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Immunotyping of radial glia and their glial derivatives during development of the rat spinal cord

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Summary

The differentiation of glia in the central nervous system is not well understood. A major problem is the absence of an objective identification system for involved cells, particularly the early-appearing radial glia. The intermediate filament structural proteins vimentin and glial fibrillary acidic protein have been used to define the early and late stages, respectively, of astrocyte development. However, because of the non-specificity of vimentin and the temporal overlap in expression patterns of both proteins, it is difficult to refine our view of the process. This is especially true of the early differentiation events involving radial glia. Using the developmentally-expressed intermediate filament-associated protein IFAP-70/280 kD in conjunction with vimentin and glial fibrillary acidic protein markers, a comprehensive investigation of this problem was undertaken using immunofluorescence microscopy of developing rat spinal cord (E13–P28 plus adult). The phenotypes of the cells were defined on the basis of their immunologic composition with respect to IFAP-70/280 kD (I), vimentin (V) and GFAP (G). A definitive immunotype for radial glia was established, viz, $I^+/V^+/G^-$; thus reliance upon strictly morphological criteria for this early developmental cell was no longer necessary. Based upon the immunotypes of the cells involved, four major stages of macroglial development were delineated: (1) radial glia ($I^+/V^+/G^-$); (2) macroglial progenitors ($I^+/V^+/G^+$); (3) immature macroglia ($I^-/V^+/G^+$); and (4) mature astrocytes ($I^-/V^+/G^+$ primarily in white matter and $I^-/V^-/G^+$, the predominant type in gray matter). It is of interest to note that the cells of the floor plate were distinguished from radial glia by their lack of IFAP-70/280 kD immunoreactivity. Introduction of the IFAP-70/280 kD marker has therefore provided a more refined interpretation of the various differentiation stages from radial glia to mature astrocytes.

Introduction

The nature of astrocytic cell development within the CNS is poorly understood due to the difficulty of tracking individual cell lineages through the complexity of CNS development. Radial glia are generally considered pivotal for deciphering macroglial differentiation (Ramon y Cajal, 1911; Rakic, 1972). They have been classically defined as those early, elongated glial cells that span in a radial fashion from the ventricular to the subpial surface. Traditionally it has been difficult to identify these cells definitively based on morphologic criteria because of the technical problem of showing continuity of cell length via microscopy. Moreover, radially disposed astrocytes exist in many regions of the adult CNS (Bignami & Dahl, 1974; Schnitzer *et al.*, 1981; Liuzzi & Miller, 1987; Schnitzer, 1988), thereby throwing into doubt the accuracy of strict morphologic criteria in defining these cells and their interrelationships. Clearly, there is a need for more rigorous definition of radial glia and other early differentiation stages leading to macroglia.

One way to do this is through the use of multiple protein markers to derive a specific, composite phenotype for a cell at any given developmental stage. This would permit not only cataloguing of the various members of the astrocytic cell lineage but also the delineation of a probable sequence of progenitor to derivative cells during differentiation. In this context, intermediate filament (IF) structural proteins, which exhibit a high degree of tissue specificity (Lazarides, 1980; Steinert & Roop, 1988; Goldman & Steinert, 1990; Fliegner & Liem, 1991; Albers & Fuchs, 1992), have proven useful. Thus, glial fibrillary acidic protein (GFAP), the glia-specific IF structural protein, has been widely used as a marker for astrocytic cells (Eng *et al.*, 1971; Bignami *et al.*, 1972; Goldman *et al.*, 1978; Bongcam-Rudloff *et al.*, 1991; Weinstein *et al.*, 1991). However, the IF composition of cells of astrocytic lineage undergoes dramatic changes during development (for review, see Fedoroff, 1986; Lukas *et al.*, 1989; McDermott & Lantos, 1989). For example, the keratin

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IF proteins initially expressed in rodent neuroectodermal cells are replaced by vimentin after the formation of the neural tube (Jackson *et al.*, 1981; Houle & Fedoroff, 1983; Lehtonen *et al.*, 1983; Chisholm & Houliston, 1987). In the subsequent developmental stage of radial glia, vimentin becomes the predominant, if not sole, IF structural protein (Dahl *et al.*, 1981b; Schnitzer *et al.*, 1981; Bignami *et al.*, 1982). As development proceeds to the stage when immature astrocytes can be recognized, both vimentin and GFAP are now present (Dahl, 1981; Raju *et al.*, 1981; Pixley & De Vellis, 1984; Gasser & Hatten, 1990). Finally, in many astrocytes of adult rodent CNS, especially those of gray matter, the expression of vimentin ceases, while that of GFAP continues (Schnitzer *et al.*, 1981; Bovolenta *et al.*, 1984; Landry *et al.*, 1990).

Despite the usefulness of IF structural proteins as differentiation markers in the study of astrocyte development, their utility is limited due to extensive overlap in their temporal expression patterns. Thus, the use of vimentin as a marker for early (radial) glia is compromised by the fact that its expression continues in many mature, GFAP-containing astrocytes, as well as in most astrocytes in primary cultures (Chiu *et al.*, 1981; Dahl *et al.*, 1981a; Osborn *et al.*, 1981; Schnitzer *et al.*, 1981; Shaw *et al.*, 1981; Valentino *et al.*, 1983; Bovolenta *et al.*, 1984; Schiffer *et al.*, 1986; Yang *et al.*, 1992b). Therefore, these proteins alone are unable to provide a highly resolved view of glial cell differentiation.

Intermediate filament-associated proteins (IFAPs) represent another group of IF proteins which hold promise in this regard (IFAP-48 kD: Abd-El-Basset *et al.*, 1988a,b; IFAPa-400: Chabot & Vincent, 1990). Recently, we have identified a new glial IFAP, IFAP-70/280 kD, in a subpopulation of non-stellate astrocytes in primary cultures prepared from neonatal rat brain (Yang *et al.*, 1992b). In the present immunofluoresence study, the expression of IFAP-70/280 kD in the context of the known vimentin/GFAP IF transition which occurs during astrocyte differentiation was investigated in rat spinal cord during development. In this manner a refined definition of radial glia and their macroglial derivatives *in situ* was obtained, based on antigenic phenotypes defined by the presence or absence of these three IF protein markers. Thus, our data presents a detailed description of the differentiation of radial glia into their derivatives in the spinal cord.

Materials and methods

Animals

Pregnant Sprague-Dawley rats of known gravid stage were purchased from Charles River Breeders (Wilmington, MA). The day of conception was designated as embryonic day 0 (E0) and that of birth as postnatal day 0 (P0). Developing rats from E13 to P28 and adult male rats were utilized for the study. Spinal cord specimens were collected at 12 h intervals in order to capture events at intermediate times of each developmental day. The spinal cord samples at the lower thoracic level were surgically removed and frozen immediately in Freon/liquid N_2 for cryostat sectioning.

Antibodies

The mouse monoclonal antibody to IFAP-70/280 kD (anti-IFAP-70/280 kD) was produced and characterized as previously described (Yang *et al.*, 1992a,b). This antibody was produced using as antigen native IF preparations from baby hamster kidney cell line (BHK-21C) (Yang *et al.*, 1992a). The antibody has been shown to stain differentiating, non-stellate astrocytes in cultures from neonatal rat brain and radial glia in developing rat cerebral cortex (Yang *et al.*, 1992b). Rabbit anti-vimentin was produced against the same antigen purified from BHK-21 cells and characterized previously (Yang *et al.*, 1985). Rabbit and anti-GFAP (Incstar Corp; Stillwater, MN), mouse monoclonal anti-vimentin (ICN ImmunoBiologicals, Lisle, IL), and mouse monoclonal anti-68 kD neurofilament protein (Hybritech, Inc., San Diego, CA) were purchased commercially. The specificity of these antibodies was tested by immunoblotting on IF-enriched preparations and immunofluorescence microscopy on sections in various tissues and cell lines which contain specific IF proteins. Fluorescein isothiocyanate (FITC)- and rhodamine-conjugated secondary goat anti-mouse or anti-rabbit antibodies were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Immunofluorescence microscopy

Cryostat sections of spinal cord specimens were mounted on gelatin-coated slides, fixed in methanol (−20° C) for 5 min and air dried. Immunostaining was performed as previously described (Yang *et al.*, 1985, 1992b). In brief, each slide was overlaid with 60 µl of primary antibody and incubated overnight at 4° C in a humidified chamber. Following extensive rinsing with phosphate buffered saline, the slide was incubated with 60 µl of appropriate fluorochrome-conjugated secondary antibody at 37° C for 1 h. Slides were subsequently washed with distilled water and mounted with coverslips in Fluoromount (Sigma; St Louis, MO). For double-label immunofluorescence, a mixture of designated mouse and rabbit antibodies was applied as primary antibodies while a mixture of appropriate rhodamine-conjugated and FITC-conjugated antibodies was used as secondary antibodies. Various controls were used to check the specificity of the immunostaining as previously described (Yang *et al.*, 1985).

Preparations were examined with a Zeiss Axiophot photomicroscope equipped with epifluorescence optics employing a mercury lamp (Carl Zeiss, Inc.; Thornwood, NY). Fluorescence micrographs were photographed on Kodak Plus-X film and developed with Diafine (Acufine, Inc., Chicago, IL).

