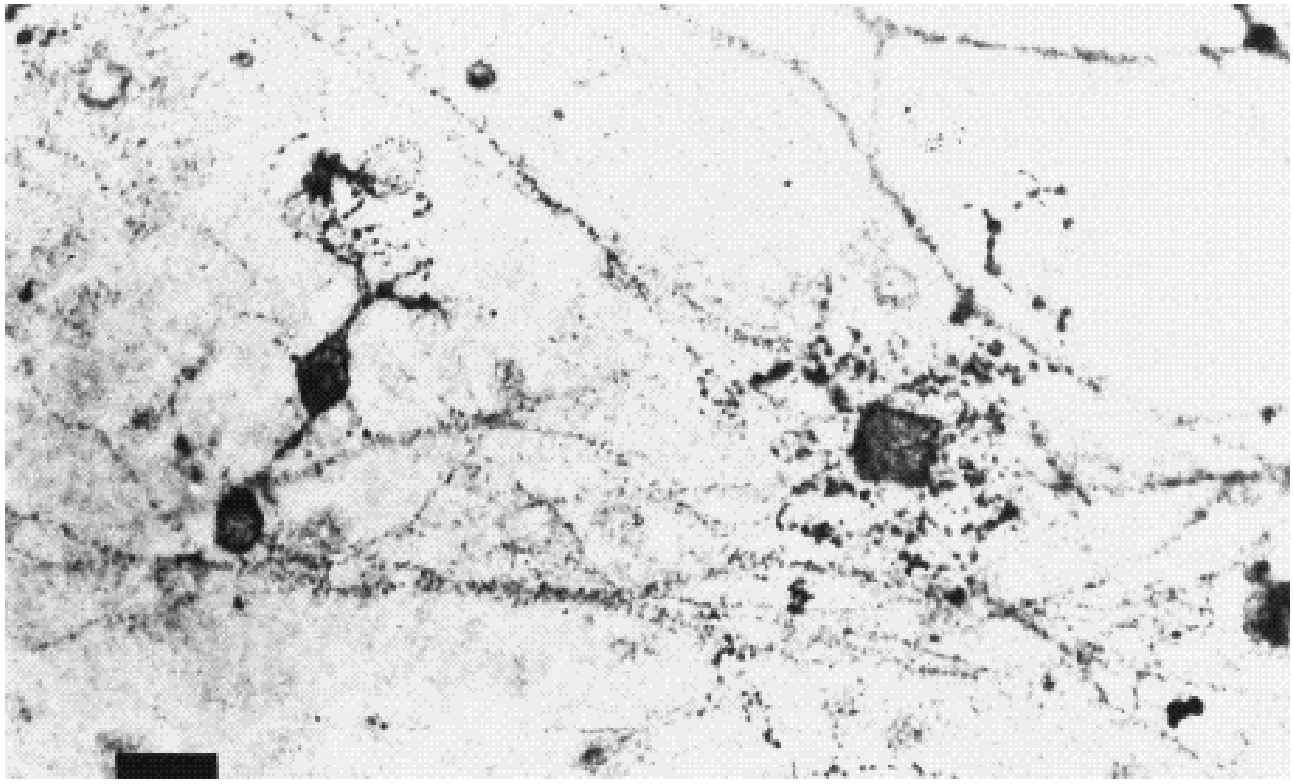

Fig. 2. Light microscopic view of one of the preparations subsequently used for scanning electron microscopy. The preparation was stained with anti-L1 and anti-galactocerebroside, onto which gold was deposited, which in turn was silver-intensified. Axons can be seen passing under three oligodendrocytes. Bar, 10 μm.

cells died, but flat glia could be seen adhering to the culture surface. In the gaps in between the flat cells, or on top of them, were some cells with small cell bodies and several processes; these mostly stained with A2B5 or tetanus toxin, and were therefore cells of the oligodendrocyte-type 2 astrocyte lineage (Miller et al. 1989). By 1 week the layer

