In Vivo Analysis of Glial Cell Phenotypes during a Viral Demyelinating Disease in Mice

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Abstract. C_{57} BL/6N mice injected intracranially with the A59 strain of mouse hepatitis virus exhibit extensive viral replication in glial cells of the spinal cord and develop demyelinating lesions followed by virus clearing and remyelination. To study how different glial cell types are affected by the disease process, we combine three-color immunofluorescence labeling with tritiated thymidine autoradiography on 1-μm frozen sections of spinal cord. We use three different glial cell specific antibodies (a) to 2', 3' cyclic-nucleotide 3' phosphohydrolase (CNP) expressed by oligodendrocytes, (b) to glial fibrillary acidic protein (GFAP) expressed by astrocytes, and (c) the O_4 antibody which binds to O-2A progenitor cells in the rat. These progenitor cells, which give rise to oligodendrocytes and type 2 astrocytes and react with the O4 antibody in the adult central nervous system, were present but rare in the spinal cord of uninfected mice. In contrast, cells with the O-2A progenitor phenotype (O4+ only) were increased in number at one week post viral inoculation

(1 WPI) and were the only immunostained cells labeled at that time by a 2-h in vivo pulse of tritiated thymidine. Both GFAP+ only and GFAP+, O4+ astrocytes were also increased in the spinal cord at 1 WPI. Between two and four WPI, the infected spinal cord was characterized by the loss of (CNP+, O4+) oligodendrocytes within demyelinating lesions and the presence of O-2A progenitor cells and O4+, GFAP+ astrocytes, both of which could be labeled with thymidine. As remyelination proceeded, CNP immunostaining returned to near normal and tritiated thymidine injected previously during the demyelinating phase now appeared in CNP+ oligodendrocytes. Thus O_4 positive O-2A progenitor cells proliferate early in the course of the demyelinating disease, while CNP positive oligodendrocytes do not. The timing of events suggests that the O-2A progenitors may give rise to new oligodendrocytes and to type 2 astrocytes, both of which are likely to be instrumental in the remyelination process.

IN small rodents, regeneration of central nervous system (CNS)[1] myelin is often efficient after a demyelinating episode (reviewed in Ludwin, 1981). In contrast, in multiple sclerosis, the paradigm of demyelinating diseases in human CNS, remyelination is often insufficient (reviewed in Silberberg, 1986). Thus it appears important to elucidate the exact cellular and molecular events underlying CNS remyelination.

The antigenic phenotype and mitogenic potential of the glial cell types involved in CNS remyelination of small rodents has not yet been studied in detail. In contrast, recent studies have elucidated the nature of the cell lineage involved in myelination during development (reviewed in Raff and Miller, 1984; and Raff, 1989). A bipotential progenitor cell has been demonstrated to give rise to oligodendrocytes and to type 2 astrocytes which are both involved in the construction of myelinated tracts (ffrench-Constant and Raff, 1986). Therefore this progenitor is called the O-2A progenitor and the cells derived from these cells constitute, with their progenitor, that O-2A lineage (Raff et al., 1983b). An antibody crucial to the identification of this lineage in optic nerve has been the A2B5 antibody which reacts with a subset of gangliosides on the cell surface (Eisenbarth et al., 1979) and was used both in vitro and in vivo to stain O-2A progenitors (Raff et al., 1983b, 1984b; David et al., 1984; Miller et al., 1985; Behar et al., 1988). In vivo studies with this antibody in other parts of the nervous system have been difficult because these specific gangliosides are also expressed in neuronal components (Eisenbarth et al., 1979).

Recent studies on the O-2A lineage have also made use of

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1. *Abbreviations used in this paper:* CNP, 2',3', cyclic-nucleotide 3' phosphohydrolase; CNS, central nervous system; GFAP, glial fibrillary acidic protein; WPI, weeks post inoculation.

the O₄ antibody (Sommer and Schachner, 1981; Schachner et al., 1981; Keilhauer et al., 1985) which reacts with the O-2A progenitor at a later stage of its development before it expresses galactocerebroside, a differentiation marker for oligodendrocytes (Dubois-Dalcq, 1987). When O₄ positive cells are sorted from the murine neonatal brain and cerebellum, they can differentiate into type 2 astrocytes or oligodendrocytes (Trotter and Schachner, 1989). In addition, differentiated oligodendrocytes and type 2 astrocytes isolated from the CNS of adult rats and mice express the O₄ antigen (Wolswijk and Noble, 1989). The O₄ antibody reacts only weakly with neurons (Schachner et al., 1981) and appears to stain glial cells of different species in a consistent way (rat, mouse, chicken, human; Sommer and Schachner, 1981; our own unpublished observations). In contrast, A2B5 antibody does not consistently stain putative glial precursor cells in mouse (our own unpublished observations) and human (Kim et al., 1986). For all these reasons, we have used the O₄ antibody to study O-2A lineage cells in the normal mouse spinal cord and in mice affected with a demyelinating disease.

The A₅₉ strain of mouse hepatitis virus reliably caused demyelination in C₅₇ black mice, with a predominance of lesions in the spinal cord. (Woyciechowska et al., 1984; Lavi et al., 1984a,b; Kristensson et al., 1986). The virus replicates exclusively in glial cells of both white and grey matter of the spinal cord in the early phase of the disease (Godfraind, C., unpublished observations; Jordan et al., 1989b). At two weeks post inoculation (WPI), the virus becomes restricted to the white matter where demyelination occurs and, at 4 WPI, virus is mostly cleared probably because the infected mice develop a vigorous immune response to the virus (Woyciechowska et al., 1984). Clinical recovery and myelin repair proceed efficiently in the following weeks (Woyciechowska et al., 1984). Thus cellular events related to demyelination and to successful remyelination can be followed closely in the spinal cord of these infected mice. We have previously used in situ hybridization to study myelin basic protein gene expression in this model (Kristensson et al., 1986). At the start of remyelination, a subset of MBP transcripts which are highly expressed in developing animals during normal myelination are increased in abundance (Jordan et al., 1989a,c).