Results

The use of antibodies to three IF structural proteins (i.e, vimentin, GFAP and 68 kD neurofilament

protein) and anti-IFAP-70/280 kD in multiple double-label immunofluorescence microscopy enabled us to delineate radial glia and their derivatives in the developing rat spinal cord. The immunoreactivities of anti-IFAP-70/280 kD in conjunction with those of anti-vimentin and anti-GFAP allowed subdivision of the E13–P28 time period for four distinct developmental stages with respect to macroglial differentiation. It should be noted that the developmental program for individual cells varies in the spinal cord, showing a ventral to dorsal differentiation gradient. Thus, the cells of the ventral cord usually mature two days before those of the dorsal cord. Within the context of these developmental stages the events visualized through the use of antibodies to IF proteins are described.

Anti-neurofilament protein 68 kD (anti-68 kD) reactivity was employed in this study as another indicator of spinal cord development (Altman & Bayer, 1984). The results also showed that developing macroglia were immunologically distinct and spatially separate from the anti-68 kD reactive neurons.

Stage I: radial glia ($I^+/V^+/G^-$)

Radial glia (Ramon y Cajal, 1911; Rakic, 1972) were the spinal cord cells that morphologically typified this stage. In the present study, these cells were characterized immunologically by the expression of vimentin and IFAP-70/280 kD, with no evidence of GFAP ($I^+/V^+/G^-$). It is interesting to note that immunofluorescence microscopy showed limited expression of this IFAP during development; while most cells of the

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Figs 1–3. Immunofluorescence staining in serial sections of an E13 rat spinal cord with monoclonal antibodies directed to IFAP-70/280 kD (Fig. 1), vimentin (Fig. 2), and 68 kD neurofilament protein (Fig. 3). While immunoreactivity of 68 kD protein was localized in the post-mitotic neuronal elements in the mantle and marginal layers of the developing rat spinal cord, those of both vimentin and the IFAP were found in the radial glia. Note that the expression of the IFAP was specifically found in the radial glia. In contrast, vimentin was widely expressed in the E13 rat embryo (e.g., the meninges surrounding the spinal cord). Figures are oriented with dorsum of spinal cord at top. Scale bar = 100 μm.

Figs 4 and 5. Double-label immunofluorescence staining in an E13 rat spinal cord with monoclonal anti-IFAP-70/280 kD (Fig. 4) and rabbit anti-vimentin (Fig. 5). Co-localization of these two IF proteins was shown in most radial glia. The cells of the floor plate region do not contain the IFAP. Scale bar = 100 μm.

Fig. 6. Immunofluorescence pattern of IFAP-70/280 kD in an E15 rat spinal cord. See text in Results for changes in the radial glia and the floor plate at this late phase of the pre-GFAP spinal cord. Scale bar = 100 μm.

Fig. 7. Immunofluorescence pattern of the 68 kD neurofilament protein in an E15 rat spinal cord. See text for changes in the neuronal elements at this late phase of the pre-GFAP spinal cord. Scale bar = 100 μm.

Figs 8 and 9. Double-labelling with the monoclonal anti-IFAP-70/280 kD (Fig. 8) and rabbit anti-GFAP (Fig. 9) demonstrated the absence of the GFAP immunoreactivity in the IFAP-containing E15 radial glia in the ventrolateral portion of the spinal cord. Scale bar = 100 μm.

Figs 10 and 11. Immunofluorescence patterns of IFAP-70/280 kD (Fig. 10) and the 68 kD neurofilament protein (Fig. 11; dorsum of spinal cord located to right) in an E16 rat spinal cord at which time the GFAP immunoreactivity was first observed. See text for details. Scale bar = 100 μm.

Figs 12 and 13. Double-labelling with the monoclonal anti-IFAP-70/280 kD (Fig. 12) and rabbit anti-GFAP (Fig. 13) in an E16 rat spinal cord demonstrated the first expression of GFAP immunoreactivity in the IFAP-containing radial glia in the marginal zone of the ventrolateral spinal cord (arrow heads). Scale bar = 100 μm.

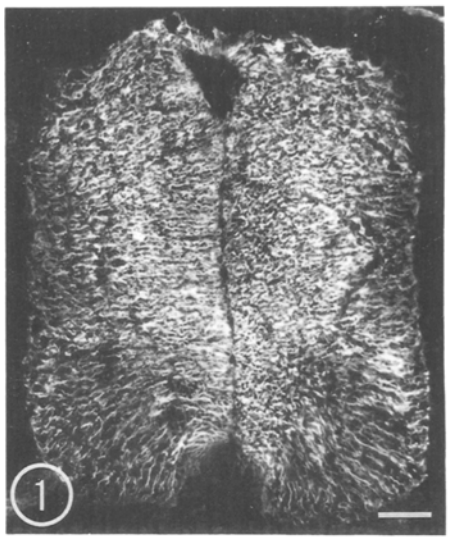
Figs 14 and 15. Double-labelling with monoclonal anti-vimentin (Fig. 14) and rabbit anti-GFAP (Fig. 15) in an E16 rat spinal cord demonstrated the appearance of GFAP in these vimentin-containing macroglial cells of the ventrolateral region (arrow heads). Scale bar = 100 μm.

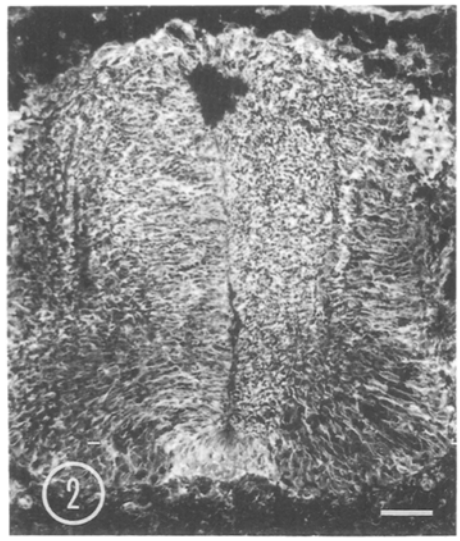
Fig. 16. Immunofluorescence staining of anti-GFAP in an E18 rat spinal cord demonstrated that the expression of GFAP in the developing macroglial cells was expanding from the ventrolateral to the lateral region of the marginal zone. Note the two processes that are sent to the subpial surface by a GFAP-containing cell (upper right) which is located in the marginal zone of the lateral spinal cord. Scale bar = 100 μm.

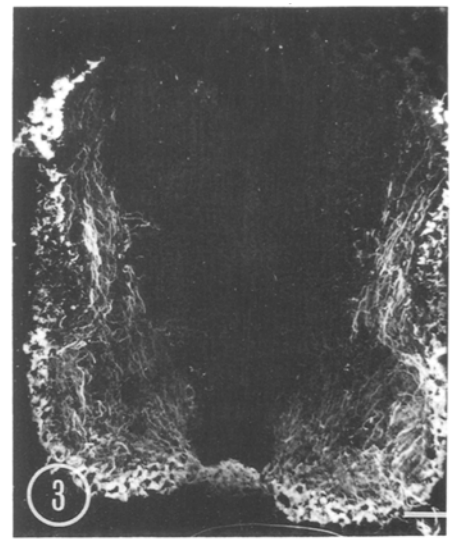
Fig. 17. Immunofluorescence staining of anti-GFAP in an E21 rat spinal cord demonstrated that the expression of GFAP in the developing macroglial cells had now expanded into the dorsolateral regions of the spinal cord (dorsum of spinal cord located to the left). The GFAP-containing cells were also observed deeper in the spinal cord. Note the lack of GFAP immunoreactivity in the central regions, including the dorsal median septum and the dorsal white columns (left). Scale bar = 100 μm.

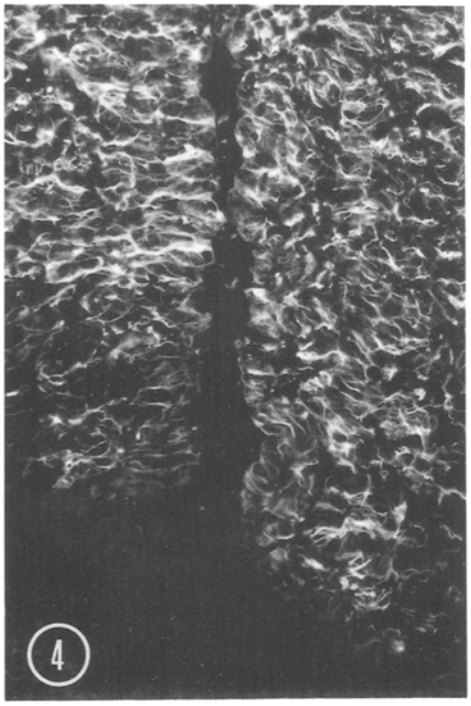
Figs 18 and 19. Double-labelling with the monoclonal anti-IFAP-70/280 kD (Fig. 18) and rabbit anti-GFAP (Fig. 19) in an E18 rat spinal cord demonstrated that the expression of GFAP occurred in IFAP-70/280 kD-containing radial glia as macroglial development expanded into the marginal zone of the lateral spinal cord. Scale bar = 100 μm.