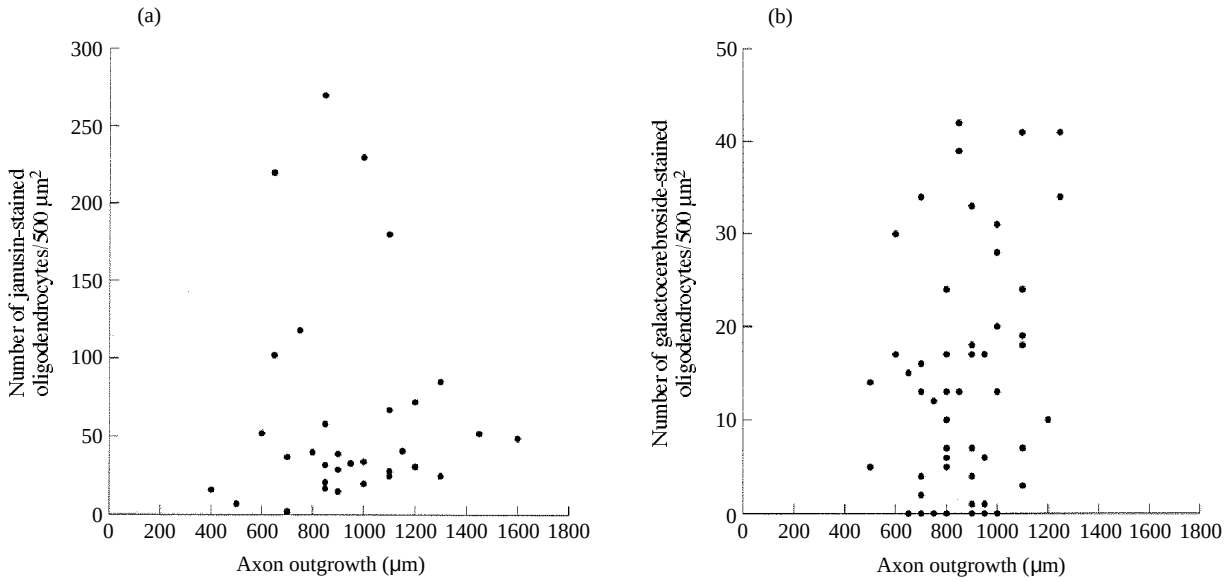

Fig. 3. Length of outgrowth of axons from individual dorsal root ganglia plotted against the density of galactocerebroside- (a) or janusin- (b) stained oligodendrocytes underlying the axons. Each point represents the outgrowth from one dorsal root ganglion. There is no correlation between axonal outgrowth and oligodendrocyte density in either graph, confirmed by linear regression analysis.

