Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 10959–10963, November 1995
Neurobiology

Myelin-associated neurite growth-inhibitory proteins and suppression of regeneration of immature mammalian spinal cord in culture

(*Monodelphis domestica*/neurite outgrowth)

Z. M. VARGA*, M. E. SCHWAB†, AND J. G. NICHOLLS*‡

*Department of Pharmacology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland; and †Institut für Hirnforschung, CH-8029 Zürich, Switzerland

Communicated by Dale Purves, Duke University Medical Center, Durham, NC, August 30, 1995

ABSTRACT **Neurite outgrowth across spinal cord lesions *in vitro* is rapid in preparations isolated from the neonatal opossum *Monodelphis domestica* up to the age of 12 days. At this age oligodendrocytes, myelin, and astrocytes develop and regeneration ceases to occur. The role of myelin-associated neurite growth-inhibitory proteins, which increase in concentration at 10–13 days, was investigated in culture by applying the antibody IN-1, which blocks their effects. In the presence of IN-1, 22 out of 39 preparations from animals aged 13–17 days showed clear outgrowth of processes into crushes. When 34 preparations from 13-day-old animals were crushed and cultured without antibody, no axons grew into the lesion. The success rate with IN-1 was comparable to that seen in younger animals but the outgrowth was less profuse. IN-1 was shown by immunocytochemistry to penetrate the spinal cord. Other antibodies which penetrated the 13-day cord failed to promote fiber outgrowth. To distinguish between regeneration by cut neurites and outgrowth by developing uncut neurites, fibers in the ventral fasciculus were prelabeled with carbocyanine dyes and subsequently injured. The presence of labeled fibers in the lesion indicated that IN-1 promoted regeneration. These results show that the development of myelin-associated growth-inhibitory proteins contributes to the loss of regeneration as the mammalian central nervous system matures. The definition of a critical period for regeneration, coupled with the ability to apply trophic as well as inhibitory molecules to the culture, can permit quantitative assessment of molecular interactions that promote spinal cord regeneration.**

As the central nervous system (CNS) matures in embryonic and neonatal mammals, a sharp decrease occurs in the ability of nerve fibers to grow across spinal cord lesions, to effect repair, and to restore functions (1–5). The exact time at which regeneration ceases varies from species to species (1–4, 6) and within an animal, depending on the maturity of the tract that has been disrupted (7–9, 33). Eventually all CNS regeneration fails except in the olfactory system. Several new strategies have been developed with the aim of prolonging the capacity for regeneration and restoration of function after lesions in adult CNS. These include the use of grafts of peripheral nerve (10, 11), embryonic tissue (12–14), and Schwann cells (15, 16), as well as the application of trophic molecules such as neurotrophin 3 (17) to promote fiber outgrowth across CNS lesions or brain-derived neurotrophic factor to promote survival of axotomized cells (18).

In pathways that become myelinated there is a correlation with the development of myelin and the appearance of myelin-associated inhibitory proteins known as NI-35/250 that prevent neurite outgrowth (19–21). After the effects of these molecules have been blocked by a specific monoclonal antibody (IN-1) neurons can once again grow across spinal cord lesions at stages when regeneration would normally be impossible (17, 22, 23). Thus, in rats aged 3 months, application of IN-1 permitted fibers to grow several millimeters beyond a lesion. One difficulty in analyzing and defining the precise mechanisms that bring regeneration to a close is the difficulty of applying molecules to the CNS in known concentrations at different sites for prolonged periods. Another is the slow time course of regeneration, usually requiring weeks or months for unambiguous fiber outgrowth beyond the lesion. Thus, it is not possible at present to follow outgrowth of neurites across a lesion in real time in an animal.