In the present study, we investigate the possible role of precursor cells in remyelination events using in vivo tritiated thymidine autoradiography combined with immunocytochemistry at regular intervals after viral inoculation. We used three-color immunofluorescence labeling to show simultaneously the O₄ antigens, the 2′, 3′, cyclic-nucleotide 3′ phosphohydrolase (CNP) protein (expressed by oligodendrocytes) and the glial fibrillary acidic protein (GFAP) (expressed by astrocytes). The use of the 1-μm frozen section technique (Tokuyasu, 1973, 1980; Griffith et al., 1984) allows detailed localization of each antigen in different glial cell types (David et al., 1984; Miller et al., 1985). Using these techniques, we have identified O-2A lineage cells (Raff et al., 1984a) in the spinal cord of normal and diseased mice and followed how these cells are affected by the demyelinating disease. Our results demonstrate that rare O₄ precursors present in the normal adult CNS are triggered to divide early in the disease and may give rise to new oligodendrocytes and type 2 astrocytes which both appear instrumental in the reconstruction of myelinated white matter.

Materials and Methods

Animals

MHV-free adult C₅₇Bl/6N mice were obtained from the National Institutes of Health animal facility (Frederick, MD). At 28 d of age, mice were infected by a single intracerebral injection with 1,000 plaque forming units of MHV-A59 in PBS prepared as previously described (Kristensson et al., 1986). Control mice, obtained from the same source, were housed separately from the infected ones. Most controls were not subjected to intracerebral injection; however, some were injected intracerebrally with PBS without virus to confirm that intracerebral injection did not itself cause spinal cord lesions. During the acute infectious phase less than half of the mice died from acute hepatitis. The surviving mice were tested neurologically at 1 WPI and those without neurological symptoms were excluded from further study. Mice were killed 1-9 wk after infection along with age-matched control animals.

Tissue Preparation

Mice were anesthetized by methoxyfluorane inhalation followed by chloral hydrate (0.4 mg/g body weight, intraperitoneal). They were then fixed by a 10-min transcardiac perfusion of a solution of 4% formaldehyde (from paraformaldehyde) and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Spinal cords were removed 1-4 h after perfusion and divided into pieces for further processing. Transverse slices ∼0.5 mm thick were cut from cervical, thoracic, and lumbar regions of each spinal cord, postfixed in buffered osmium tetroxide, and embedded in epoxy resin. Sections 1-2 μm thick were later cut from these blocks, stained with toluidine blue, and examined to confirm the presence of virus-induced demyelinating lesions in each infected animal.

The remaining tissue was processed for immunocytochemistry of 1 μm frozen sections following the Tokuyasu technique (1973, 1980). Transverse slices of spinal cord 0.5-0.7 mm thick were postfixed overnight at 4°C in the aldehyde fixative solution described above. Afterwards the blocks were immersed sequentially for 12 h each in 5%, 15%, 50%, and 2.3 M sucrose in PBS at 4°C. In some cases, 30% polyvinylpyrrolidone (molecular mass 10 kD; Sigma Chemical Co., St. Louis, MD) was added to the 2.3-M sucrose solution; this improved the cutting properties of the blocks and reduced artifactual separation of tissue elements within the white matter. Nevertheless, some separation of elements was unavoidable in actively demyelinating specimens with abundant vacuolization. After sucrose infiltration, each piece was trimmed to an appropriate size, mounted on a specimen holder, and frozen by immersion in liquid nitrogen. The specimens were stored in liquid nitrogen until use. Transverse sections 1 μm thick were cut from those blocks using glass knives and a Reichert-Jung Ultracut E microtome with ultracryotomy system FC4D. The sections included in most cases at least a complete half face, from the midline to the lateral edge of the spinal cord. Sections were transferred on 2.3 M sucrose drops to slides coated with a dried aqueous solution of gelatin (1% pig skin gelatin; Sigma Chemical Co.) and chromium potassium sulfate (0.1%) in PBS. Slides bearing sections were then immersed in 4% formaldehyde in PBS for 5 min and stored in PBS at 4°C for 1-2 d before processing for immunocytochemistry.

Immunocytochemistry

Before immunolabeling, the sections were treated as follows: 10 min in 100% ethanol at −20°C; three rinses in PBS at room temperature (1-2 min each); 5 min in 1% sodium borohydride in PBS; and PBS rinses until bubbles were no longer observed (∼5-10 min). Both ethanol and sodium borohydride treatments improved staining for CNP antigen and O4 antibody. In addition, sodium borohydride treatment served to eliminate an otherwise substantial tissue autofluorescence. The sections were then incubated for 3 h at room temperature in a solution containing 0.5 M Tris buffer, pH 7.6 (TB), 10% BSA, 3% normal goat serum, 0.1% gelatin, and 0.05% sodium azide to reduce nonspecific antibody binding. They were then exposed overnight to the primary antibody solution at 4°C. The primaries, applied singly or in combination, were O₄, a mouse monoclonal IgM (dilution 1/2 of culture supernatant, from hybridoma kindly provided by I. Sommer; Sommer and Schachner, 1981); rabbit anti-CNP (dilution 1/100 of whole serum kindly provided by F. A. McMorris; McMorris et al., 1984; Sprinkle et al., 1980); and a monoclonal rat anti-GFAP (an IgG; dilution 1/10 of culture supernatant from hybridoma 2.2B10.6 kindly provided by V. Lee; Lee et al., 1984).

One to three of the primary antibodies were mixed together in a solution containing 0.1% gelatin, 1% BSA, and 0.05% sodium azide in TB (TGBA).