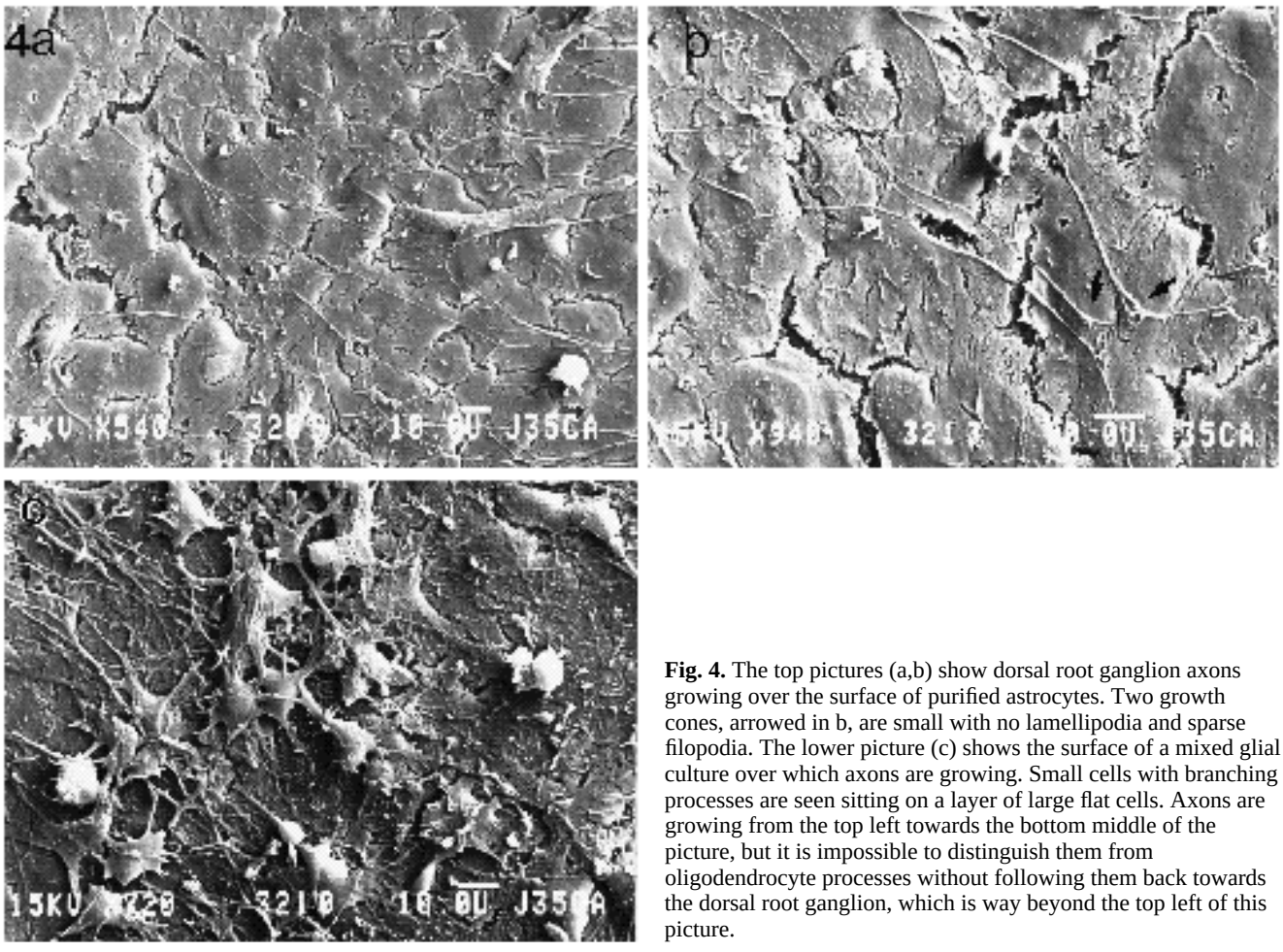

Fig. 4. The top pictures (a,b) show dorsal root ganglion axons growing over the surface of purified astrocytes. Two growth cones, arrowed in b, are small with no lamellipodia and sparse filopodia. The lower picture (c) shows the surface of a mixed glial culture over which axons are growing. Small cells with branching processes are seen sitting on a layer of large flat cells. Axons are growing from the top left towards the bottom middle of the picture, but it is impossible to distinguish them from oligodendrocyte processes without following them back towards the dorsal root ganglion, which is way beyond the top left of this picture.