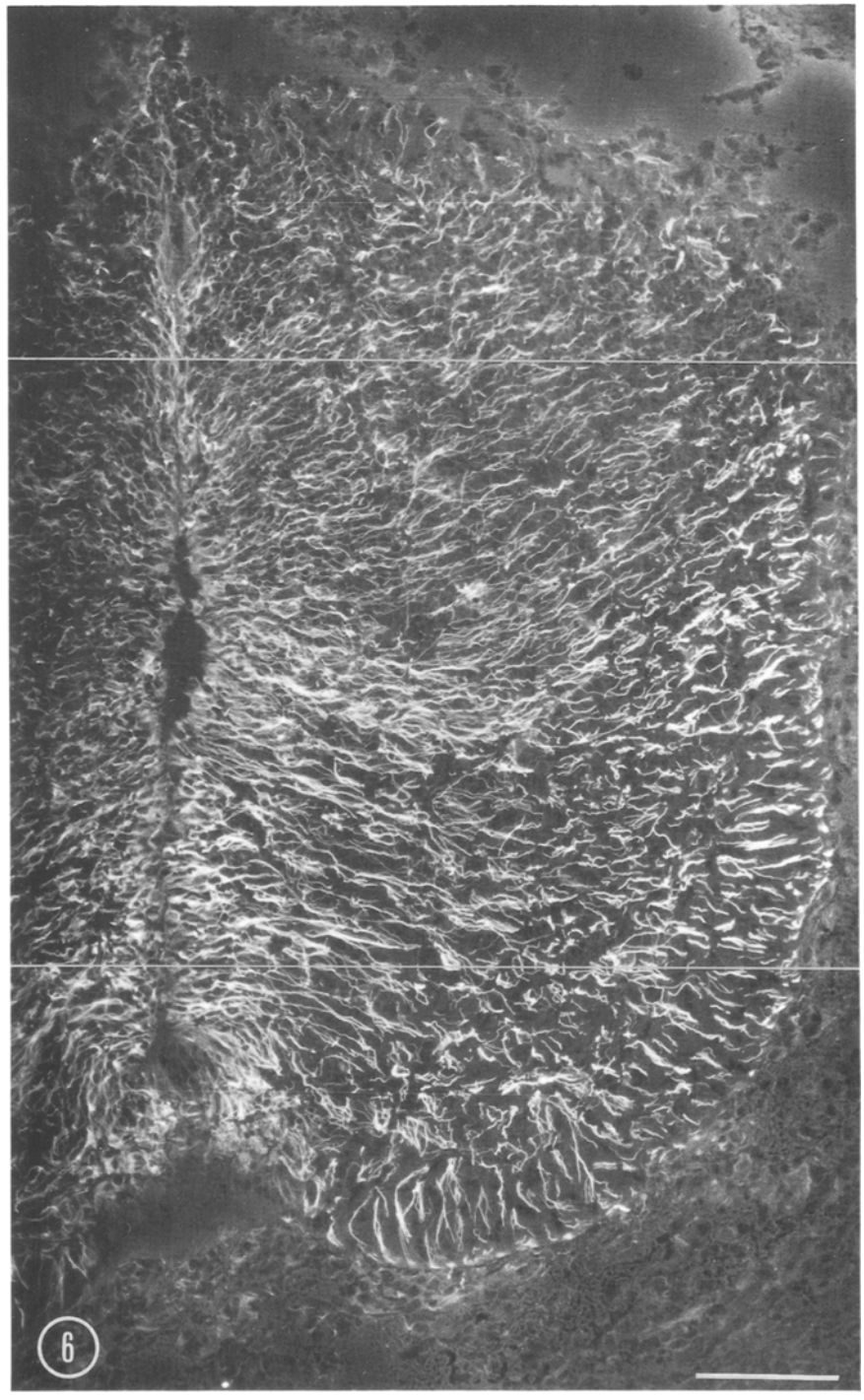
Figs 20 and 21. Double-labelling with the monoclonal anti-IFAP-70/280 kD (Fig. 20) and rabbit anti-GFAP (Fig. 21) in a PO rat spinal cord demonstrated that the expression of GFAP occurred in IFAP-70/280 kD-containing radial glia as macroglial development expanded into the deep mantle gray region. Scale bar = 100 μm.

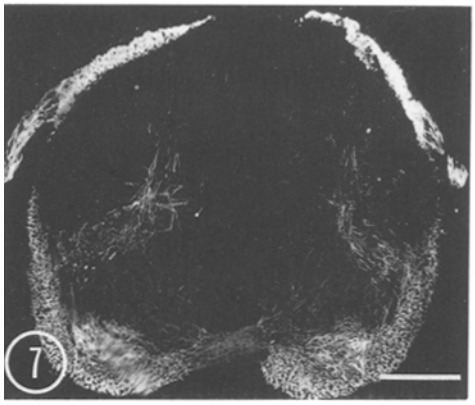








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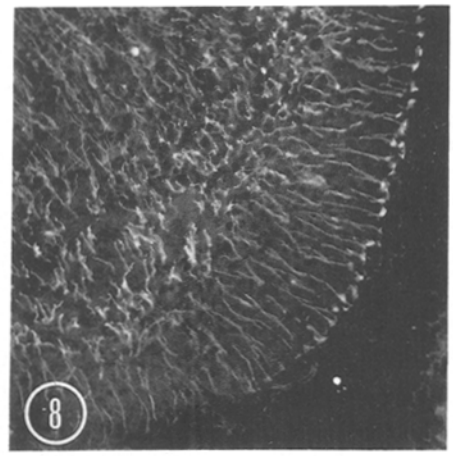
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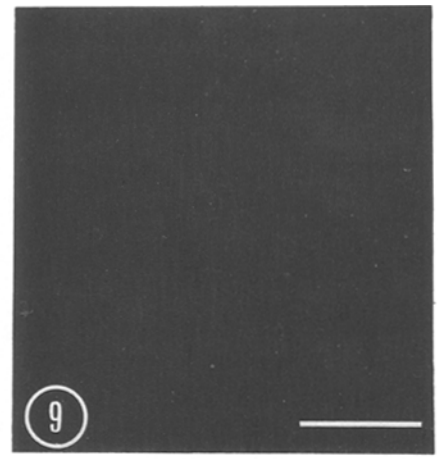
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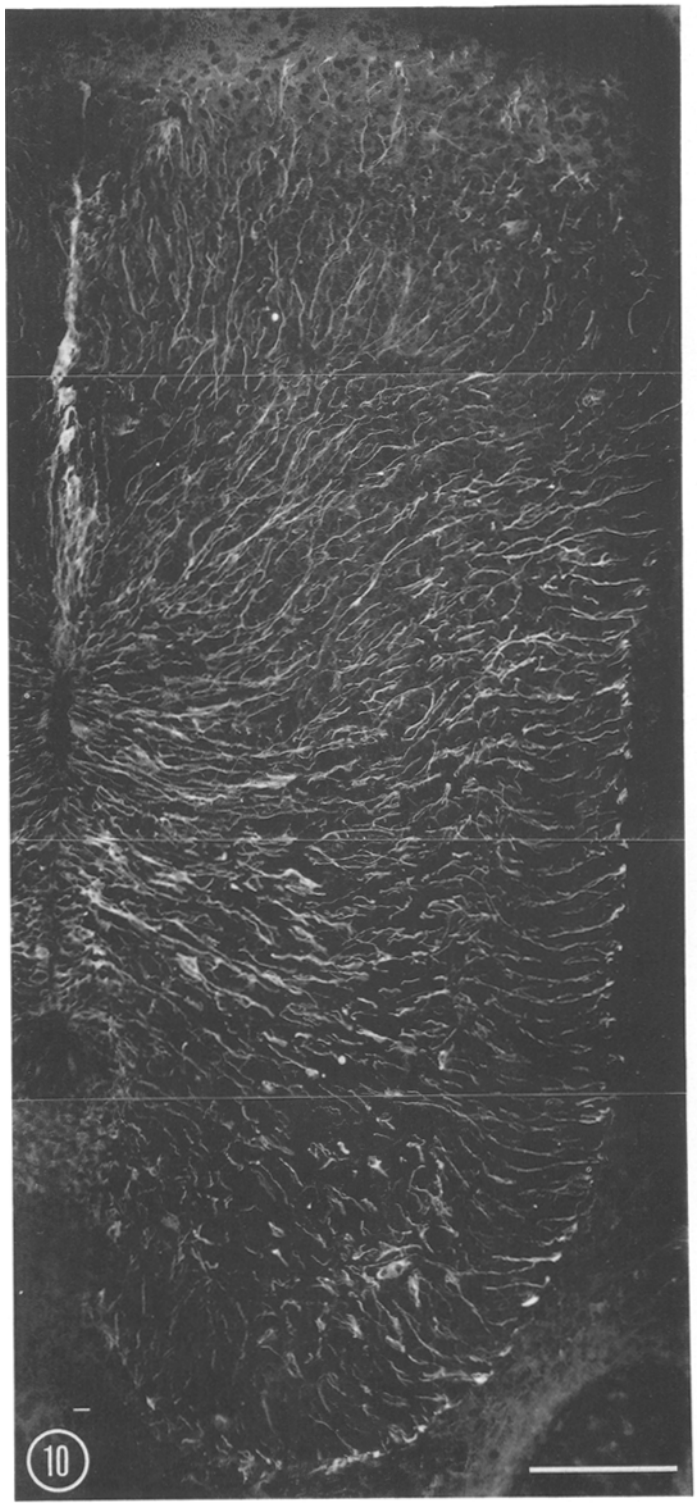
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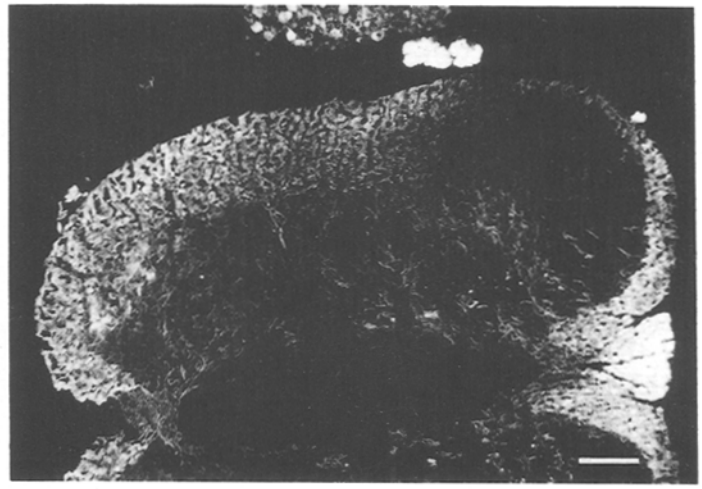

