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Cultivation of rat fetal spinal cord slices in a semi-solid medium: a new approach to studying axonal outgrowth and regeneration

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A soft agar culture system was used for the cultivation of spinal cord slices with the purpose of improving the evaluation of the dynamics of axonal outgrowth and development. Slices of the spinal cord of 15-day-old fetal Wistar rats were cultured in a 0.5% agar culture medium. The sprouting and outgrowth of axons from the slices was observed at 6–24-h intervals. The morphology and growth rates of axons could be easily investigated by light microscopy. Quantification of growth parameters of individual neurites is made easy because no cells migrate out of the slices, so that the outgrowth is not masked by migrating neurons, fibroblasts, glial cells etc. The axons had well-developed growth cones, comparable to those observed in liquid medium; the daily growth rate was on average 318 μm during the 6 days of observation, with a maximum of 1050 μm per day. Back-labelling with a fluorescent dye (DiI) indicated that the longest neurites originated from motoneurons. Our experiments show that axons can develop and grow in a soft agar medium without the need for adding any growth promoting factor or substrate molecule.

Introduction

Cultivation of spinal cord slices (SCS) *in vitro* enables the study of neurite outgrowth, the formation of neuronal interconnections, and bundling and fascicle formation (Letourneau, 1975; Dribin and Barrett, 1980). Thus, SCS cultures contribute to a better understanding of the dynamics of axonal development and peripheral nerve regeneration. Moreover, *in vitro* conditions represent a favourable system to influence neurons and the development of their processes (neurites) by biologically active substances

(Tanaka et al., 1982; Van der Neut et al., 1988), as well as the cell and tissue interactions in organotypic cultures (Braschler et al., 1989; Jaeger et al., 1989). Culture conditions, especially the composition of the culture medium and the quality of the substrate, determine for a great deal both the survival of neurons and the character of neurite outgrowth (Dribin and Barrett, 1980; Tanaka et al., 1982). The combination of a liquid culture medium and a two-dimensional substrate covered with different substances (collagen, poly-L-lysine (PLL), polyornithine (PORN)) is most commonly used so far for the cultivation of SCS (Tanaka et al., 1982; Van der Neut et al., 1988).

The semi-solid culture medium (soft agar) is especially suited to study cell differentiation and organ development. This culture system, with its three-dimensional biological matrix surrounding

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the tissue explants, approaches in vivo conditions and was used successfully to study colony formation by lymphocytes (Bartkova et al., 1987), growth of tumour stem cells (Hamburger and Salmon, 1977) and tooth germ morphogenesis (Dye and Kollar, 1978). An agar culture medium was also used to study nerve fibre elongation in cultures of dissociated dorsal root ganglia (Letourneau, 1978) and cellular differentiation in cultures of brain cell aggregates (Bjerkvig et al., 1986). However, this culture system has not been used so far for the cultivation of spinal cord explants.

In this study we established the basal culture conditions for the cultivation of rat fetal spinal cord slices in a soft agar culture system, morphological characteristics and the growth rate of neurites growing out from these slices. The results show that different processes can grow in semi-solid medium and that this culture system is suitable especially for axon propagation. We therefore consider SCS cultures in soft agar to be a useful model for the study of axon development and regeneration.

Methods

Preparation of slices

Spinal cord slices were prepared as described before (Van der Neut et al. 1988). In short, spinal cords (SC) from 15-day-old fetal Wistar rats were freed from meninges and dorsal root ganglia, and cut into 0.4–0.5 mm slices by using 2 scalpels with No. 15 blades. Usually 20–25 slices were obtained from one SC.

Culture procedures

A slightly modified two-layer culture, previously described by Bartkova et al. (1982), was used for the experiments. The lower agar layer, consisting of 1.5 ml of culture medium supplemented with 0.5% agar (Difco Agar Noble), was pipetted to 35-mm petri dishes (Nunc). The SCS were placed on top of this agar layer and then the upper agar layer, 0.5 ml of medium supplemented with 0.5% agar, was added. Care was taken that the slices were not covered by the upper agar layer; the best result was obtained when the top

of the slices was level with the surface of the upper agar layer, so that the surface of the slice was exposed to the humidified air/CO₂ atmosphere. The culture medium consisted of DMEM (Gibco), supplemented with 10% FCS (Gibco), 100 U/ml sodium benzylpenicilline (Centrachemie, The Netherlands), 100 µg/ml streptomycin sulphate (Pharmachemie, The Netherlands). The pH was adjusted to 7.2–7.4 with a 7.5% solution of NaHCO₃ (Gibco). Cultures were kept in a humidified atmosphere of 6.5% CO₂ in air at 37°C. Without changing the (semi-solid) medium, cultures remained in good condition for at least 2 weeks.

Evaluation of neurite outgrowth

The processes which grew out from slices were evaluated morphologically under phase contrast. The length of individual processes and their fascicles was measured in 10 different experiments during 4–6 days with 6, 8 or 24 h intervals by using an OLYMPUS OSM micrometer. In this way the development in time of individual processes could be followed, resulting in a detailed description of their growth.