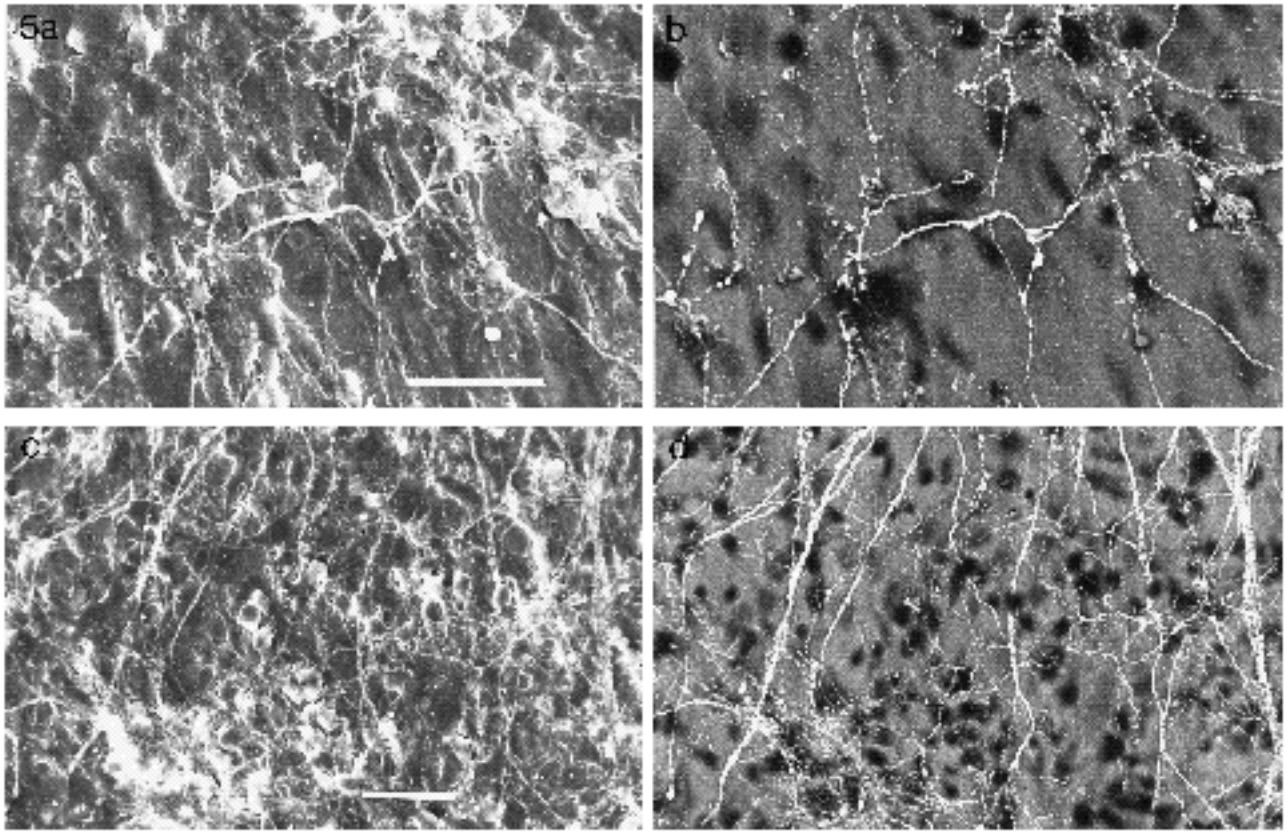

Fig. 5. Scanning electron micrographs of the surface of mixed glial cultures in which the axons have been silver stained using anti-L1. (a and c) are taken using the secondary electron detector, which provides an image of the surface topography of the culture, while (b and d) are images of the same areas taken using the backscatter detector, which provides information about the composition of the specimen. The backscattered images clearly show L1-stained axons growing in the cultures. Reference to a and c shows that these axons are found on the surface of the cultures, except where they pass under small process-bearing cells as in the top right of a and the centre of c. Bars, 50 μm.

of flat astrocytes was confluent, and cells of the oligodendrocyte-type 2 astrocyte lineage were seen on top of the astrocytes (Keilhauer et al. 1985). As the cultures aged, the numbers of oligodendrocytes which could be stained for galactocerebroside or janusin steadily increased. The number of cells which stained for janusin was larger than the number which could be stained for galactocerebroside, and included cells with a less highly branched morphology. This is consistent with previous observations that janusin is found on both mature and less-mature oligodendrocytes (Pesheva et al. 1989b; Jung et al. 1992). The underlying astrocyte layer changed little over time, except that the cells changed from a simple polygonal shape to a more process-bearing morphology. The cultures for the present experiments were between 3 and 4 weeks old. Values for the numbers of galactocerebroside-and janusin-staining oligodendrocytes per unit area are given on the graphs in Fig. 3; those cultures with few or no oligodendrocytes had been shaken to remove the surface-dwelling cells.

Growth of dorsal root ganglion axons: light microscopy

Dorsal root ganglia adhered readily to the glial surface, and axons could be seen by phase-contrast starting to radiate over the culture within a few hours. Cultures were fixed 48 hours after the DRGs had been placed on them, and stained with antibodies to L1, janusin and galactocerebroside to visualise the axons and the oligodendrocytes. Axonal growth was extensive, radiating out from the DRGs in all directions for 1 to 2 mm. We saw no evidence that the growing axons had stopped or changed their direction of growth to avoid oligodendrocytes; the pathway taken by individual axons was mostly a straight line, and often appeared to pass directly under oligodendrocytes (Figs 1 and 2). In order to see whether the presence of oligodendrocytes affected the extent of axon growth, we counted the janusin and galactocerebroside-stained oligodendrocyte density under the growing axons on our cultures, to see whether there was any correlation between variations in oligodendrocyte density and length of axon growth, and we also measured axonal growth on cultures depleted of oligodendrocytes to see whether axons would grow further on oligodendrocyte-free cultures. When the extent of axon growth from each DRG was plotted against the underlying oligodendrocyte density, measured with both galactocerebroside and janusin staining, the points appeared randomly scattered (Fig. 3), and linear regression analysis revealed no significant correlation between the presence of oligodendrocytes and the extent of axon growth.