Certain advantages for approaching these problems are provided by the CNS of the neonatal opossum isolated and maintained in culture. The CNS in its entirety survives well for periods of a week or longer and continues to exhibit reflex behavior, cell division, and growth in culture (24, 25). Moreover, repair that is rapid (5 days), extensive, and reliable occurs after a crush or a cut has been made to the spinal cord *in vitro*, provided the animal is less than 12 days of age (26–28). In this preparation it has been shown that the capacity for regeneration across a cervical spinal cord lesion comes to an abrupt end at 12 days, the stage at which astrocytes, oligodendrocytes, myelin and myelin-associated growth-inhibitory proteins have developed (5). The present experiments were designed to use the neonatal opossum CNS (*i*) to establish whether growth observed in younger animals corresponds to regeneration (that is, fibers that had been transected growing across the lesion) or to growth of developing fibers that had not been cut but that reached the lesion and grew through it, and (*ii*) to determine whether application of IN-1 antibody in this system could promote outgrowth of fibers and regeneration across a crush at stages after the end of the critical period 12 days after birth.

MATERIALS AND METHODS

Dissection, Lesioning, and Tissue Culture. Opossum (*Monodelphis domestica*) pups were taken from the colony at 6, 13, 15, and 17 days after birth, anesthetized by methoxyflurane and cooling on ice, and killed by rapid section of heart and lungs (5, 24–27). The entire CNS was removed and cultured in basal medium Eagle (BME; GIBCO) gassed with 95% O_2/5% CO_2. In some preparations dorsal root ganglia (DRGs) were retained in the cervical regions. The spinal cords were crushed with fine watchmaker's forceps at the sixth cervical segment and were cultured for 5 days in BME with 1% fetal bovine

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Abbreviations: BME, basal medium Eagle; CNS, central nervous system; DRG, dorsal root ganglion; DiI $C_{18}(3)$, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; DiI $C_{18}(5)$, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine; DiO $C_{18}(3)$, 3,3′-dioctadecyloxacarbocyanine; HRP, horseradish peroxidase.

‡To whom reprint requests should be addressed at: Department of Pharmacology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland.

serum, insulin (Actrapid, 10 μg/ml; Novo-Nordisk Pharma, Küsnacht, Switzerland), 7S nerve growth factor (7S NGF, 30 ng/ml; Sigma), and gentamicin (0.1 mg/ml; Sigma). In DRG preparations the culture medium contained NGF at 120 ng/ml. The crushes caused complete disruption of all neurites on that side of the spinal cord (26, 27).

Carbocyanine Dye Labeling. Three different carbocyanine dyes were used (as the perchlorate salts): 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine [DiI $C_{18}(3)$], 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine [DiI $C_{18}(5)$], and 3,3′-dioctadecyloxacarbocyanine [DiO $C_{18}(3)$; Molecular Probes; ref. 29]. Saturated solutions of dyes in 100% ethanol for DiI or 100% propylene oxide for DiO were used to coat glass microelectrodes with thin layers of crystals. The dye-coated tips were inserted into the ventrolateral spinal cord or into the dorsal columns and were left embedded in the tissue. The dyes were visualized under a Leica DM fluorescence microscope using the following filters: 450–490 nm, DiO; 515–560 nm, DiI $C_{18}(3)$ and DiI $C_{18}(5)$; 630–640 nm, DiI $C_{18}(5)$. The fluorescence emitted from DiO (500 nm) and DiI $C_{18}(5)$ (665 nm) did not overlap with the filter sets we used.

Tests for Axonal Regeneration. Two methods were used for determining whether axons that grew across a lesion had been cut or had simply grown into it. Labeling of DRG cell bodies was used to test for regeneration by identified cells. After dissection the dorsal columns of spinal cords from 6-day-old opossums (n = 8) were labeled with DiO. The preparations were cultured uncrushed to allow retrograde labeling of DRG cell bodies. Twenty-four to 36 hr after labeling, a crush was made in the segment rostral or caudal to the dye pipette and the dye electrode was removed. After four more days in culture, preparations were fixed in phosphate-buffered 4% paraformaldehyde and labeled with DiI $C_{18}(5)$ near the site of DiO labeling. The preparations were left 3–4 days in the fixative at room temperature. The DRGs adjacent to the crush were removed, mounted on glass slides, and photographed. Double labeling of DRG cell bodies indicated axonal regeneration. DRG cells could not be used for testing regeneration in the presence of antibody IN-1 under our conditions. Like caudal regions of the spinal cord (5), these tracts become myelinated late and regeneration continues until day 16 (5, 33).