Indirect immunoperoxidase (IPx) staining

For indirect NF-immunoperoxidase staining, the cultures were fixed with ice-cold methanol, washed 3 times with PBS and incubated with monoclonal mouse anti-neurofilament (NF) IgGs (clone 2F11, Dakopatts, Denmark, 1:50). As 2nd antibody horseradish peroxidase (HRP) conjugated F(ab')₂ fragments of sheep anti-mouse IgGs were used (Sigma, 1:40). The immunoreactivity product was visualized with aminoethylcarbazole.

DiI treatment

After 72 h of cultivation, 0.25 µg of C-18-DiI (Molecular Probes), dissolved in 0.5 µl of ethanol, was added to the top of the upper agar layer in the proximity of outgrowing axons under visual control with an inverted microscope. The results were evaluated under a fluorescence microscope (incident wavelength 540 nm) in native cultures after 5 days.

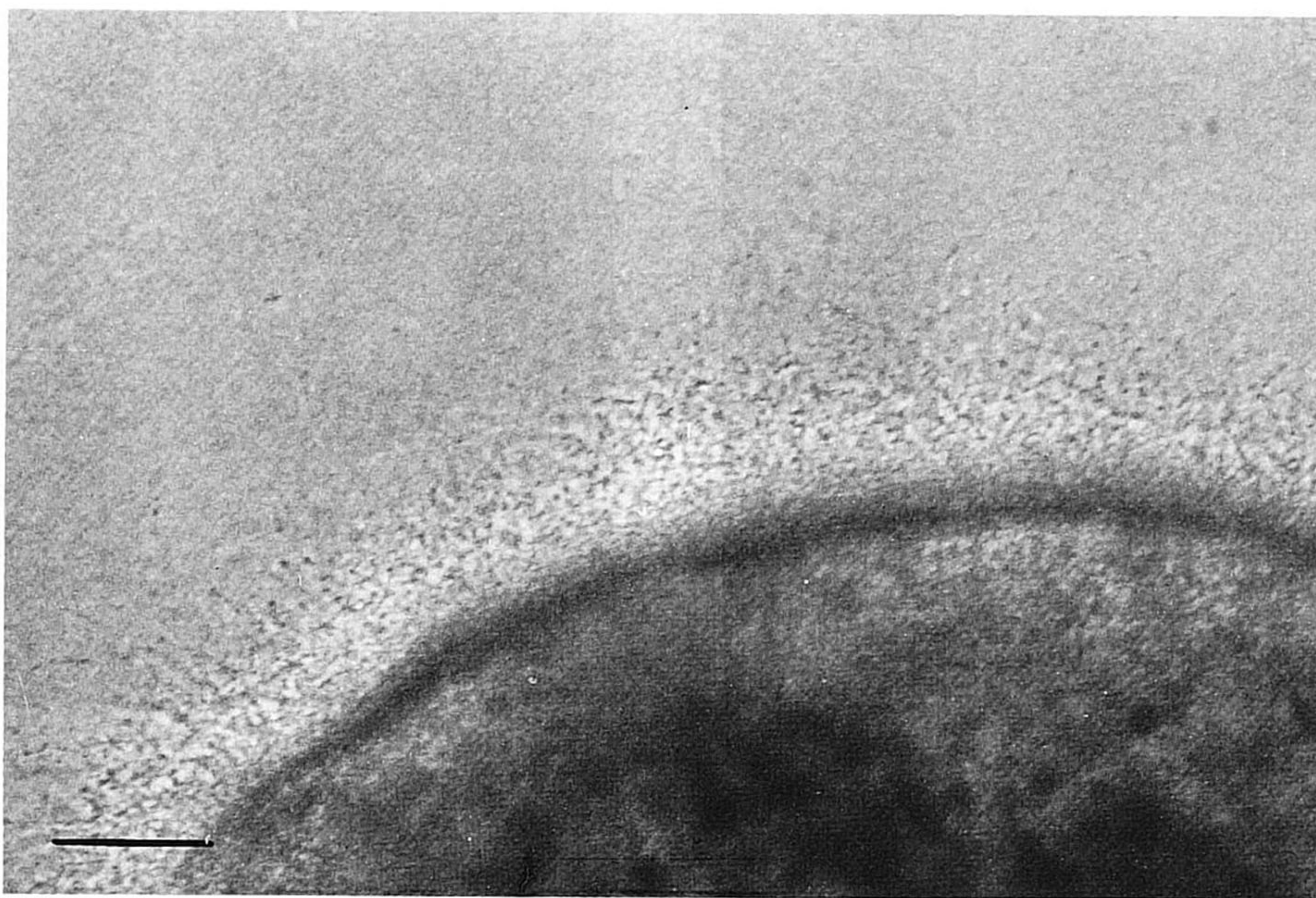


Fig. 1. Light microscopical picture of a rat spinal cord slice after 4 days in culture. Short "hairy" processes are visible around the perimeter of the slice. Bar = 50 μ m.