Scanning electron microscopy

We wished to see whether axons growing in mixed glial cultures actually came into contact with oligodendrocytes,

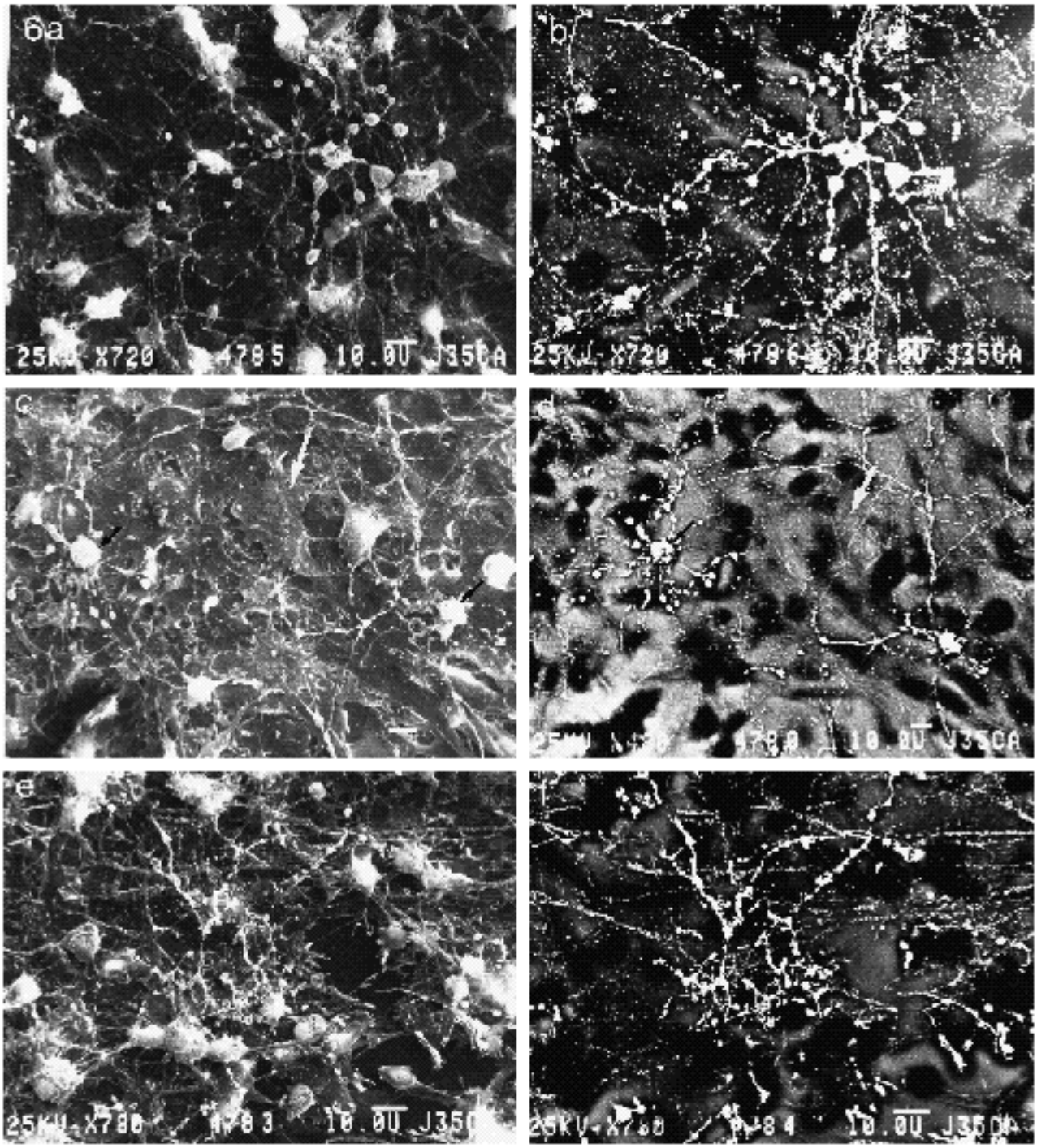

Fig. 6. Three preparations which have been stained with antibodies to both L1 and galactocerebroside; the secondary electron images on the left, and the backscatter images on the right. This staining protocol caused some degradation in surface structure, but the overall relations of cells and axons can be clearly seen. (a and b) show an oligodendrocyte with axons passing under it from top to bottom; in c and d (c is at a slightly higher magnification to show details) there are two stained oligodendrocytes, arrowed in black, with many axons coming into contact with their processes. In the centre there is a large flat cell, presumably an astrocyte, arrowed in white, which has many silver-stained axons associated with it, but these are not visible on the surface of the cell, and