Fibers in the ventral fasciculus were used to test the effects of IN-1 on regeneration. The ventrolateral spinal cord was labeled with DiI $C_{18}(3)$ at cervical segments 7 and 8. In 16 preparations, neurites of cell bodies located in the brainstem were retrogradely labeled. The preparations were cultured for 24–36 hr, then crushed two segments rostral to the dye electrode and recultured for 3 days. At the end of the culture period the CNS was fixed and examined for growth cones growing from rostral to caudal in the crush site.

Blockade of Growth Inhibition with IN-1 Antibody. Preparations from opossums aged 13, 15, and 17 days, in which growth of ventral neurites through crushes never occurs, were crushed and cultured in BME (n = 34) with added IN-1 (21) (n = 39; hybridoma of supernatant at 50 μl/ml in BME) or with anti-horseradish peroxidase (HRP) antibodies (23) (n = 8) at the same dilution. The IN-1 (IgM) supernatant was taken from mouse hybridoma subclone 8E1-10; anti-HRP supernatant was taken from mouse hybridoma cell lines. All preparations were recultured with antibody 1 and 3 days after dissection. Preparations that were cultured in the presence of IN-1 antibody were tested for regrowth of DiI-stained axons into the crush as described above. IN-1 was not tested for its effect on DRG fibers, since they grow until postnatal day 16.

Control Staining. To test the penetration of antibodies into the tissue, preparations were incubated for 2 days with IN-1 or anti-HRP. Preparations were then briefly rinsed in phosphate-buffered saline, fixed as whole mounts in 4% paraformaldehyde, embedded in 30% sucrose, and cryosectioned. Twenty-micrometer sections on glass slides were labeled with goat-derived secondary antibodies (Jackson ImmunoResearch) for 2 hr at room temperature at a dilution of 1:100 in the blocking solution (2% normal goat serum in phosphate-buffered saline). The biotinylated mouse μ-chain-specific secondary antibodies were detected with fluorescein-conjugated streptavidin (Jackson ImmunoResearch) applied for 1 hr at room temperature after 1:500 dilution in the blocking solution. Controls were carried out by (*i*) omitting the primary antibody, (*ii*) omitting the secondary antibody but not the primary antibody and avidin-coupled fluorescein (Fig. 3*B*), (*iii*) omitting primary and secondary antibody but not the avidin-coupled fluorescein, or (*iv*) testing for tissue autofluorescence due to paraformaldehyde fixation without fluorescein. In all controls insignificant background was detected.

RESULTS

Evidence for Regeneration of Transected Axons. Dye labeling experiments were made to determine whether growth across a lesion in neonatal spinal cord was due to regeneration of fibers that had been cut or to new growth of developing axons that arrived at the crush. DRG cells constituted a population of identified neurons that could be labeled before and after regeneration (Fig. 1). The carbocyanine dye DiO was first injected into a 5-day-old spinal cord caudal to the site of the future lesion to label dorsal tract fibers and their cell bodies in the ganglion. After 1 day a crush was made and 4 days were allowed for regeneration, after which the preparation was fixed. A second dye electrode coated with DiI $C_{18}(5)$ was inserted caudal to the crush to label the DRG axons that had grown through the lesion. Fig. 1 *a–c* show the effectiveness of filters that separated DiO and DiI labeling. In Fig. 1 *d–f* one doubly labeled cell stands out. In addition, DRG cells can be recognized that were stained by one or the other dye but not both. The DRG cells that were labeled only with the first dye, DiO (Fig. 1 *a* and *d*) had either not regenerated or failed to pick up dye applied after growth had occurred. The axons of cells labeled only with DiI (Fig. 1 *b* and *e*) had grown toward, into, and through the crush after the lesion had been made, or they had been transected but without having taken up the first label. The axons of DRG cell bodies that were doubly labeled had been cut and had regenerated. Exact determinations of regeneration compared with newly grown axons were difficult because the limited time in culture (5–7 days) and the localized application of dyes did not allow for complete labeling of all DRG cells by DiO or DiI. One rough estimate made by counting 89 cell bodies is that no fewer than 20% of the fibers had regenerated (18 cell bodies were doubly labeled). That all fibers are broken by such a crush and that growth occurs in both directions have been fully documented elsewhere (26, 27).