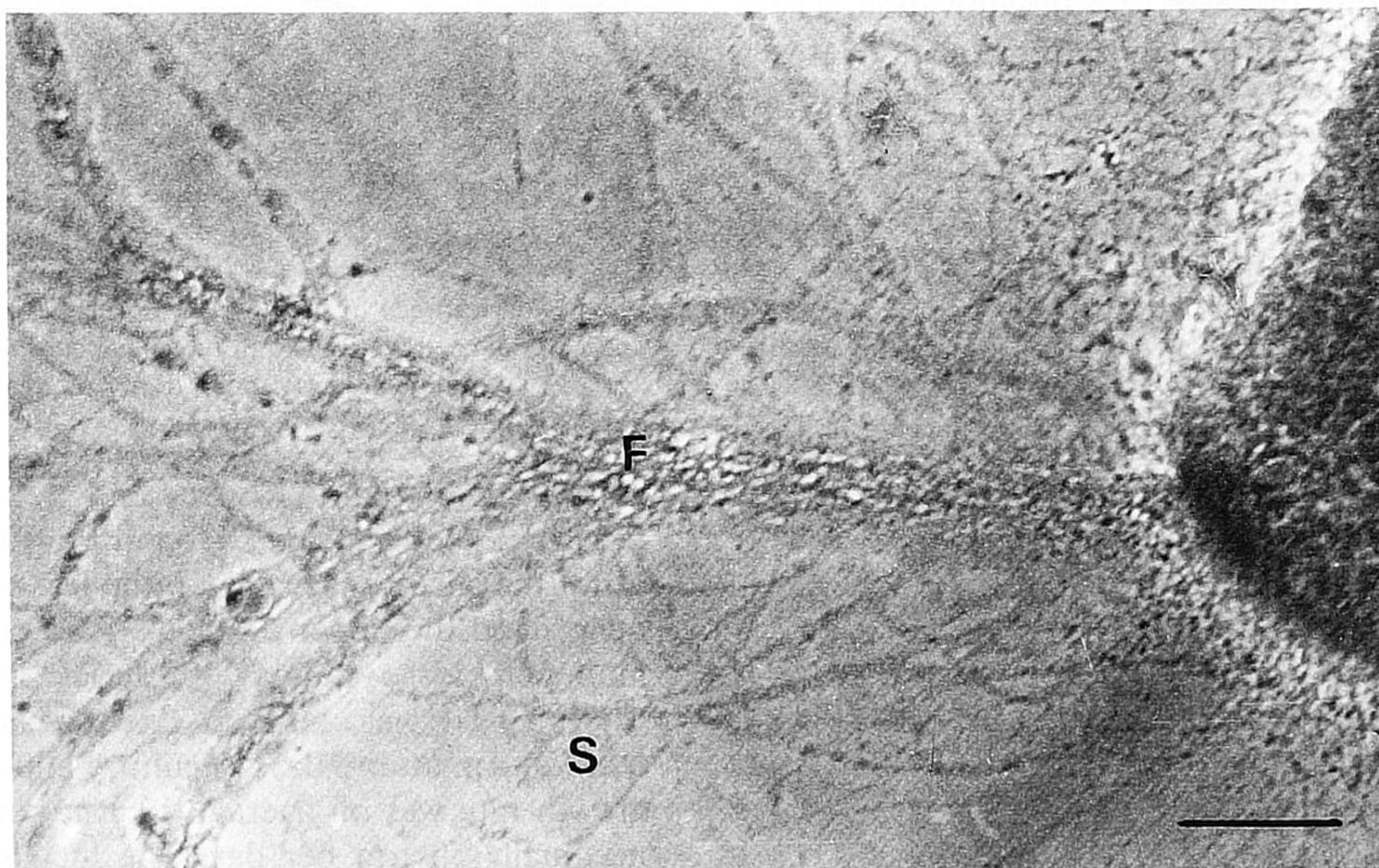


Fig. 2. Rat spinal cord slice at 4 days in culture. Large neurites, growing on top of the agar layer can be seen. Note single processes (S) and the formation of fascicles (F). Bar = 100 μ m.