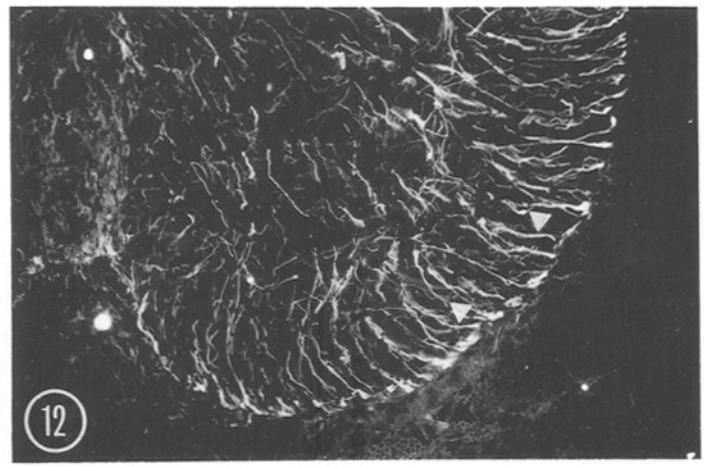
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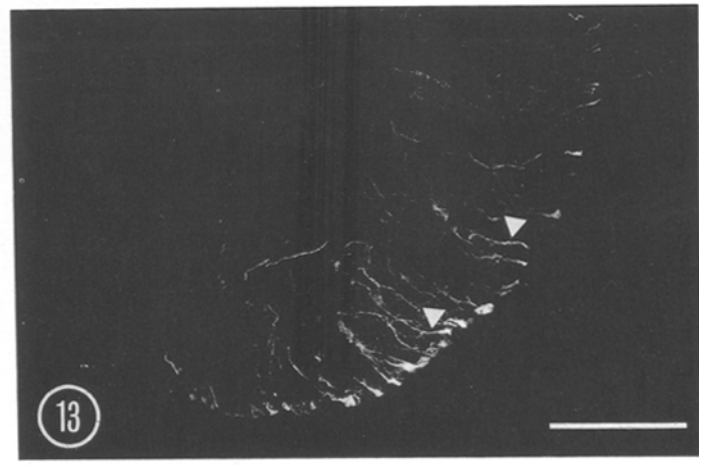
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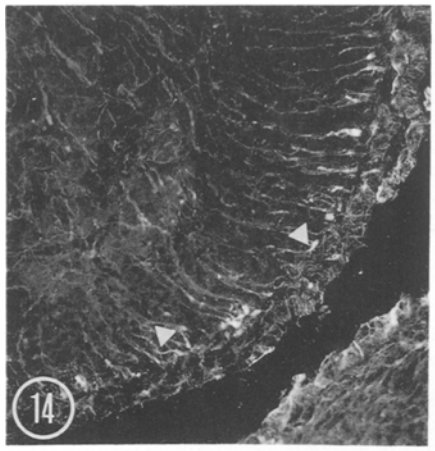
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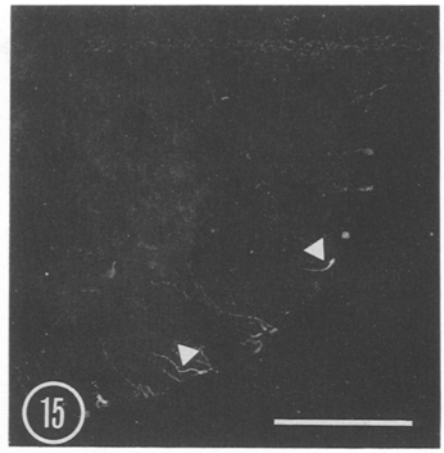
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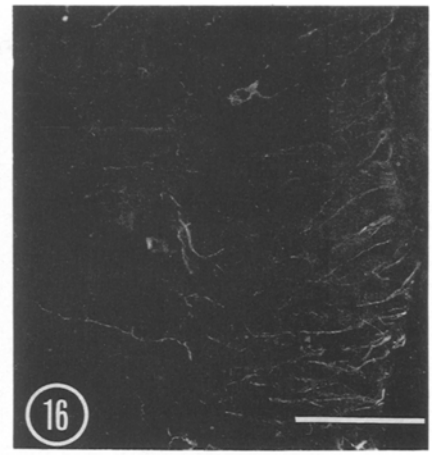
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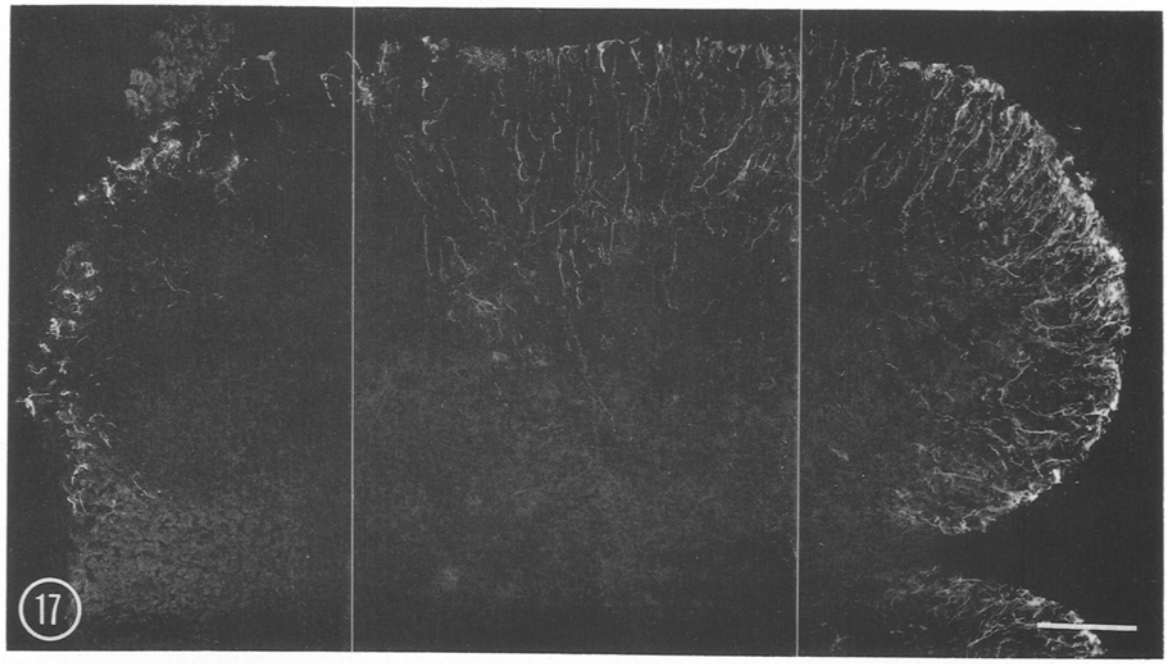
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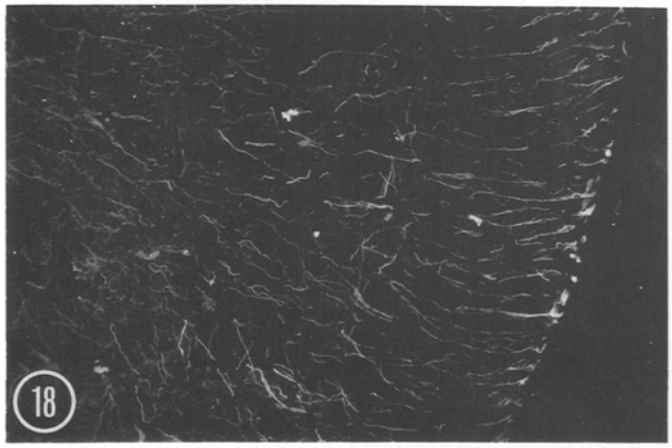
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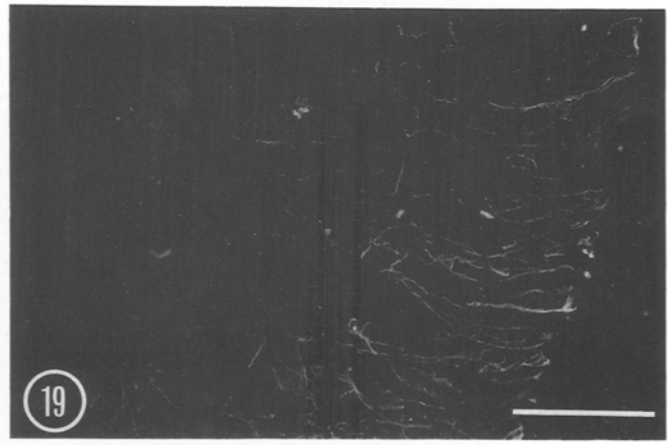
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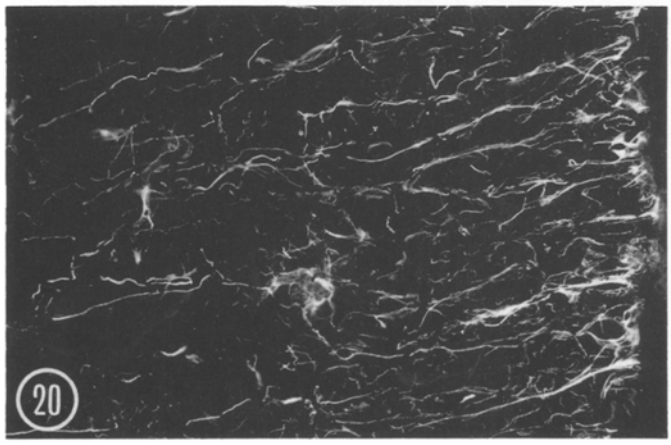
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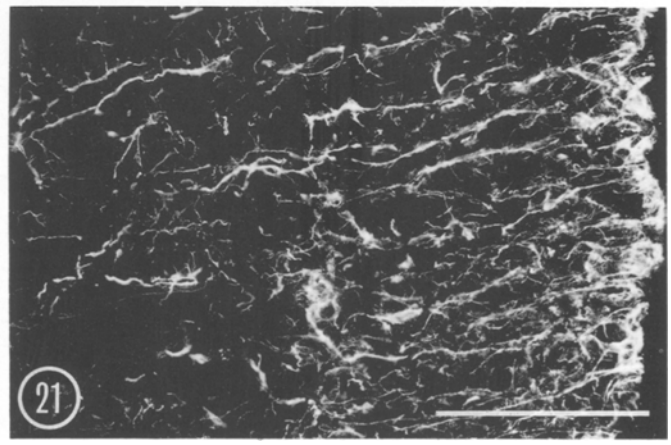
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embryo were vimentin-positive, only radial glia and developing muscle cells were IFAP-positive. In the spinal cord, IFAP-70/280 kD and vimentin had very similar distribution patterns which were different from that of the 68 kD neurofilament protein (Figs 1–3). Further studies by double-label immunofluorescence microscopy demonstrated that all IFAP-positive cells were vimentin positive with the exception of the obvious absence of the IFAP from the floor plate (Figs 1, 2, 4, 5) (cf. Hirano *et al.*, 1991). In the late phase of this stage, the size of the floor plate was decreased (Fig. 6). In addition, the overall organization of the radial glia changed as differentiation advanced. The formation of distinct bundles from the IFAP-positive radial glia (Fig. 6) now contrasted greatly with the dispersed distribution of the radial glial cells seen in the early phase. The cell density in the spinal cord also appeared to be decreased, especially in the ventral mantle and marginal zones, presumably due to the massive increase in neuronal cell bodies and fibres (Fig. 7). This developmental stage was in existence in the earliest embryos examined in this study (E13) and persisted as long as the radial glia remained GFAP-negative (E16 and later) (Figs 8, 9).