After washing the sections with TB, secondary antibodies diluted in TGBA were applied to the sections for 3 h at room temperature. For double and triple stains, appropriate secondary antibodies were applied simultaneously. O_4 was visualized with rhodamine-conjugated goat anti-mouse IgM (μm chain specific; Jackson Immunoresearch Laboratories, Inc., Westgrove, PA; 58 μg/ml), the rabbit anti-CNP with fluorescein-conjugated donkey anti-rabbit Ig (Amersham Corp., Arlington Heights, IL; 1:15), and the rat anti-GFAP with biotinylated sheep anti-rat Ig (Amersham Corp.; 10 μg/ml). After washing in TB, sections were treated with 25 μg/ml of streptavidin conjugated to 7-amino-4-methyl-coumarin-3-acetic acid (Molecular Probes Inc., Junction City, OR; Khalfan et al., 1986; Appel et al., 1987) in TGBA to mark the biotinylated secondary antibody, washed again, fixed 1–4 min in 4% formaldehyde in PBS, and coverslipped with a solution of .02 M Tris, pH 8.6, and 80% glycerol. Sections were examined by epifluorescence with a Zeiss Photoscope III equipped with three simultaneously mounted filter sets for rhodamine, fluorescein, and coumarin fluorescence and photographed with Kodak T-Max-400 film, developed for ASA 1,600.

The absence of cross-reaction between the three antibody channels was tested by omitting in turn each one of the three primary antibodies while leaving the other two in the primaries mixture and while including all three secondaries in the secondaries mixture. Omission of each primary completely abolished binding of the corresponding secondary, while signals in the other two channels remained unchanged. In addition, each section provided its own further demonstration of the specificity of the three channels, since each of three primaries exhibited unique and characteristic regional and intracellular patterns of staining. Identification of all double or triple labeled cells was not possible. We identified cells as multiply labeled only when we saw coincident, overlapping, or concentric multiple labeling and when the corresponding phase contrast image was consistent with that interpretation.

We stained mouse tissue with a mouse monoclonal IgM (O_4); fortunately, the inflammatory reaction did not confound our results. When 10 and 100 μg/ml of nonspecific IgM (Calbiochem-Behring Corp., San Diego, CA) were applied to sections with processing as for O_4 immunolabeling, we saw either no staining, or infrequently a diffuse signal lacking cellular structure. Rarely, bright oval cells were seen at the pia or in white matter; those cells, probably B lymphocytes, were also stained with the secondary antibody alone. Foamy macrophages, observed only in some specimens, were stained by O_4 but not by nonspecific IgMs; these were easily recognized on morphological grounds and, as with the B lymphocytes, were excluded from descriptions presented here.

Tritiated Thymidine Autoradiography

At 1, 2, 3, and 4 WPI, some infected mice as well as their matching controls were injected intraperitoneally 2 h before killing with [methyl-^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA), 10 μCi/g body weight. Subsequent tissue preparation and the immunolabeling was performed as described in the previous sections. After immunolabeling, the sections were dehydrated in 70% ethanol and air dried. They were then dipped in Kodak NTB-2 photographic emulsion diluted 1/2 in distilled water, dried, and stored 1–3 wk at 4°C before being developed. The developing procedure was performed at 14°C: sections were first immersed in Kodak D-19 developer (1:2 in water) for 4 min, rinsed in distilled water for 1 min, treated with Kodak fixer for 5 min, rinsed in distilled water, and coverslipped with tris-glycerol as described above.

In a few cases 2-μm-thick sections of epoxy embedded spinal cord were also processed for autoradiography and stained with toluidine blue. To determine the fate of dividing cells, animals were injected with the above dose of tritiated thymidine four times, at 12-h intervals, from 12 to 14 dpi or 19 to 21 dpi, and were killed 5 mo later. Cryostat sections from these animals were immunostained for CNP by a peroxidase anti-peroxidase procedure described elsewhere (Jordan et al., 1989b), with diaminobenzidine as the chromogen. After intensification with osmium tetroxide, immunostained sections were washed in water 1–2 h, passed through graded ethanols and air dried, and processed for autoradiography as above.

Lipid Extraction and Immunodetection

Lipids were extracted from adult C_{57} BI/6 whole brain and whole spinal cord as previously described (Magnani et al., 1982; Rougeon et al., 1986). Briefly, 500 mg (wet weight) of tissue was homogenized with 1.5 ml H_2O, the homogenate was mixed with 5 ml methanol and 2.5 ml chloroform and stirred at room temperature for 30–45 min. The mixture was centrifuged for 15 min at 1,500 g. For the spinal cord, pellets were resuspended and

recentrifuged a second time. The supernatants were collected, evaporated to dryness, and suspended in chloroform/methanol (1:1).

For thin layer chromatography, lipid extracts and standards were loaded on Baker thin layer chromatography silica-gel plates and run in a chloroform/methanol/0.2% $CaCl_2$ (60:40:9) solvent system. The plates were air dried and subjected to routine iodine or orcinol staining or to O_4 immunostaining. For the immunostaining, plates were blocked for 1 h at room temperature with 10% FBS and 3% BSA in MEM Hepes medium and incubated overnight at 4°C with O_4 culture supernatant diluted 1:4 in MEM Hepes with 10% FBS. After washing in cold PBS, the plates were incubated 90 min with biotinylated goat anti-mouse IgG (H + L specific; dilution, 1/200; Cappel Laboratories, Malvern, PA), washed, incubated with streptavidin-peroxidase complex (Amersham Corp., dilution 1/200), and treated with diaminobenzidine and hydrogen peroxide. Control plates followed the same procedure except that O_4 antibody was omitted. The silica gel plates used remained intact throughout the prolonged immersion in aqueous solutions and no special treatment for stabilization was required.