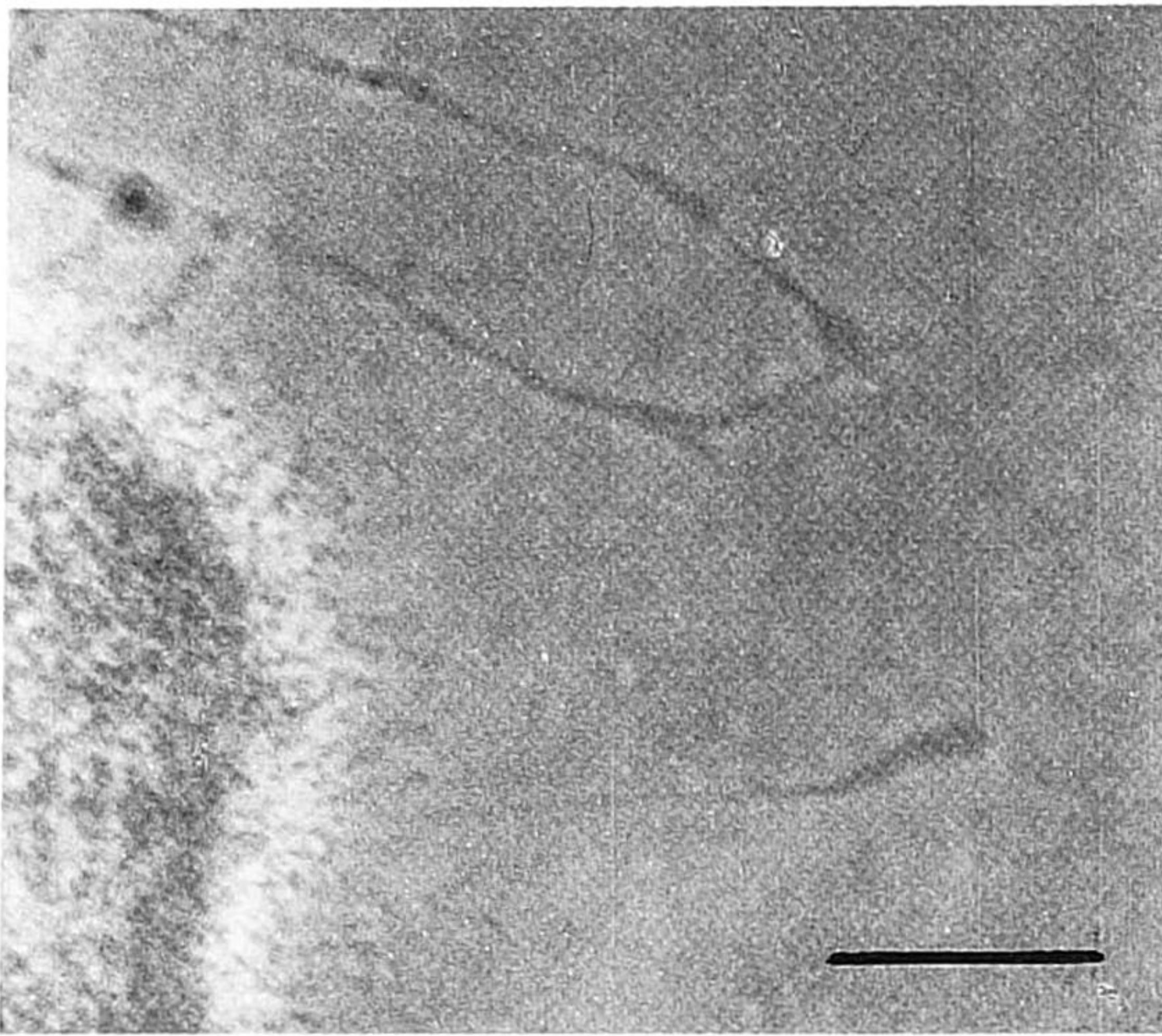


Fig. 3. Growth cones of neurites growing out of slices after 2 days in culture. Bar = 50 μ m.

Results

Two basic, distinguishable types of cell protrusions appeared during cultivation around the perimeter of the slices. The first type (Fig. 1) consisted of relatively short processes which were absent when the slices were explanted and appeared within the first 12 h of cultivation. These processes were very thin, remained relatively