have therefore presumably passed underneath it. (e and f) show a large oligodendrocyte whose cell body has become dislodged during the staining procedure, making it easier to see the axonal process, going from right to left, coming into contact with and apparently passing under the oligodendrocyte processes.

or whether they somehow avoided touching them. We first looked at axon growth on purified astrocytes, and saw that axons almost all grew on the surface of the astrocyte monolayer, very few penetrating and passing between cells and culture surface. The axons on this astrocytic surface nearly all had small growth cones with few filopodia and no lamel-

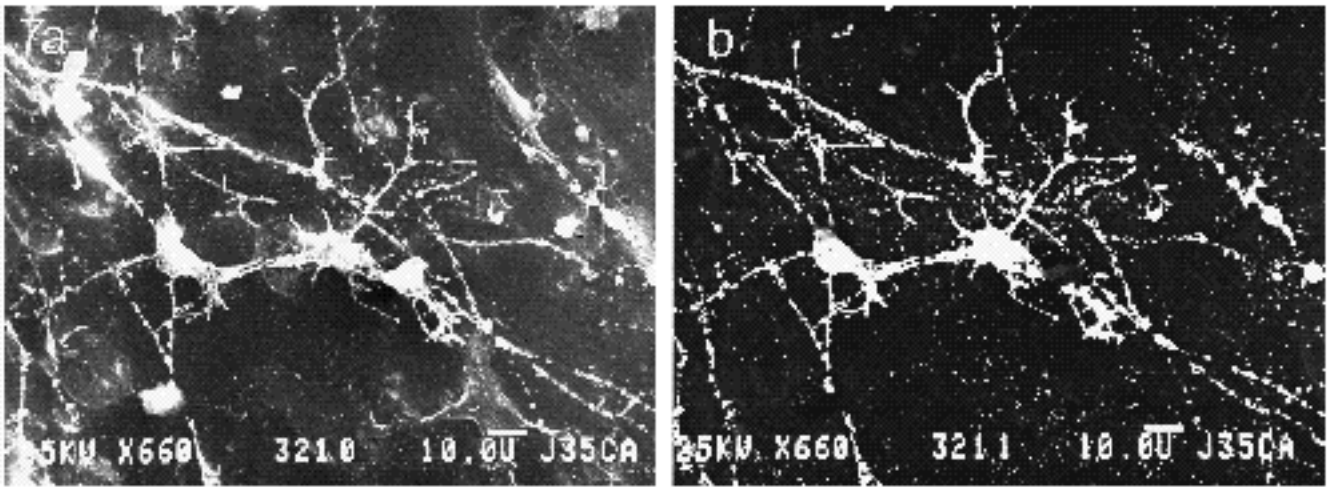

Fig. 7. Janusin-stained oligodendroctes with axon passing from top left to bottom right under the cell processes; (a) is the secondary electron image; (b) the backscattered image. Bar, 10 μm.

lipodia, as previously described (Johnson et al. 1991) (Fig. 4 a,b).