Results

Specificities of Antibodies

The specificity of the antibodies against the myelin protein CNP and the glial fibrillary acidic (intermediate filament) protein has been described before (Lee et al., 1984; McMorris et al., 1984; Sprinkle et al., 1980). The O_4 antibody stains white matter in cryostat sections (Schachner et al., 1981) and has been shown to react with sulfatides (Singh
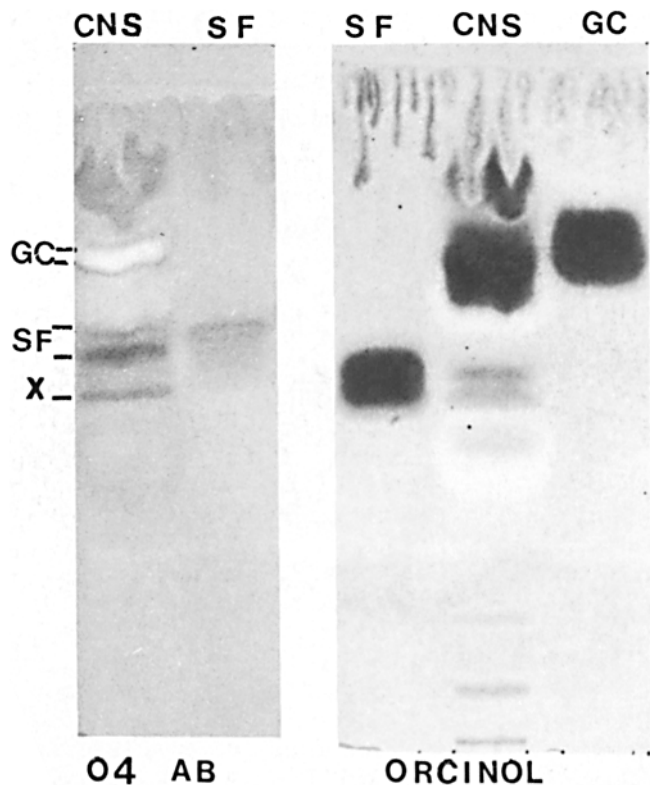

Figure 1. O_4 immunoreactivity for CNS lipids analyzed by thin layer chromatography. (Left) O_4 immunostaining; (right) visualization of glycolipids by orcinol staining. In CNS extracts (CNS), O_4 reacts strongly with a pair of bands identified by their migration pattern on TLC plates as sulfatides (SF). O_4 reacts much more weakly with bands identified galactocerebroside (GC). The identity of a fifth band (X), intermediate in intensity, is not known. Lanes marked SF and GC contain purified sulfatide and galactocerebroside standards, respectively.
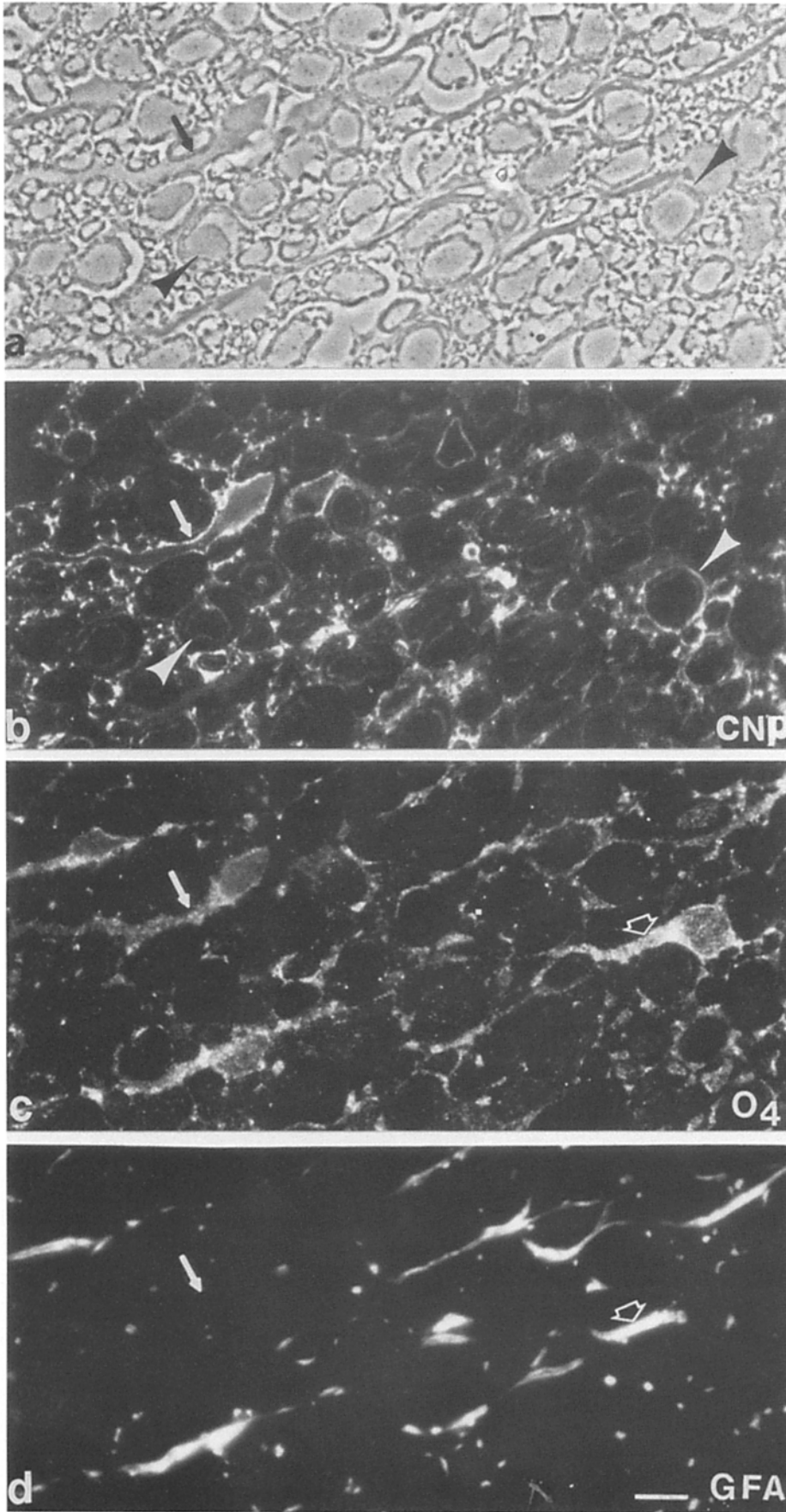
Figure 2. 1-μm-thick section of spinal cord white matter from normal mouse stained with three-color immunofluorescence. The same field is shown by phase contrast (*a*) and illuminated for each of the three antigens studied (*b–d*). Myelin sheaths can be recognized in phase contrast; they remain reasonably well compacted (*a*). CNP immunoreactivity (*b*) is absent from compact myelin but is present in rings immediately inside (*left arrowhead*) and outside (*right arrowhead*) of compact myelin. An oligodendrocyte in the section shows a rim of CNP immunoreactivity along the plasmalemma of its cell body and large process (*solid arrow*). O₄ immunoreactivity (*c*) is somewhat granular within processes and often surrounding the nuclei of stained cells (*solid* and *open arrows*). GFAP immunoreactivity (*d*) appears as bands or small dots representing radially and longitudinally oriented processes, respectively, but the label does not fill the entire cytoplasm (e.g., *open arrow* in *c* and *d*). Note that the O₄+ GFAP+ cells have rather thin GFAP containing processes (*c* and *d*). Bar, 10 μm.