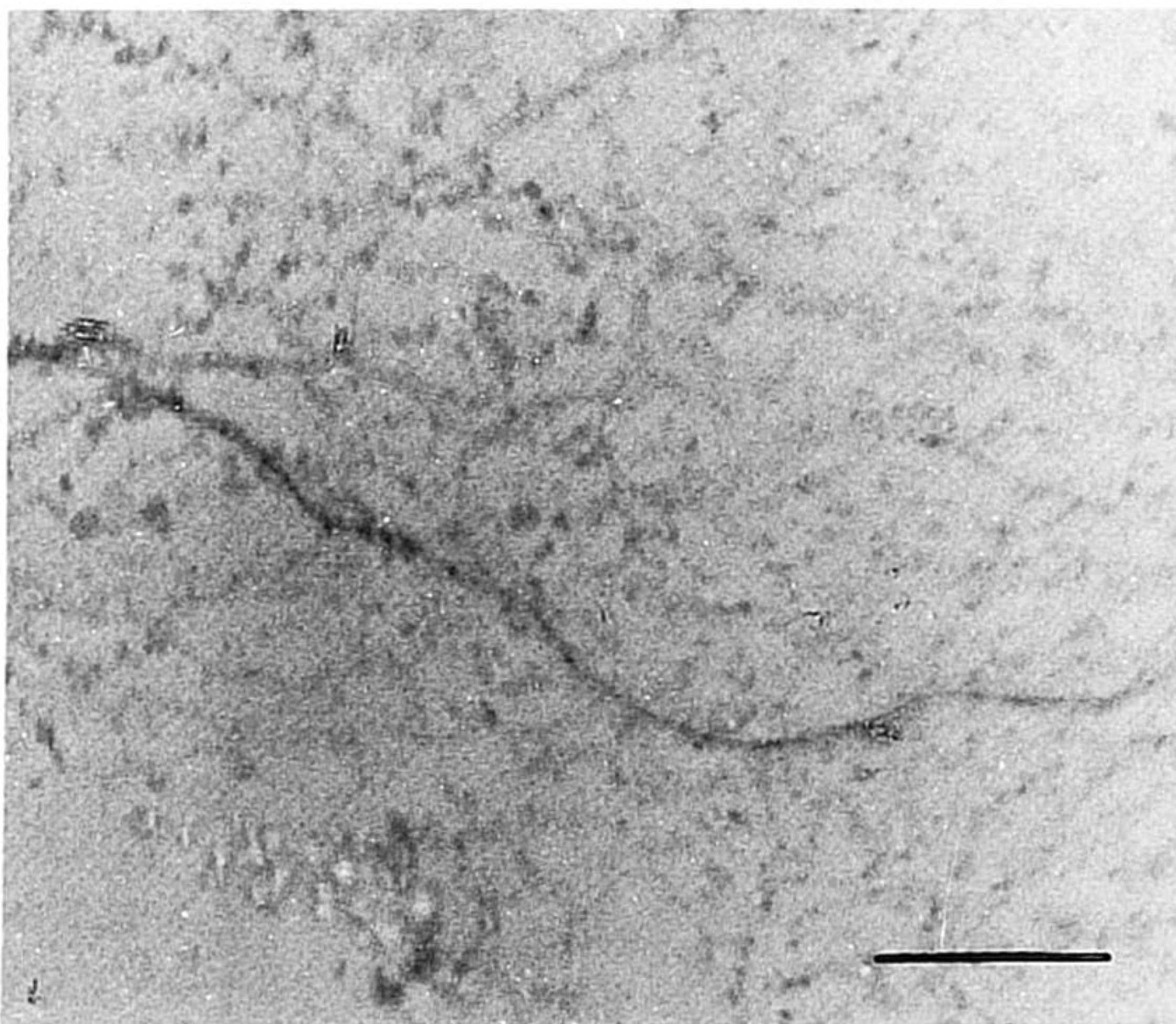


Fig. 4. Neurofilament (immunoperoxidase) staining of neurites growing on the agar layer after 6 days in culture (slice outside field of vision). Bar = 50 μ m.