Simple SEM observations of axon growth in mixed glial cultures were extremely hard to interpret, because the surface of such cultures is covered by a profusion of fine processes, some presumably axonal, others oligodendrocytic (Fig. 4c). We therefore developed a technique by which we could immunostain axons, oligodendrocytes, or both. This technique resulted in some degradation of the anatomy of the cultures, but allowed the various processes to be unequivocally visualised and identified. Preparations in which axons alone were stained with anti-L1 showed that axons in mixed glial cultures grew over the surface of the astrocytic layer, but underneath the small cells with branching processes, and as far as we could determine under their fine processes as well (Fig. 5). In preparations in which both L1 and oligodendrocyte markers were applied, many of these small surface-dwelling cells with fine branching processes bound antibodies to galactocerebroside or janusin, and were therefore oligodendrocytes. These axons must, therefore, have grown while in contact with oligodendrocytes (Figs 2,6,7). Oligodendrocyte precursors are motile, although motility is lost with the start of differentiation (Small et al. 1987; Kachar et al. 1986; Fawcett et al. 1989b; Miller et al. 1989): some of the oligodendrocytes we saw in contact with axons could therefore have moved into position after the growth cone had passed by. However, we looked at the very furthest extent of axon growth, and still saw fine axons growing between the astrocyte layer and oligodendrocytes (Fig. 8). In some cultures we observed instances of axons growing underneath astrocyte processes (e.g. Fig. 6c,d); the great majority of axons, however, were always found on the surface of the astrocyte layer.

Discussion

The main finding of this set of experiments is that axons growing over a mixed population of astrocytes and oligodendrocytes show no evidence of avoiding the oligodendrocytes, or of inhibition of growth. We have shown in a previous set of experiments that growth of these same axons is inhibited by oligodendrocytes alone (Fawcett et al. 1987).

There can be three main classes of explanation for the difference between the present result and the inhibitory effects seen when axons contact solitary oligodendrocytes: first, the oligodendrocytes might have been changed by the presence of astrocytes so that they no longer expressed their inhibitory molecules; second, interactions with astrocytes might have changed the axons so that they no longer responded to oligodendrocytes; and third, the astrocytes might locally neutralise any inhibitory molecules on the oligodendrocyte surface. The first possibility, that oligodendrocytes no longer secrete inhibitory molecules, is probably not the case: we have demonstrated the presence of one oligodendrocyte-inhibitory molecule, janusin, in our cultures, and it has previously been shown that expression of janusin is in fact enhanced by the presence of astrocytes (Jung et al. 1992). Another indication of oligodendrocyte differentiation is that many oligodendrocytes expressing galactocerebroside, a marker for relatively mature cells, are present. However, it is still possible that axons might have affected the local expression of inhibitory molecules where they contact oligodendrocytes, and there is some evidence for a modulation of janusin expression by axonal contact (Jung et al. 1992). The second possibility, that interactions with astrocytes change the axons so as to render them insensitive to oligodendrocytes could be true. It is certainly the case that dorsal root ganglion cell axons growing on astrocytes have a very different growth cone shape to those growing on laminin, collagen or Schwann cell surfaces. We have recently observed that axons growing on an astrocytic monolayers have predominantly very small growth cones, with little in the way of filopodia or lamellipodia, rather like the growth cones often seen in vivo (Johnson et al. 1991; Tosney and Landmesser, 1985; Gorgels, 1991; Kim et al. 1991; Easter et al. 1984; Harris et al. 1987). It is possible, although there is at present no evidence, that these compact growth cones respond differently to oligodendro-