Stage II: glial progenitors ($I^+/V^+/G^+$)

In lower thoracic spinal cord, GFAP immunoreactivity was first detected at E16. The major morphologic feature of the spinal cord when GFAP expression started was the beginning of obliteration of the dorsal portion of the neural canal in the formation of the dorsal septum. This was highlighted by the condensation of radial glia to form a thick, vertically oriented IFAP-positive band at the site of the dorsal septum (Fig. 10) (cf. Gilmore, 1971; Skoff, 1990). This apparent medial movement of glial cells in the dorsal half of the cord was also indicated by the medial disposition of the dorsal roots and the first appearance of the dorsal column as demonstrated by anti-68 kD (Fig. 11, as compared with Figs 3, 7).

This period was characterized by the acquisition of GFAP by the presumptive macroglial cells, thereby transforming them into $I^+/V^+/G^+$ cells, which are considered the immediate macroglial derivatives of the radial glia. The GFAP expression was first observed in the ventral spinal cord (Figs 12, 13).

This transformation always began in the marginal white matter in cells that apparently lose their attachment to the central ventricular surface (Figs 12–16), indicating an intimate temporal association with apparent loss of continuity of the radial glia with the ventricular surface. During the following week, GFAP expression advanced dorsally in the marginal zone of the cord, and subsequently proceeded deeper into the mantle gray matter (Figs 16–21). Expression of GFAP continued to appear in detached cells since it was not observed in cells abutting the venticular surface (Fig. 17). Individual macroglia could be visualized within the marginal zone, i.e., the developing white matter (Fig. 16). It is of interest to note that individual glial fibres did not necessarily represent separate cells since macroglial cells at this stage were often seen to have multiple cellular processes (Fig. 16).

By double-label immunofluorescence it was shown that GFAP expression was always initiated within I^+/V^+ cells (Figs 12–15, 18–21). Thus, these macroglial cells ($I^+/V^+/G^+$) were shown to be the direct derivatives of the radial glial. Moreover, radial glia ($I^+/V^+/G^-$) were definable immunologically by their IFAP expression and morphologically by their central attachment. Double-label analysis with anti-GFAP and anti-68 kD demonstrated that the glial cells were distinct from the neurons.

The last structures to be encompassed by the wave of GFAP acquisition were the dorsal funiculi and the dorsal septum (Figs 22, 23). GFAP extension centrally into the ventral gray zone was already well advanced by the time IFAP-positive cells in these dorsal structures were just initiating expression of GFAP. As in the more ventral cord areas, the acquisition of GFAP in these dorsal elements proceeded from the periphery

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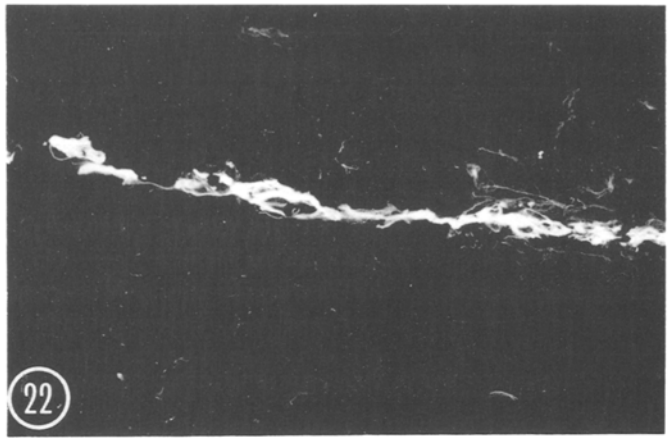
Figs 22 and 23. Double-labelling with the monoclonal anti-IFAP-70/280 kD (Fig. 22) and rabbit anti-GFAP (Fig. 23) in a P4 rat spinal cord demonstrated the expression of GFAP finally occurring in the IFAP-containing dorsal median septum, which was surrounded by GFAP-containing cells that had previously ceased IFAP expression. Dorsum of spinal cord is located to left. Scale bar = 50 μm.

Figs 24 and 25. Double-labelling with the monoclonal anti-IFAP-70/280 kD (Fig. 24) and rabbit anti-GFAP (Fig. 25) in an E18 rat spinal cord revealed that the expression of the IFAP had begun to cease in some of the GFAP-containing glial cells in the marginal zone of the ventrolateral spinal cord. Scale bar = 50 μm.

Figs 26 and 27. Immunofluorescence staining of IFAP-70/280 kD in a P2 spinal cord. By birth, the immunoreactivity of the IFAP was significantly decreased in most parts of the neonatal spinal cord except the dorsal white column and the dorsal median septum (Fig. 26). Note that the IFAP staining has disappeared from the central canal region (Fig. 27). Scale bar = 50 μm.