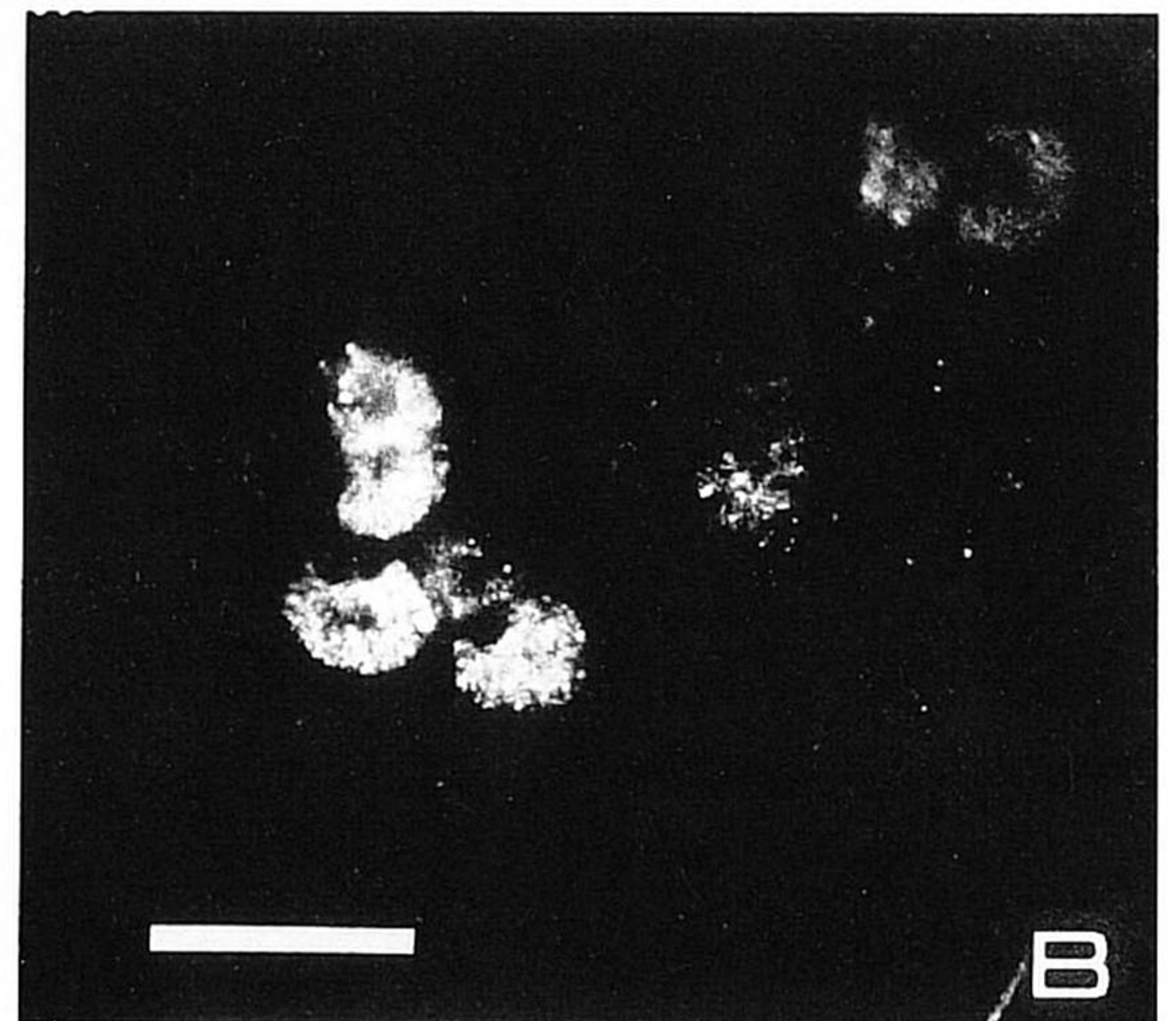
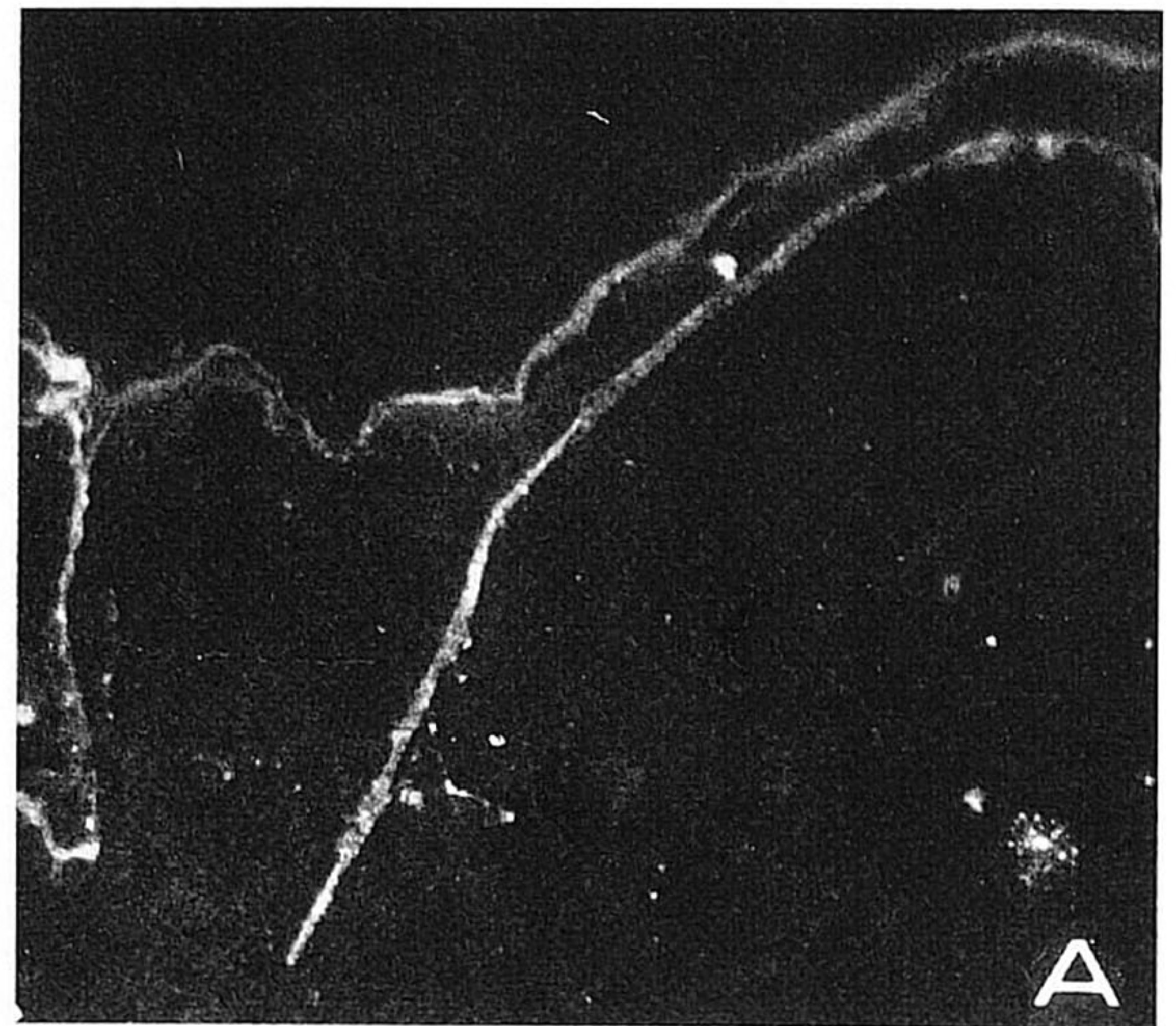