CNP
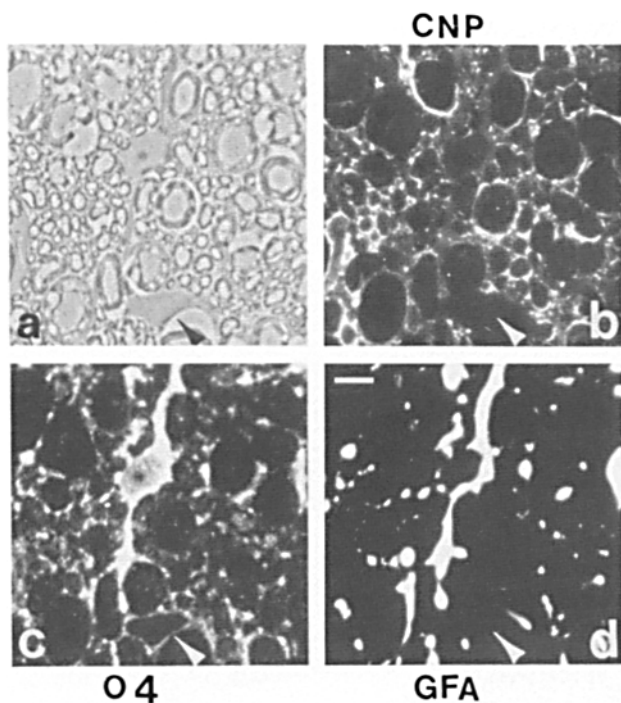
O 4 | **GFA**

Figure 3. Normal mouse spinal cord. A cell near the bottom of the field whose nucleus is visible in phase (*a, arrowhead*) is stained by O₄ (*c*), but not by CNP (*b*) or GFAP antibody (*d*). Near the center of the field is an O₄+ GFAP+ astrocyte. Bar, 10 μm.

and Pfeiffer, 1985); however, it is not known whether it reacts with lipids other than sulfatide in the rodent CNS. Therefore, we performed thin layer chomatography on lipid extracts prepared from brain or whole spinal cord and reacted the separated lipids with the O₄ antibody. Lipids from both brain and spinal cord yielded five bands which were recognized by the O₄ antibody. Lipids from both brain and spinal cord yielded five bands which were recognized by the O₄ antibody (Fig. 1). The two upper most bands, which were moderately faint, migrated at the same rate as galactocerebroside (*GC*) standards (Fig. 1, *right*). The next two bands (*SF*), which are dark, comigrated with sulfatide standards (which are also shown in Fig. 1). The lowest band (*X*) recognized by O₄ antibody was less intensely labeled than the sulfatide bands, yet it showed substantial reactivity (*X*; Fig. 1, *left*). We found orcinol staining on a band approximately at the same location as the X band, suggesting that the latter might be a glycolipid. It is not known to what extent this as yet unidentified component contributes to staining of our semi-thin frozen sections.

Spinal Cord in Normal Mice

With the three antibodies used in this study, we have detected four different antigenic phenotypes among the spinal cord glial cells: oligodendrocytes (CNP+ O₄+, and CNP+ only) two types of astrocytes (GFAP+ only, and GFAP+ O₄+), and the putative O-2A progenitor (O₄+, CNP− and GFAP−) (Wolswijk and Noble, 1988). Cells with these exact same antigenic phenotypes can also be isolated from these spinal cords and cultured (Armstrong, R., V. Friedrich, K. V. Holmes, and M. Dubois-Dalcq, manuscript in preparation). The in vivo morphology of the cells with each anti-

genic phenotype is illustrated in Figs. 2 and 3. The oligodendrocytes displayed CNP staining along the plasmalemma of their cell bodies and processes but not in compact myelin, as described before (Braun et al., 1988: Trapp et al., 1988). Thus, of the myelin sheaths, only the outer and inner cytoplasmic loops were stained (Fig. 2 *b*). Most oligodendrocytes also bound O₄ within the perikaryal cytoplasm (Fig. 2, *filled arrow*). As expected, more CNP+ O₄+ oligodendrocytes were detected in the white than grey matter. Interestingly, a few cells which only reacted with CNP antibody were present in the grey matter, mostly in the dorsal horn. The GFAP antiserum showed intense filament staining in cytoplasmic bundles, both in cell bodies and in processes, as described (Levine and Goldman, 1988). Many cells were stained only for GFAP; these are thought to represent type 1 astrocytes (Raff et al., 1983a). In addition we found numerous astrocytes which stained for O₄ in addition to GFAP. We identify these as type 2 astrocytes (Raff et al., 1983a) (Fig. 2, *c* and *d* and Fig. 3, *c* and *d*). O₄ staining was distributed in these cells as fine cytoplasm granules often prominent in regions of intense GFAP staining. Finally, we found O₄+ CNP− GFAP− putative progenitors with a shape similar to that of oligodendrocytes. These cells were smaller than oligodendrocytes and their processes could never be followed to myelin sheaths (Fig. 3, *bottom*). They were very rare in normal animals (average one per section and present only in the white matter [Fig. 3]).