A second, simpler, unambiguous test for regeneration was used to demonstrate regeneration after treatment with IN-1 antibody (see below). In such experiments one label, DiI, was inserted into ventral tracts at a distance from the site of the future crush and then allowed to diffuse for 24–36 hr. The needle was then removed and the crush was made, breaking all fibers. Three to 4 days later, labeled fibers that had picked up the dye before the crush had been made grew into the crush site. Such fibers that had not yet reached the far side of the crush could not have picked up dye except by prior labeling. The cell bodies were within the spinal cord at various levels up to the brainstem.

Regeneration After the "Critical Period" in the Presence of IN-1 Antibody. Fig. 2*A* shows the failure of outgrowth in a control preparation from a 13-day-old animal; in comparison, Fig. 2*B* shows the regeneration that occurred in the presence of IN-1, an antibody which blocks the growth-inhibitory effect of myelin-associated protein NI-35/250 (21). Similar results with clear outgrowth were obtained in 22 out of 39 injured spinal cords that were isolated from 13- to 17-day-old animals

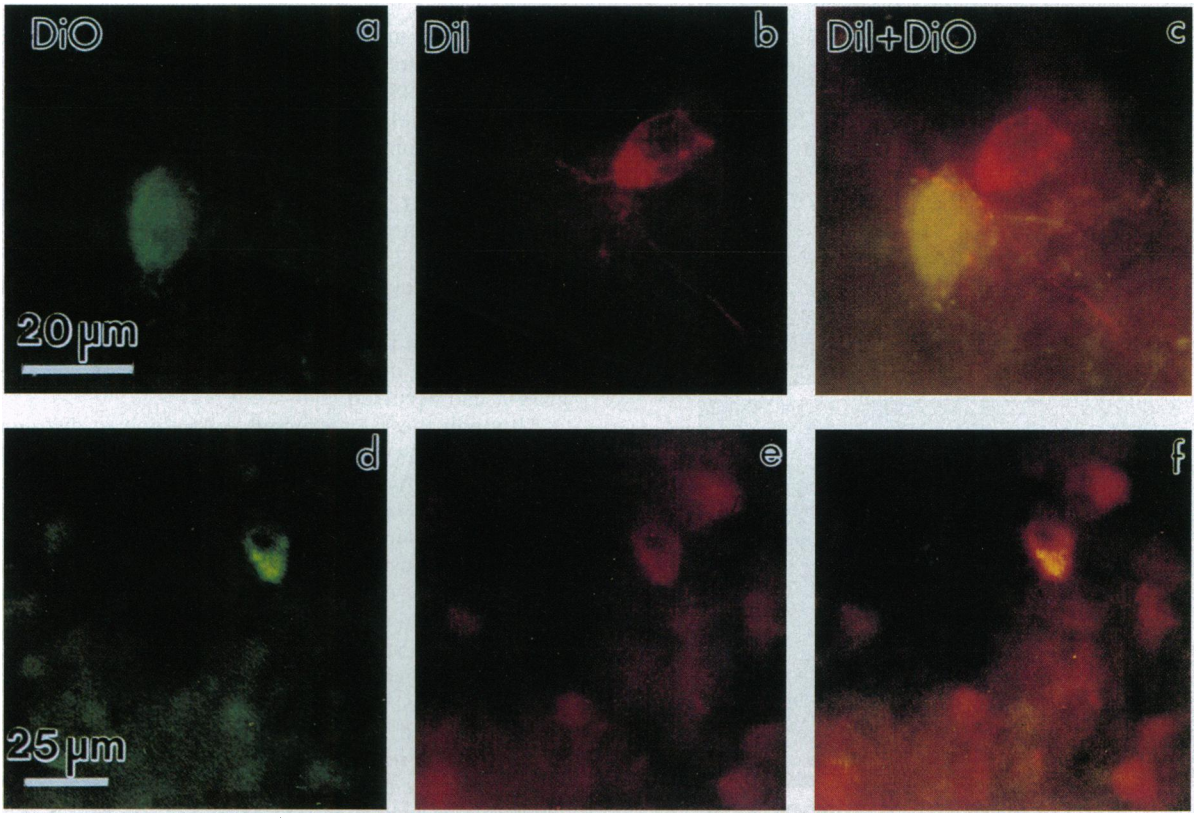

FIG. 1. Double labeling of regenerating DRG cells. DRG cells were labeled first by DiO applied immediately after the CNS of a 5-day-old opossum had been placed in culture. After 1 day in culture the spinal cord was crushed between the dye electrode and the DRG. Four days later the preparation was fixed and DiI was applied close to the original DiO injection. Cells were either red (DiI) or green (DiO), if singly labeled and viewed in their appropriate filter, or bright orange, if doubly labeled and doubly exposed. *a–c* show that separation was complete for DRG cells in ganglion whole mounts that had been labeled with DiO (*a*) or DiI (*b*). A single DRG cell which was clearly labeled by both dyes in *d* and *e* appears as bright orange in *f*. The axon of this cell had been labeled by the first dye, DiO, and had regenerated to be stained by the second dye, DiI. Other cells labeled by DiO or DiI, but not both, are also visible (see text). Color values change with double exposure.

and incubated in culture medium that contained IN-1 (see *Materials and Methods*). The outgrowth was unambiguous; it was, however, shorter and less abundant than that seen in spinal cord lesions of younger animals. The proportion of animals showing successful regrowth was comparable to that seen in animals aged 3–9 days (5). As late as 17 days, when the spinal cord is larger and more developed but continues to survive well in culture, outgrowth still occurred when IN-1 had been added. The results are summarized in Table 1.