Fig. 5. Fluorescence of neurites on top of the agar layer (A) and neuronal cell bodies within the spinal cord slice (B) after back-labelling with DiI. Bar = 50 μ m.

short, had a "hairy" aspect and never formed fascicles. They appeared around the full perimeter of the slices, especially on the lateral side and penetrated the upper agar layer. No positive immunoperoxidase staining of these "hairy" processes for NF was observed. Their maximal length was 120 μ m after 6 days in culture and their daily growth rate was on average 12 μ m per day, and maximally 50 μ m per day. The localisation and morphology of these processes in slices in culture was similar to that of glial cell processes in histo-

logical sections prepared of the spinal cord of rat fetuses of the same developmental stage, E15 (data not shown).

The second type of processes appeared later, usually after 24 h in culture. These processes showed all characteristics of axons: they grew either as a single process or they formed fascicles (Fig. 2). They appeared only on distinct parts of the slices, most often on ventro-lateral and dorso-lateral corners and grew only on top of the upper agar layer, and did not penetrate the agar. The processes were thicker than the ones mentioned above and growth cones could be observed (Fig. 3). Immunoperoxidase staining for NF was positive (Fig. 4). Back-labelling with DiI stained

both the neurites (Fig. 5A) and the cell bodies of motoneurons within the slices (Fig. 5B).

The maximal length of axonal processes growing on the upper agar layer was $2300\ \mu\text{m}$ after 6 days in culture, their average daily growth rate was $318\ \mu\text{m}$ with a maximum $1050\ \mu\text{m}$ per day. The growth rate fell during the course of cultivation; a maximum was observed usually between 48 and 72 h after seeding the slices on to the agar. Formation of new fascicles from these processes could be seen especially in later phases of cultivation. The daily distribution of the average length of these processes is demonstrated in Fig. 6. Growth over 24 h, when studied with 6 and 8 h intervals, was not equal and varied within this