The in vivo distribution of the type 1 and type 2 astrocytes in the spinal cord somehow differs from that described in the rat optic nerve (Miller and Raff, 1984; Miller et al., 1985). GFAP+ only type 1 astrocytes were scattered in grey matter as well as in deep regions of the white matter. The white matter appeared to be divided in two zones with respect to the GFAP and O₄ staining. A number of radially oriented O₄+ GFAP+ astrocytes were detected in the peripheral regions of the white matter. By analogy with the optic nerve (Miller and Raff, 1984; Miller et al., 1985), we had expected that the radially oriented astrocytes forming the glial limiting membrane of the spinal cord would be type 1 astrocytes unreactive with the O₄ antibody. Instead we found cell bodies, radial processes, and pial endfeet strongly O₄ as well as GFAP positive. These peripheral astrocytes have also been stained with the monoclonal antibody A2B5 as well as with the antibody to the GD3 ganglioside (Hirano and Goldman, 1988) and therefore exhibit the type 2 astrocyte antigenic phenotype. However, the radial glia of the spinal cord differentiates well before myelination and in that regard must be distinguished from the type 2 astrocytes described in optic nerve (Miller et al., 1985; Liuzzi and Miller, 1987). Except for these radially oriented astrocytes at the periphery of the white matter, the other O₄+ GFAP+ cells of spinal cord fulfilled both the antigenic and positional criteria for type 2 astrocytes since they were located deeper in the white matter (Miller et al., 1985; Liuzzi and Miller, 1987). These type 2 stellate astrocytes were more frequent in the ventral zone than the dorsal zone. Their processes were much thinner than those of the peripheral radial glia (Fig. 2 *d* and Fig. 4 *f*).

The Spinal Cord of Mice with Demyelinating Disease

The time course of demyelination and remyelination in animals infected with MHV-A59 has been described else-
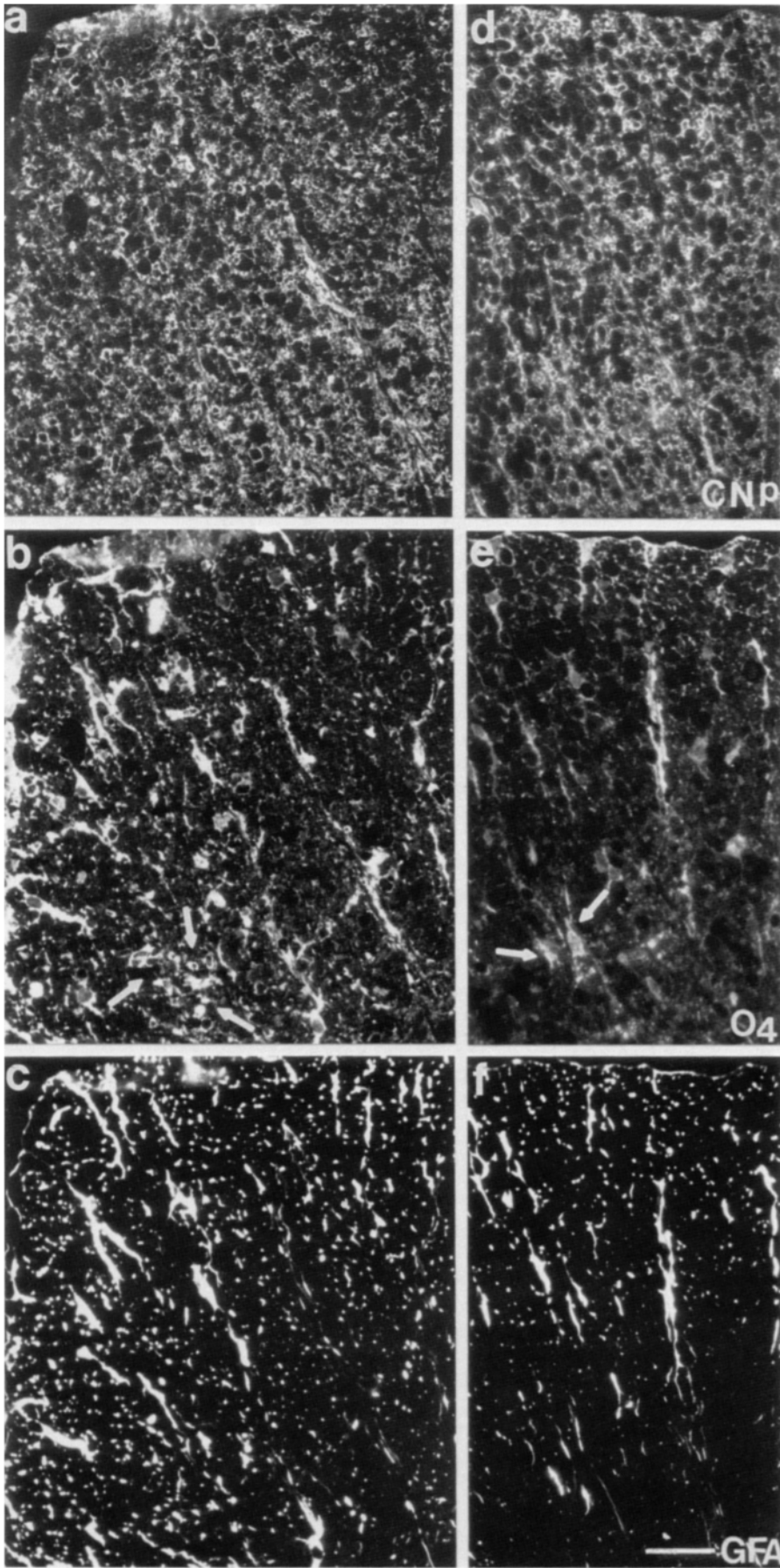
Figure 4. White matter of mouse spinal cord, 1 WPI. (*Left sections*) Infected mouse; (*right sections*) control mouse. CNP immunostaining is similar in infected (*a*) and control specimens (*d*), indicating that little destruction of myelin has occurred. However, a region at the upper left of *a* shows reduced immunoreactivity. O_4 immunostaining is dramatically increased in the infected mouse (*b*) and is associated with small process bearing cells deep in white matter as well as with many radial fibers which also stain for GFAP. Three white arrows in the lowest part of *b* have been placed around a group of cells which are strongly O_4 immunoreactive but do not contain GFAP. In contrast two O_4+ cells in the lower part of *e* (*white arrows*) also express GFAP. Note that their processes are thinner than those of the radially oriented astrocytes (*f*). Overall GFAP immunoreactivity is also slightly increased in the infected animal (*c*) as compared to control (*f*). Bar, 50 μm.