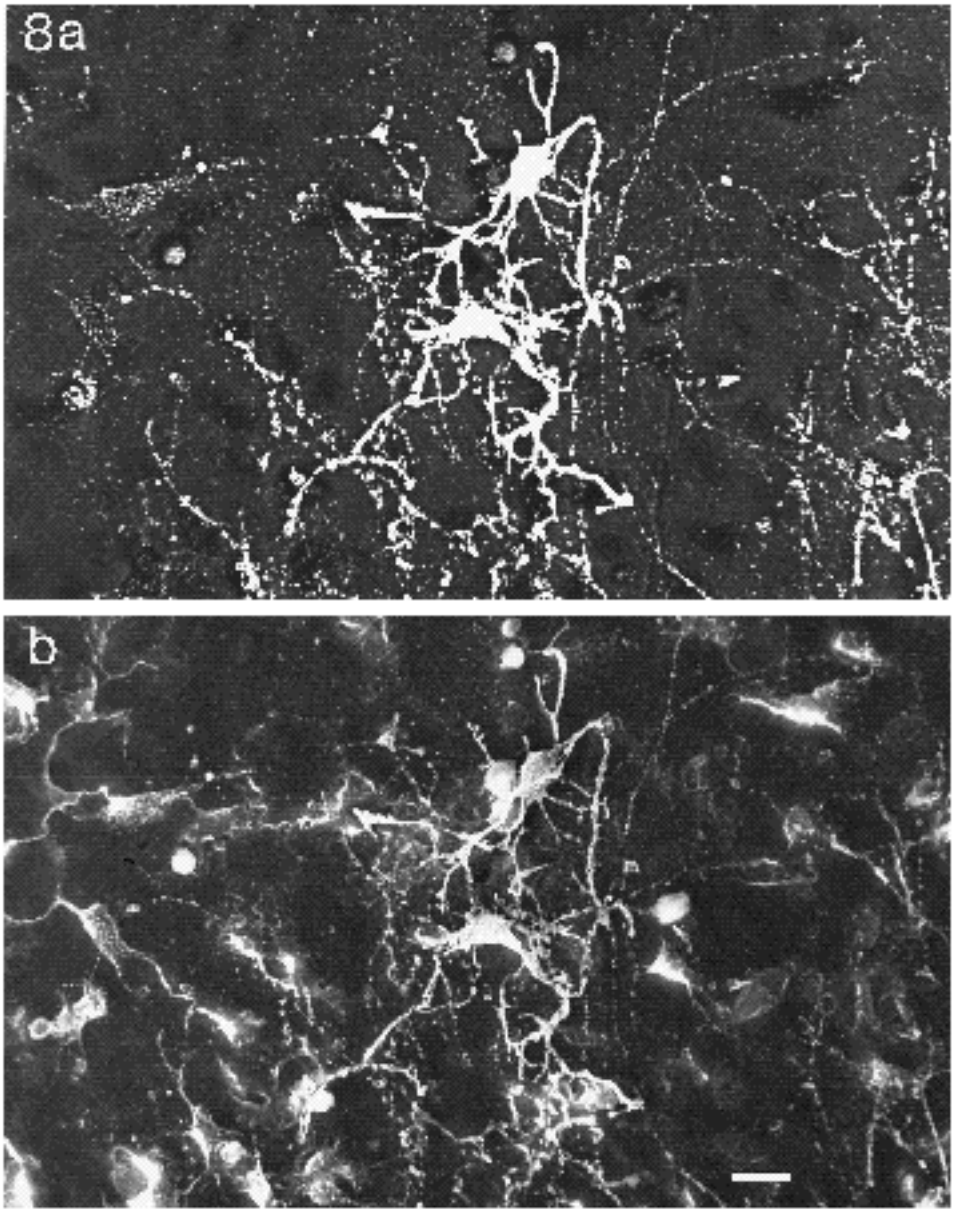

Fig. 8. Galactocerebroside-stained oligodendrocytes right at the outer limit of the axonal growth from a dorsal root ganglion; (a) is the backscattered image; (b) the secondary electron image. Some very fine axonal processes, which have been slightly disrupted by the staining procedure, can be seen passing under the cells from bottom centre to top centre. Bar, 10 μm.

cytes than the more expansive growth cones seen on laminin or collagen. There is a third possibility, that astrocytes modify the oligodendrocyte surface or the environment locally, for instance by means of proteases or protease inhibitors, although again in the absence of direct evidence any such hypothesis must be speculative. It should also be noted that not all axons have their growth inhibited by purified oligodendrocytes (Ard et al. 1991). There is some correlation with developmental stage, in that embryonic axons may not be inhibited while regenerating postnatal axons are, but the precise reasons for the different responses to oligodendrocytes remain obscure.

In practical terms these experiments demonstrate that the form of tissue-culture model used in these experiments does not accurately reproduce the inhibitory nature of the CNS glial environment; the main glial cell types making up that environment are present in our cultures, yet axon growth is not inhibited; results from this type of tissue culture model must therefore be interpreted with these limitations in mind. Three-dimensional or organ cultures provide more accurate models of axonal behaviour in vivo, but are more cumbersome and less susceptible to experimental intervention. The present results provide a demonstration of the complexity of interactions between the various cell types in the CNS, and of the ways in which the different types of cell can modulate the behaviours of others.

This work was supported by grants from the International Spinal Research Trust and the Medical Research Council.

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(Received 27 May 1992 - Accepted 23 July 1992)