Figs 28 and 29. Double-label immunofluorescence microscopy with the monoclonal anti-IFAP-70/280 kD (Fig. 28) and rabbit anti-GFAP in the ventrolateral region of an E20 rat spinal cord (Fig. 29). The morphology of most GFAP-positive glia changed from radially arranged, elongated cells to non-radial cells with various morphologies. Scale bar = 50 μm.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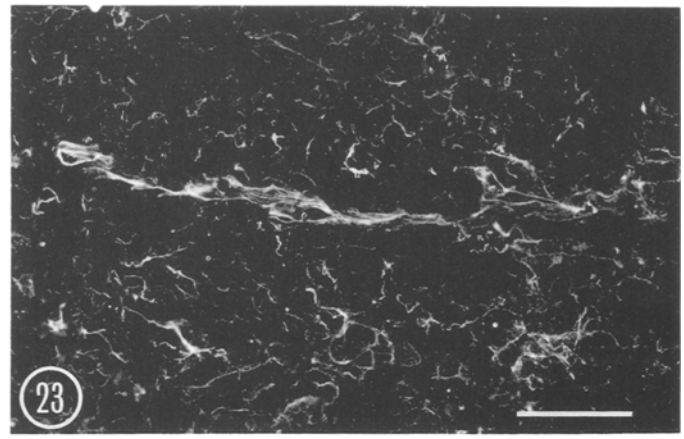
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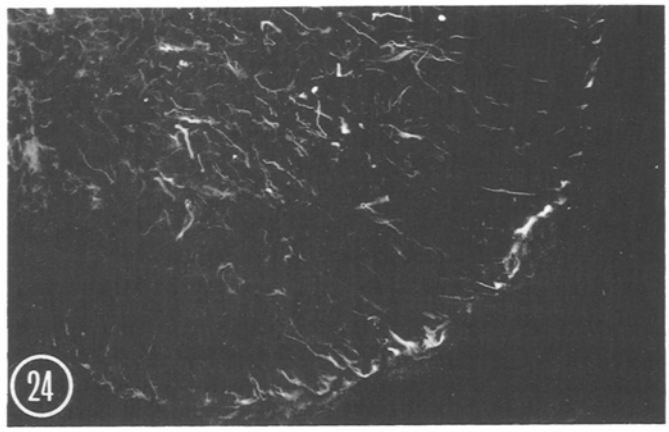
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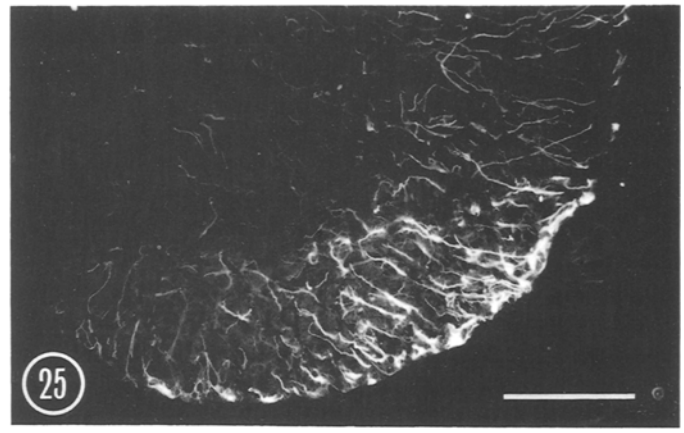
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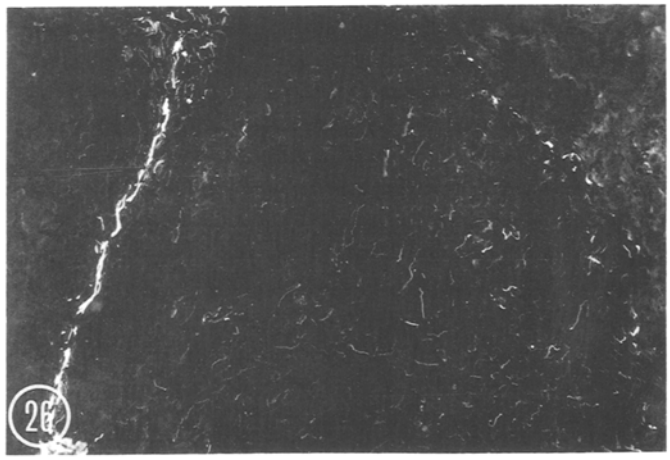
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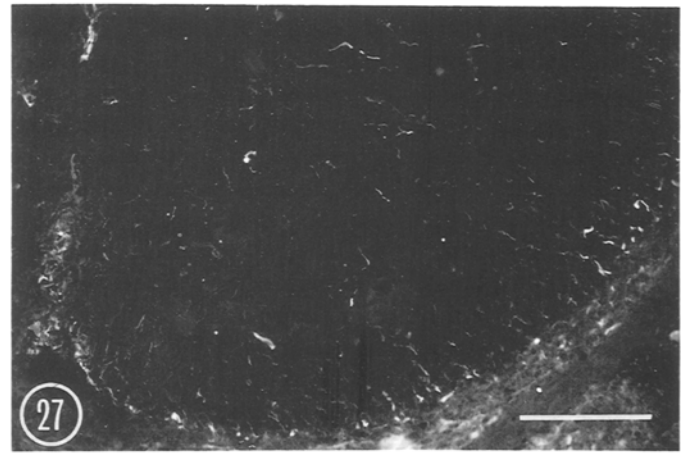
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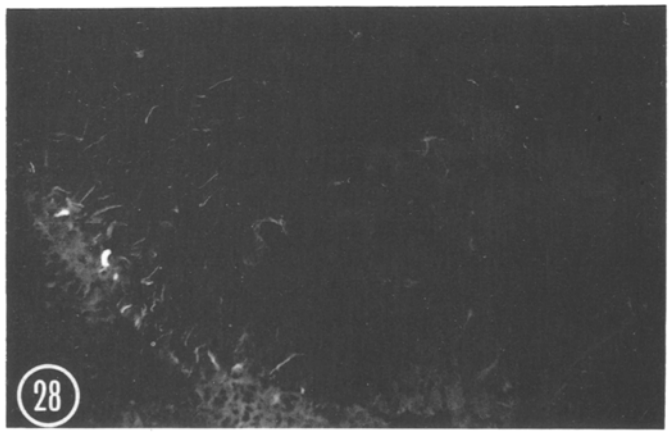
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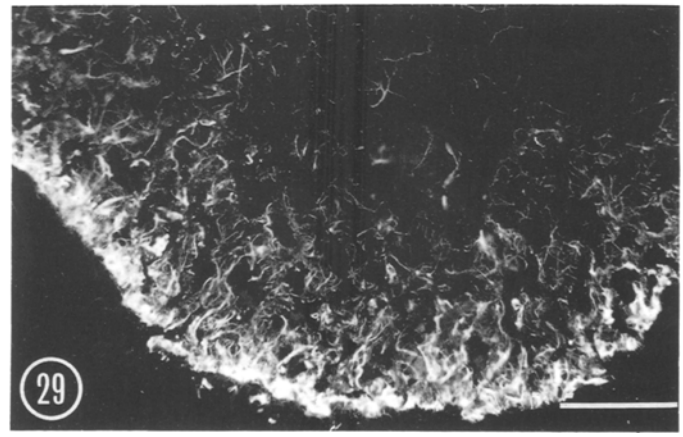
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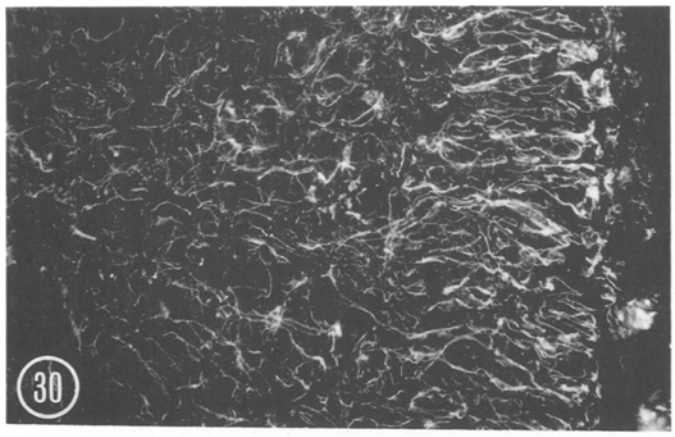
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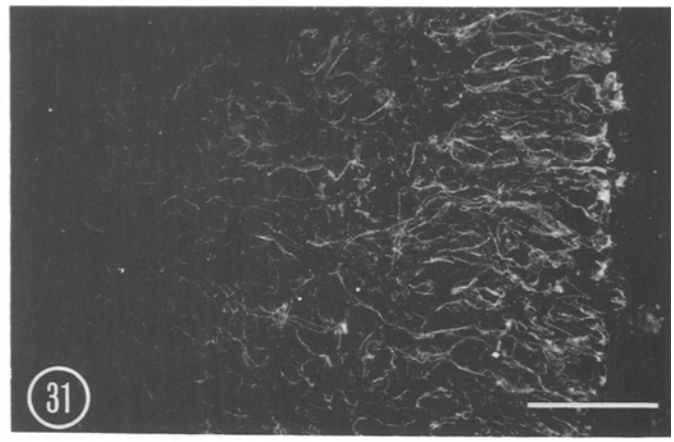
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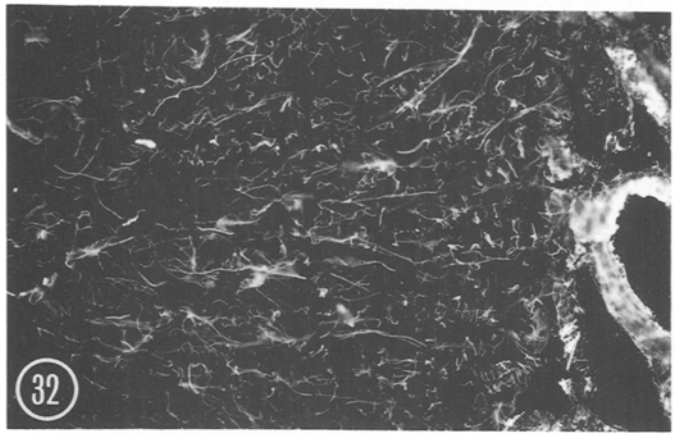
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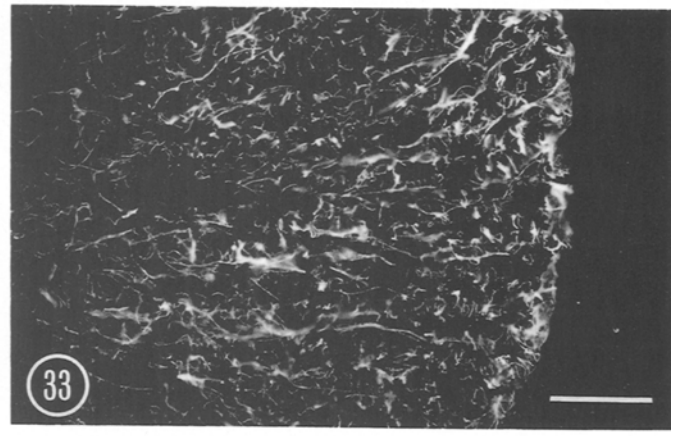
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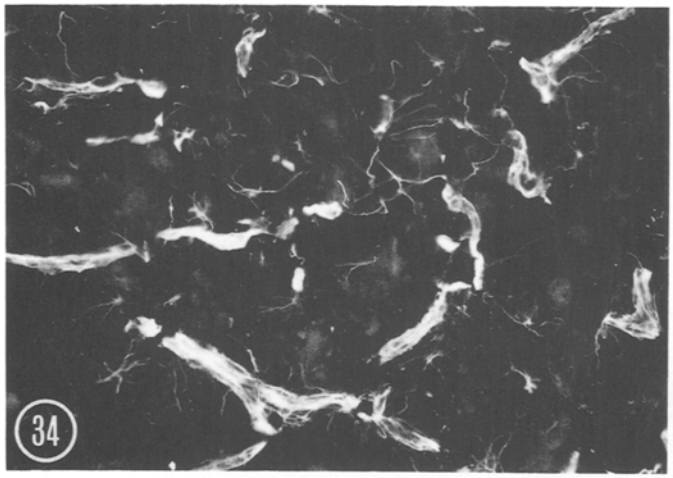
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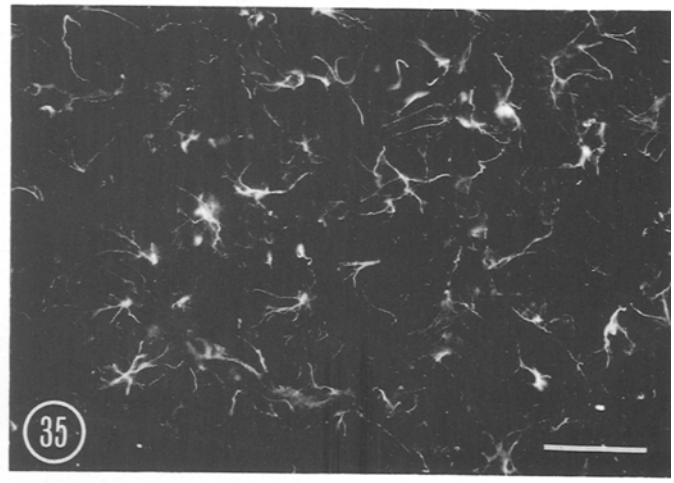
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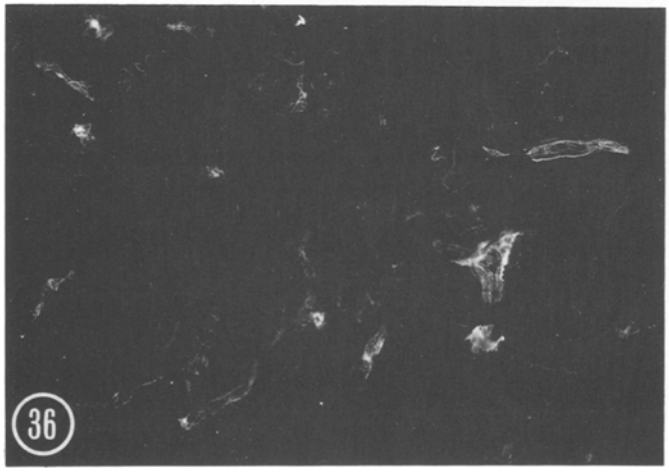
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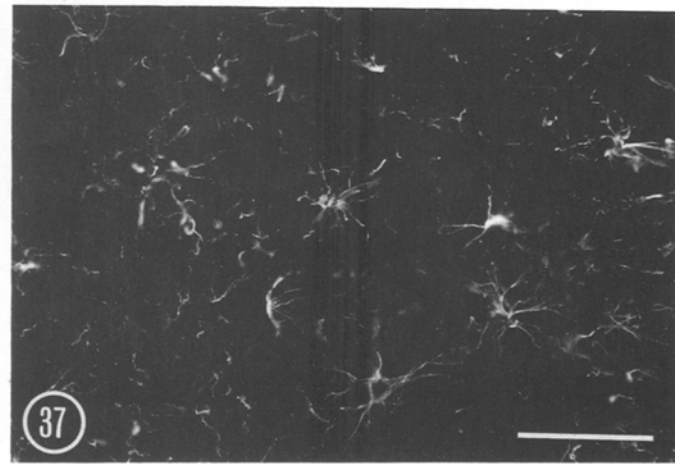
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toward the central canal zone. Thus, while the wave of GFAP expression was beginning ventrally by late E16, it was not completed dorsally until approximately P3, by which time the events of the next period (III) were essentially completed for the derivative cells of the radial glia in the region adjacent to the pia mater of the ventral cord.