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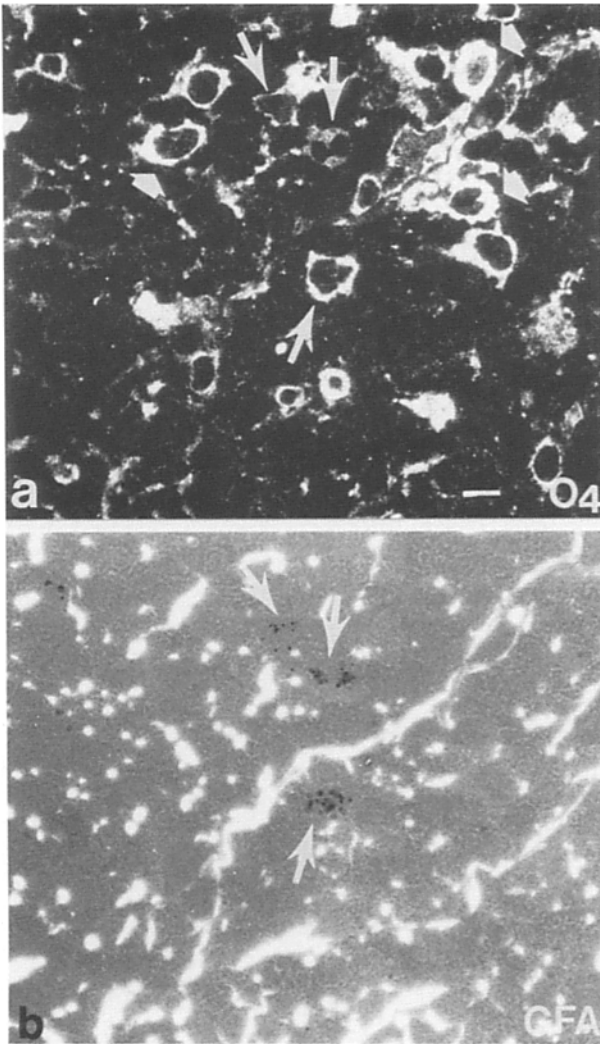
Figure 5. Spinal cord from infected mouse, 1 WPI. [³H]Thymidine was injected 2 h before killing to label the nuclei of proliferating cells. *a* shows O₄ immunofluorescence; *b* is a double exposure showing GFAP by immunofluorescence and a bright field image which reveals silver grains. Three O₄ positive cell bodies (*a, arrows*) are overlaid by silver grains (*b*). All three cells lack both GFAP (*b*) and CNP (not shown) immunoreactivity. Other O₄+ only cells (not thymidine labeled) are seen close by (*fat white arrows*). Bar, 10 μm.

where (Woyciechowska et al., 1984; Kristensson et al., 1986; Jordan et al., 1989*b*). The intensity and the evolution of lesions may vary from one animal to another as well as from one series of viral inoculation to another. Nevertheless most histopathological features follow a characteristic time sequence. Mild inflammatory lesions and very discrete areas of myelin loss are seen at 1 WPI mostly in the anterior and posterior roots. At 2 WPI, the myelin breakdown becomes apparent. Inflammatory cells infiltrate the lesions. At 3 and 4 WPI, the myelin loss is extensive with vacuolization and macrophage infiltration. At that time remyelination starts and progresses steadily in the next weeks (Jordan et al., 1989*b*).

At 1 WPI Both Antigenic Expression and Distribution of Glial Phenotypes Were Perturbed throughout the Spinal Cord. Loss of myelin was minimal, and CNP immunoreac-

tivity was generally normal (Fig. 4 *a* vs. Fig. 4 *d*). However, oligodendrocytes in focal areas of the white matter showed decreased CNP staining. The overall level of O₄ staining was substantially increased in the infected animals (Fig. 4 *b* vs. 4 *e*), while GFAP staining was increased less dramatically (Fig. 4 *c* vs. 4*f*). The number of GFAP+ stellate astrocytes was increased mostly in the grey matter and some of these cells also expressed the O₄ antigen. Control experiments with second conjugated antibodies alone showed no labeled cells within the parenchyma but some of the lymphocytes infiltrating the meninges were labeled by the antimouse IgM conjugate.

The number of O₄+ CNP− GFAP− progenitor cells was considerably increased (10–25 per section) over control animals (one per section); they sometimes formed clusters and were detected in both grey and white matter (Fig. 4, *b* and *e*, and Fig. 5 *a*). Several of these O₄ cells had incorporated tritiated thymidine given 2 h before sacrifice confirming that these cells were undergoing active DNA synthesis (Fig. 5 *b*). No other glial cells were found to be thymidine labeled at 1 WPI.

From 2 to 4 WPI the Major Antigenic and Cellular Changes Were Observed in Demyelinating Lesions. These changes consisted of a dramatic loss of CNP contrasting with an intense O₄ immunoreactivity (Figs. 6 and 7). The CNP disappeared from the cytoplasmic loops of myelin sheaths first and later from the oligodendrocyte perikarya. CNP-stained myelin debris was found in the extracellular space and in putative macrophages during later stages (Fig. 6, *b* and *d*). O₄+ only progenitor cells similar to those seen at 1 WPI (Fig. 5 *a*) were now found in these lesions (Fig. 6, *f* and *h*, *arrowheads*), and were sometimes labeled by tritiated thymidine (Fig. 7). In addition, large stellate cells expressing both O₄ and GFAP were also frequent in the lesions (Fig. 6, *f* and *h* and Fig. 7) compared to the normal (Fig. 6, *e* and *g*) and occasionally incorporated thymidine (Fig. 7). In the grey matter, the O₄+ astrocytes were less prominent than at 1 wk. Their number progressively decreased to normal levels within the next few weeks. GFAP+ only astrocytes remained common and were located mostly in regions adjacent to the demyelinated areas. An intriguing observation at this stage of the disease was the presence in the grey matter of rare cells stained for all three antigens, CNP, O₄, and GFAP (Fig. 8). None of these cells was labeled with tritiated thymidine given 2 h before killing.