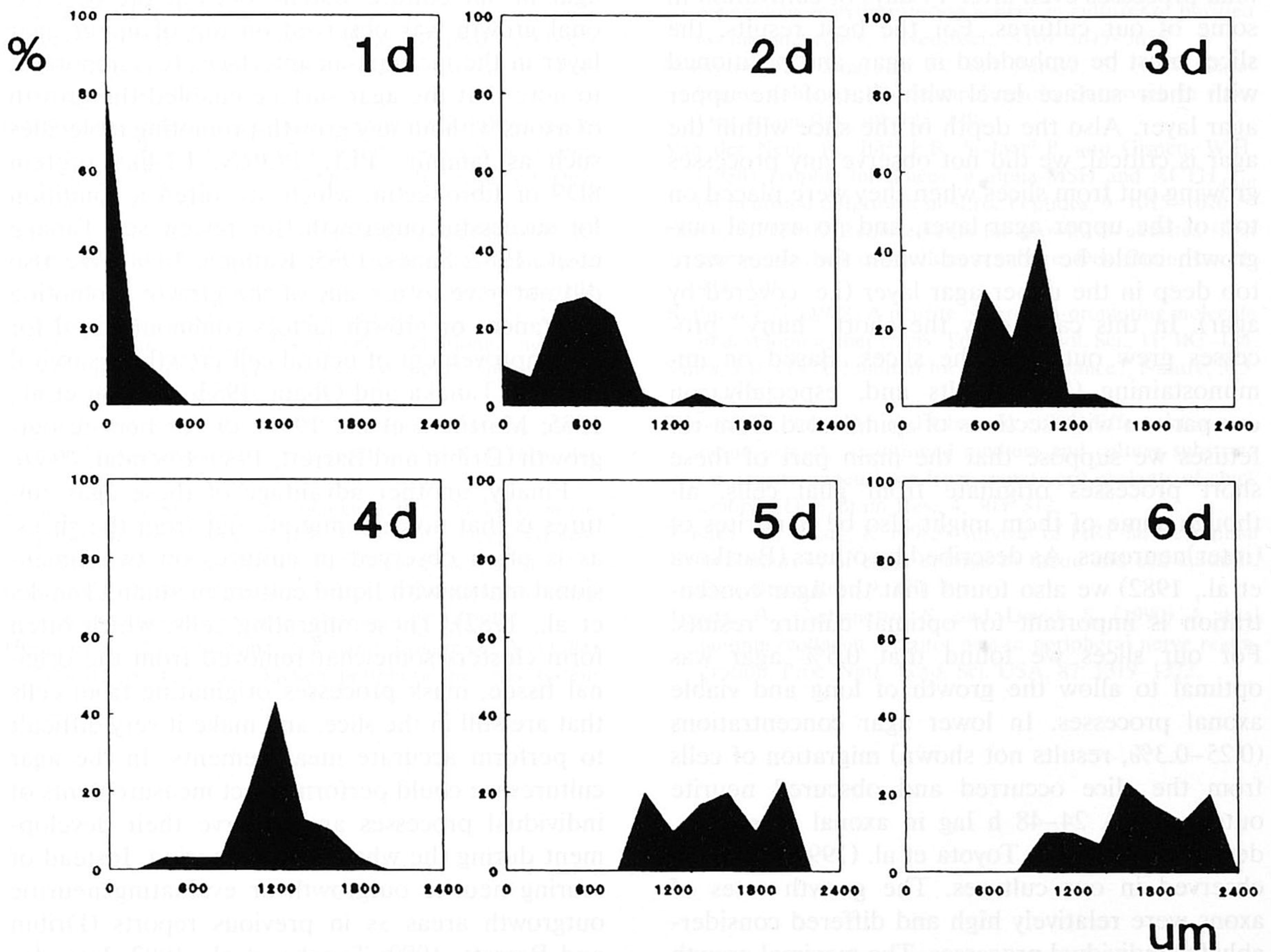


Fig. 6. Distribution of the length of neurites growing out from spinal cord slices cultured in an agar semi-solid medium from day 1 to day 6.

period, especially when some axons changed the leading position in the fascicles. These changes were more frequent than in cultures observed with longer intervals, presumably partly as a result of the repeated handling of the cultures (motion, drop in temperature and P_{CO_2} , etc.).

Discussion

The soft agar culture system described in this study provides an excellent environment for spinal cord slices and also a good substrate for neurites, as it enables long survival of neurons and their neurites without the use of growth-promoting additives. We observed the appearance of new axonal processes even after 14 days of cultivation in some of our cultures. For the best results, the slices must be embedded in agar, and positioned with their surface level with that of the upper agar layer. Also the depth of the slice within the agar is critical: we did not observe any processes growing out from slices when they were placed on top of the upper agar layer, and no axonal outgrowth could be observed when the slices were too deep in the upper agar layer (i.e. covered by agar). In this case, only the short "hairy" processes grew out from the slices. Based on immunostaining (NF) results and, especially, on comparison with sections of spinal cord from rat fetuses we suppose that the main part of these short processes originate from glial cells, although some of them might also be dendrites of (inter)neurons. As described by others (Bartkova et al., 1982) we also found that the agar concentration is important for optimal culture results. For our slices we found that 0.5% agar was optimal to allow the growth of long and viable axonal processes. In lower agar concentrations (0.25–0.3%, results not shown) migration of cells from the slice occurred and obscured neurite outgrowth. A 24–48 h lag in axonal outgrowth, described *in vivo* by Toyota et al. (1990), was also observed in our cultures. The growth rates of axons were relatively high and differed considerably for individual processes. The maximal growth rate (about 1000 μm per day) was about 3 times higher than described *in vivo* by Toyota et al.

(1990) and is comparable with the highest growth rate in sensory ganglia neurons cultured on PORN (Letourneau, 1975).

The role of the extracellular matrix in the development of neurites and especially axons in cell and tissue cultures of the nervous system has been widely discussed in previous reports (Humphries et al., 1988; Lagenaur and Lemmon, 1987; Tanaka et al., 1982; Sanes, 1985; Obata, 1981; Nakayama et al., 1989; Braschler et al., 1989). However, agar as a suitable substrate for outgrowth and fascicle formation of motoneuron axons has not been mentioned so far. Letourneau (1978) reported about the neuritic outgrowth from dissociated ganglia neurons cultured inside the agar medium. Some neurites appeared inside the agar in our culture system too, but the best axonal growth was observed on top of upper agar layer in the medium-air interface. It is important to note that the agar surface enabled the growth of axons without any growth-promoting molecules such as laminin, PLL, PORN, L1-like protein 8D9 or fibronectin, which are often a condition for successful outgrowth (for review see Tanaka et al., 1982; Sanes, 1985; Rathjen, 1988). We also did not have to use any of the growth promoting substances or growth factors commonly used for the improvement of neural cell growth or survival *in vitro* (Tanaka and Obata, 1983; Laerum et al., 1985; Martinou et al., 1989), or for neurite outgrowth (Dribin and Barrett, 1980; Ebendal, 1989).

Finally, another advantage of these agar cultures is that no cells migrate out from the slices, as is often observed in cultures on two-dimensional matrix with liquid culture medium (Tanaka et al., 1982). These migrating cells, which often form clusters somewhat removed from the original tissue, mask processes originating from cells that are still in the slice, and make it very difficult to perform accurate measurements. In the agar cultures we could perform exact measurements of individual processes and observe their development during the whole culture period. Instead of scoring neurite outgrowth or evaluating neuritic outgrowth areas as in previous reports (Dribin and Barrett, 1980; Tanaka et al., 1982; Van der Neut et al., 1988), we were able to measure very precisely the growth rates of individual neurites

as well as the fascicles they formed. This contributes to better understanding of the dynamics of the growth of processes and the formation of axonal fascicles.

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