Stage III: immature macroglia ($I^-/V^+/G^+$)

Temporally, the onset of this developmental stage extended from approximately late E18 for the ventral cord to P7 for the dorsal regions. The characterizing event with respect to the antigenic composition of cells of radial glia derivation was the loss of IFAP-70/280 kD immunoreactivity. However, these GFAP-positive cells maintained vimentin expression to yield $I^-/V^+/G^+$ cells, which, because a subsequent developmental event had yet to occur (in stage IV), were referred to here as immature macroglia.

Double-label immunofluorescence showed that the loss of IFAP-70/280 kD immunoreactivity began in the ventral white matter adjacent to the pia mater (Figs 24, 25). The IFAP was lost in cells from the ventral to the dorsal cord and secondarily from the periphery inward, following the same sequence by which GFAP expression spread through the cord. At birth, this process of IFAP loss continued until only the dorsal funiculus and the dorsal septum remained strongly immunopositive for the IFAP (Figs 26, 27). In the remainder of the cord, only sporadic IFAP-positive cells were observed. After the loss of IFAP immunoreactivity, the GFAP-positive cells were transformed from the elongated appearance reminiscent of their radial glial origin to more compact, rounded and branched cells, particularly in the ventral white matter (Figs 28, 29). It is very important to note that despite IFAP loss, the GFAP-positive cells were still immunopositive for vimentin (Figs 30, 31).

Stage IV: mature astrocytes ($I^-/V^+/G^+$ and $I^-/V^-/G^+$)

The distinguishing event of this stage, which started from approximately P12 (Figs 32–35), was the loss of vimentin from a subpopulation of the GFAP-positive cells, producing $I^-/V^-/G^+$ species. However, it should be noted that many $I^-/V^+/G^+$ cells still persisted in adult white matter.

The antigenic transformation was especially apparent in the region surrounding the central canal and in the gray matter, where the GFAP-positive cells exhibited a stellate appearance. By P28, double-labelling demonstrated the absence of vimentin reactivity in the majority of the gray matter astrocytes (Figs 36, 37). The result of this process was the production of two apparently 'mature' (as compared with astrocytes in adult spinal cord) astrocytic types: $I^-/V^+/G^+$ and $I^-/V^-/G^+$. The former characterized the cord white matter while the latter was indicative of the gray matter of the spinal cord.

Discussion

Until recently, the IF structural proteins have been the only intracellular proteins routinely used as markers of astrocyte differentiation: GFAP for relatively mature astrocytes and the non-specific vimentin for early, developing astrocytes (for a review, see Fedoroff, 1986). But the usefulness of vimentin is limited because this IF protein is also widely expressed in many mature astrocytes and in most cultured astrocytes (Chiu *et al.*, 1981; Dahl *et al.*, 1981a; Osborn *et al.*, 1981; Schnitzer *et al.*, 1981; Yang *et al.*, 1992b).