That true regeneration of transected fibers occurred in older animals after application of IN-1 was demonstrated by showing that axons that had previously been labeled grew into the crush. As before, while accurate estimates could not be made, 20% represents a lower limit for regenerating compared with newly grown fibers. In contrast to the experiments with IN-1, no fibers entered the crushes of 34 control preparations, treated in the same way but without antibody, from animals aged 13 days. The number 34 refers to the present control series, which was carried out in parallel with those preparations treated with the antibody. In previous experiments published elsewhere, failure at >12 days has been shown in >50 animals (5, 33).

Additional tests were made to determine the penetration of antibodies into the isolated spinal cord and the effect of IN-1. Fig. 3 shows the association of IN-1 with cellular structures in the ventral spinal cord of a 13-day-old opossum after application of the antibody for 2 days. The staining pattern corresponded to the distribution of myelin and oligodendrocytes at this stage, which are more prevalent in ventral regions of the cord. Diffuse staining was present in dorsal spinal cord regions. Access was presumably through the open floor of the fourth ventricle and channels to the cerebral vesicles and spinal aqueduct through cut roots. Anti-HRP antibody was applied to test for penetration and specificity. Anti-HRP antibody penetrated the spinal cord but, as expected, stained diffusely with no obvious differences among dorsal, ventral, and lateral regions of the cord. In eight experiments in which spinal cords of 13-day-old animals were treated with anti-HRP antibody, no growth of fibers occurred into the lesion.

DISCUSSION

Regeneration Compared with New Growth. The principal difference between the present experiments and those made on regeneration in cats, rats, hamsters, and the North American opossum (1–3, 6) is in the use of an isolated CNS in culture. From earlier work on *Monodelphis* it was known that nerve fibers grew through a crush or even a cut (28) over long distances, reliably and profusely. Within 5 days it becomes possible to observe the outgrowth because one can follow stained axons in the living preparation day by day and because the circumscribed lesion is uncomplicated by inflammation or bleeding. Hence, the time required for a reliable bioassay is much shorter than the days or weeks required *in vivo*. On the other hand, culture entails certain obvious disadvantages. One disadvantage is the short time available for double labeling. It is known that the fine structure, electrical activity, and viability are preserved for the 5 days required for regeneration without detectable deterioration (25, 30). Our experience with the opossum CNS in culture suggests that this time might be extended by systematically optimizing the conditions; nevertheless 5–6 days is the maximum we can safely use at present.

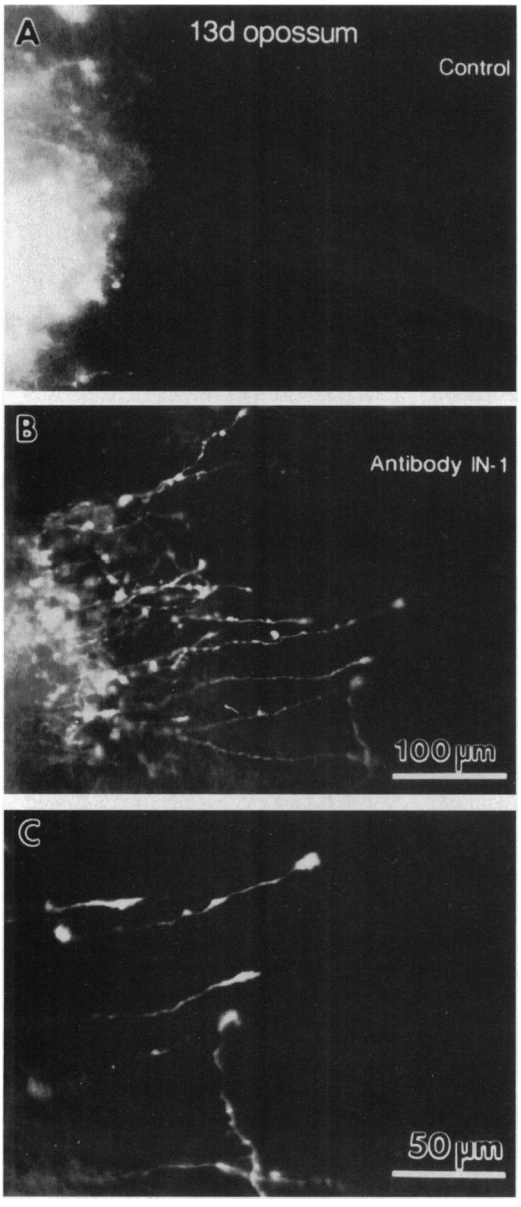

FIG. 2. Effect of IN-1 antibody on neurite outgrowth after the critical period. (*A*) Crush region in a 13-day-old opossum CNS maintained in culture for 5 days without antibody. In this and 33 other preparations no growth into the crush occurred. (*B*) A similar 13-day-old opossum CNS treated with IN-1 antibody shows outgrowth into the crush. (*C*) Growth cones in *B* at higher magnification. Such results were obtained in 22 out of 39 preparations from animals aged 13 days or more.

As a result, there is limited time available for label to be taken up by axons and transported to cell bodies before the crush is made, if sufficient time is to be allowed for the next step, regeneration. For DRG cells the distance is short. Cells whose axons had regenerated were clearly labeled by both dyes: DiO applied before the lesion had been made and DiI picked up by cut axons that had grown across the crush. Unfortunately, DRG cells are unsuited for antibody tests with IN-1 because they continue to regenerate up to postnatal day 16 (33) and because the CNS beyond postnatal day 18 becomes too large to culture reliably.

Table 1. Regeneration after the critical period with IN-1 antibody

Experiment	Age, days	Preparations with neurite growth, no./total
No IN-1	13–17	0/34
Anti-HRP	13	0/8
IN-1	13	22/39
IN-1	15–17	4/7

For other neurons that regenerated in ventral spinal cord, the single-label results were equally unambiguous. In these tests, fibers that had been labeled before the crush was made had regrown into the lesion site but not beyond it at the time of observation. Consequently they could not have picked up label after having grown, since they had not yet reached the area containing the dye-coated electrode. A large number of fibers did reach the other side in most experiments. For such neurites it was impossible to determine from which direction growth had occurred.

Although these experiments allow one to distinguish between new growth of undamaged fibers into the lesion and true regeneration of cut fibers, they do not provide reliable quantitative estimates for the relative numbers for the following reasons. First, the dye was applied for only 1 day before the crush was made, and the crystals remained attached to the electrode (this of course is an advantage in one respect, since it means that DiI does not label regions away from the site of application). Hence, not all regenerating fibers would have been labeled before the crush was made. Similarly, the second label also failed to mark all the fibers that had grown beyond the crush in double-label experiments. From our counts of doubly labeled cell bodies in the DRG, we estimate that ≈20% (as a lower limit) of the fibers that grew back represent true regeneration as opposed to new growth by undamaged neurons.