A number of associated proteins of the IF cytoskeleton have been identified within the past few years (Dale *et al.*, 1990; Yang *et al.*, 1990; Foisner & Wiche, 1991). They are a heterogeneous group of minor quantity proteins, physically associated with IF, that appear to regulate the supramolecular organization of the IF cytoskeleton. Intermediate filament-associated proteins are generally identified on the basis of the following criteria: (1) co-localization with IF *in situ*; (2) co-isolation with IF in Triton/high-salt cellular residues; (3) co-cycling with IF through repeated IF disassembly/assembly protocols; and (4) *in vitro* recombination of the purified IFAP with IF assembled from purified IF subunit protein (Yang *et al.*, 1985; Lieska *et al.*, 1985; Yang *et al.*, 1990). Recent studies have demonstrated that the expression of many IFAPs is developmentally regulated. This is

Figs 30 and 31. Double-labelling with monoclonal anti-vimentin (Fig. 30) and rabbit anti-GFAP (Fig. 31) in the lateral region of a P2 rat spinal cord demonstrated presence of vimentin in the GFAP-positive cells in which the expression of IFAP-70/280 kD had essentially ceased (see Figs. 26 and 27). Scale bar = 50 μm.

Figs 32–35. Double-labelling with monoclonal anti-vimentin (Figs 32 and 34) and rabbit anti-GFAP (Figs 33 and 35) in a P12 rat spinal cord. The expression of vimentin persisted in the GFAP-positive cells of the white matter (Figs 32 and 33), while its expression diminished in the astrocytes of the gray matter (Figs 34 and 35). Note that the astrocytes of gray matter are stellate. Vimentin can also be seen in the cells of the blood vessels (Fig. 34). Scale bar = 50 μm.

Figs 36 and 37. Double-labelling with monoclonal anti-vimentin (Fig. 36) and rabbit anti GFAP (Fig. 37) demonstrated the absence of vimentin immunoreactivity in essentially all astrocytes in the gray matter of the P26 rat spinal cord. Scale bar = 50 μm.

particularly evident for the glial IFAPs. For example, IFAP-48 kD represents a protein expressed late in the development of murine astrocytes (Abd-El-Basset *et al.*, 1988a,b), IFAPa-400 has been identified in very early derivatives of the neuroectoderm in the chick embryo (Chabot & Vincent, 1990), and IFAP-70/280 kD has been shown to be expressed in radial glia-like cells in the developing rat cerebral cortex (Yang *et al.*, 1992b) and spinal cord (present study).

Two major findings come from studies on IFAP-70/280 kD expression in astrocytes in primary cultures derived from neonatal rat brain: (1) the presence of the IFAP only in non-stellate astrocytes and (2) the identification of four subtypes of non-stellate astrocytes on the basis of the differential expression of the IFAP (I) in conjunction with the expression patterns of vimentin (V) and GFAP (G), viz., $I^+/V^+/G^-$, $I^+/V^+/G^+$, $I^-/V^+/G^+$, and $I^-/V^-/G^+$ (Yang *et al.*, 1992b). The tissue counterparts of these *in vitro* subtypes are also observed in developing rat cerebral cortex (Yang *et al.*, 1992b). In the context of developmental chronology, these subtypes of the non-stellate lineage correspond to early radial glia, to two immature astrocyte types, and mature astrocytes, respectively. Thus, this IFAP marker, considered in conjunction with two IF structural proteins (GFAP and vimentin), has provided a more clearly defined picture of the various differentiation states of non-stellate astrocytes in an *in vitro* system than has heretofore been possible.

In the present study, application of this approach for defining antigenic phenotypes has provided an equally dramatic view of astrocyte development in rat spinal cord tissue. First, the introduction of IFAP-70/280 kD expression into this analysis allows assignment of a specific immunotype, $I^+/V^+/G^-$, to radial glia. The emergence of this cell type at E13 and the distinctive radial morphology revealed by immunolabelling with anti-IFAP-70/280 kD confirms its identity as radial glia. Second, these radial glia are clearly shown to be developing species since IFAP-70/280 kD is not demonstrable in normal adult astrocytes (including those exhibiting a radial arrangement (unpublished observations)) (cf. Misson *et al.*, 1988, 1991). Indeed, some of its derivatives on the pathway to macroglia in the rat spinal cord can now be identified and immunotyped by this approach. While previously there was no objective means of identifying immature (intermediate differentiation stage) astrocytes or distinguishing them from mature cells, IFAP-70/280 kD labelling in conjunction with that of GFAP/vimentin can now define (1) a direct, immature macroglial derivative of radial glia ($I^+/V^+/G^+$), (2) immature glia ($I^-/V^+/G^+$), and (3) mature astrocytes ($I^-/V^+/G^+$ and $I^-/V^-/G^+$). These phenotypes are in agreement with those previously defined in the neonatal rat cerebral cortex (Yang *et al.*, 1992b). Furthermore, this developmental scenario for radial glia is in strong agreement with recent studies on the murine central nervous system employing the monoclonal antibody RC2 (Misson *et al.*, 1988, 1991). The RC2 antigen also appears early embryologically and is lost in the early postnatal period, but its physicochemical properties, e.g., two polypeptides at M_r 95 and 106 kD plus a single ganglioside or acidic glycolipid (Mission *et al.*, 1987), appear distinct from the IFAP-70/280 kD described here. It also should be noted that whereas Misson and colleagues utilize single-label immunofluorescence techniques in conjunction with cell morphology to assess radial glia ontogeny, we rely upon multiple antibody labelling in order to immunotype the cells involved.

On the basis of our studies to date, the following hypothesis can be proposed for the ontogenesis of non-stellate (type-1) astrocytes both *in vitro* and *in vivo*. Radial glia have the phenotype $I^+/V^+/G^-$ and appear to be attached at one end of the cell to the surface of the presumptive central canal and at the other end to the external surface of the spinal cord (Stage I). There is an apparent progressive loss of attachment of radial glia to the surface of the presumptive central canal beginning late E16 in the ventral region of the spinal cord (cf. Schmechel & Rakic, 1979). This may represent a signal for the initiation of GFAP expression in radial glia (Stage II). In culture, the phenotypic counterpart of this direct radial glia derivative ($I^+/V^+/G^+$) is a non-stellate (i.e., type-1; for a review, see Raff, 1989) astrocytic cell (Yang *et al.*, 1992b). Moreover, the demonstration of GFAP in the IFAP-containing cells constitutes biochemical evidence that radial glia are precursors of astrocytes (cf. Schmechel & Rakic; 1979; Goldman *et al.*, 1986; Culican *et al.*, 1990). Beginning late E18 in the ventrolateral region of the spinal cord, these derivative cells gradually cease IFAP expression to become $I^-/V^+/G^+$, a phenotype referred to here as immature astrocytes (Stage III). A shape change from elongated fibres to more branched cells accompanies this loss of IFAP expression (cf. Schmechel & Rakic, 1979; Edwards *et al.*, 1990; Misson *et al.*, 1988, 1991). Finally, approximately 12 days postnatally a subpopulation of immature astrocytes begins to lose vimentin to become $I^-/V^-/G^+$ (Stage IV). This occurs more prominently in the gray matter. While the $I^-/V^-/G^+$ phenotype characterizes the mature astrocytic type in spinal cord (especially those in gray matter), many of the $I^-/V^+/G^+$ phenotype cells persist to become the mature type present in the white matter of spinal cord.

In almost all instances, these waves of differentiation (as identified by antigen compositional changes in the putative radial glia lineage) are initiated in the ventral cord, proceed dorsally, and simultaneously advance from the cord periphery toward the central canal (cf. Altman & Bayer, 1984, with respect to neurogenesis). Each of the four periods of radial glia differentiation occupies several days of development

in an overlapping manner. While cells in the dorsal region may be just initiating GFAP expression (Stage II), others in the ventral cord periphery have already entered the next stage (Stage III), ceasing expression of IFAP-70/280 kD and changing cell shape to become immature astrocytes.

Finally, it should be noted that astrocytes may not be the only derivatives of radial glia. Some radial glia which remain attached to the central canal (and detach from the subpial surface of the cord) may represent stem cells for neurons (Levitt *et al.*, 1981; Hockfield & McKay, 1985; Frederiksen & McKay, 1988; Evrard *et al.*, 1990). Furthermore, it has been suggested that while the lineages of astrocytes and oligodendrocytes are already distinct postnatally (Vaysse & Goldman, 1990; Carnow *et al.*, 1991; Skoff & Knapp, 1991), some radial glia or their immature prenatal derivatives may represent progenitors of oligodendrocytes/type-2 astrocytes (Choi & Kim, 1984; Hirano & Goldman, 1988; Choi, 1990; Goldman & Vaysse, 1991). Inasmuch as the sources and pathways of these cell lineages have not yet been identified, such possibilities cannot be unequivocally discounted. By providing a more exact definition of radial glia and more clearly resolving the early differentiation period of this cell type along the macroglia lineage, the present study indicates many fruitful areas for future investigation.

Acknowledgements

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