Regeneration After the Critical Period in the Presence of IN-1 Antibody. A clear advantage of cultured spinal cord is that molecules can be simply applied to the bathing fluid in known concentrations and tested rapidly for their effects. By contrast, in animals the application by antibody-secreting cells or by micropumps is technically demanding and difficult to control with precision for prolonged periods. What was clear was that in spinal cords of 17-day-old animals, fibers grew into and through a crush in the presence of IN-1. In no preparation without IN-1 dissected from animals aged 13 days or more have we ever seen growth of CNS neurons after cervical spinal cord lesions. Moreover, the effect was specific for IN-1 and was not reproduced by an antibody against HRP. Both antibodies were shown to enter the cord but stained differently as predicted. The large molecules presumably entered the cord through leakage sites along roots, in pia, and over ventricles.

That the outgrowth in a 13- to 17-day-old CNS in medium with IN-1 was less profuse than that in younger animals was not surprising. It has been shown elsewhere that growth-inhibitory proteins associated with myelin accumulate after postnatal days 9–12 in *Monodelphis* spinal cord (5). The inhibitory action is not, however, completely blocked by IN-1 (5), suggesting the presence of additional inhibitors such as myelin-associated glycoprotein (31, 32).

These results confirm that one of the mechanisms that prevents spinal cord regeneration at the time that repair comes to an end is the development of myelin-associated growth-inhibitory proteins. That these are not the only factors seems likely. Mechanisms that might be involved include decreased capacity for growth in more mature neurons, changes in receptors on their surfaces, changes in concentrations of trophic factors and guidance molecules, and the development of scars and other repellent mechanisms. In culture, one can hope to test rapidly and quantitatively such factors singly and in combination.

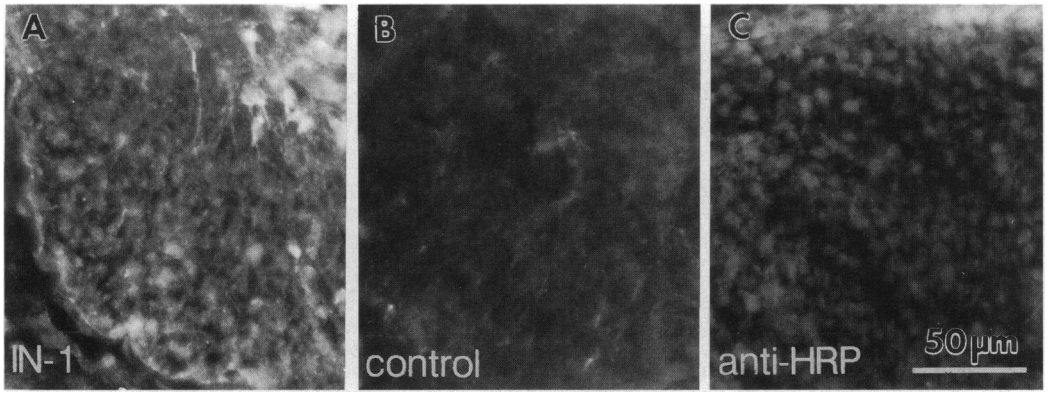

FIG. 3. Penetration of IN-1 antibody into spinal cord in culture. (*A*) Staining pattern of IN-1 showing oligodendrocytes, myelin, and patchy stain in the ventral part of the spinal cord after application of antibody for 1 day. (*B*) Control experiment in the absence of secondary antibody. (*C*) An IgM antibody against HRP also shows penetration of the spinal cord. The presence of anti-HRP antibody did not promote regeneration in animals aged ≥13 days. Anti-HRP was chosen as a control because, like I-N1, it is an IgM antibody.

We are grateful to Dr. W. B. Adams for his suggestions and for critical reading of this manuscript. We are grateful to Mr. P. Bättig for photography. This work was supported by grants from the Swiss National Fund and the International Research Institution for Paraplegia to J.G.N.

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