The distribution of antigenic phenotypes among cells dividing at 2 WPI was determined by counting (Table I). Nearly 3/4 of labeled immunoreactive cells were O⁴+ only, while most of the remainder were O₄GFAP+. The small number shown for the O₄⁺ CNP⁺ category represents a single cell found in the sample used for quantitation. Qualitative examination of a larger group of specimens showed this category to be quite rare. A comparable group of control sections contained no immunostained, radiolabeled cells.

From 7 to 10 WPI Remyelination Was Observed. CNP immunoreactivity returned to normal levels in most regions of the spinal cord while some O₄+ GFAP+ stellate cells still persisted at 7–10 wk (Fig. 9). O₄+ CNP− cells were now as rare as in controls.

To study the fate of cells dividing in MHV-infected spinal cord, we injected tritiated thymidine at 3 and 4 WPI, and killed them 5 mo later, when remyelination was completed.
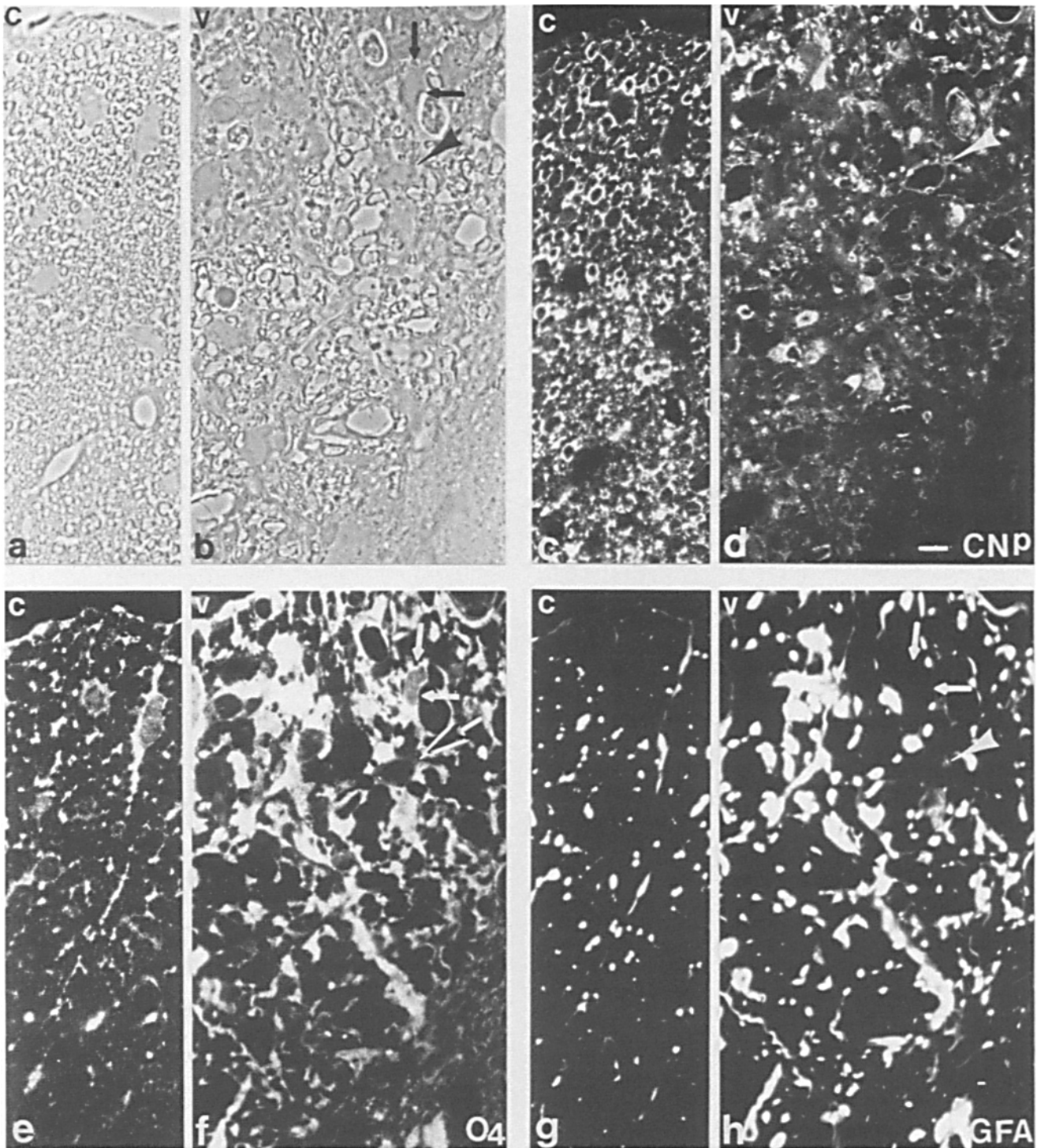
Figure 6. Spinal cord dorsal funiculus of control (*c*, in *a*, *c*, *e*, and *g*) and virus infected (*v*, in *b*, *d*, *f*, and *h*) animals at 2 WPI, near the dorsal root. Phase contrast and CNP immunostaining show massive destruction of myelin in infected animals (compare *a* and *c* with *b* and *d*). Only myelin debris and a few oligodendrocytes (*b* and *d*, *arrowhead*) show CNP immunoreactivity. Above the arrowhead, an O_4+ only cell is seen (*double white arrows* in *f* and *h*). A massive increase in processes and cell bodies stained with O_4 and GFAP is evident in the infected spinal cord (compare *e* with *f* and *g* with *h*). Bar, 10 μm.

Cryostat sections of spinal cord were immunostained for the oligodendrocyte-specific marker CNP and processed for autoradiography. Thymidine labeled CNP+ oligodendrocytes were found in all specimens (data not shown). This is in agreement with the earlier study by Herndon et al. (1977), showing thymidine-labeled oligodendrocytes by electron mi-

croscopy after long survival of animals injected with another strain (JHM) of mouse hepatitis coronavirus.

Discussion

In the present study, we have used triple label immunocy-
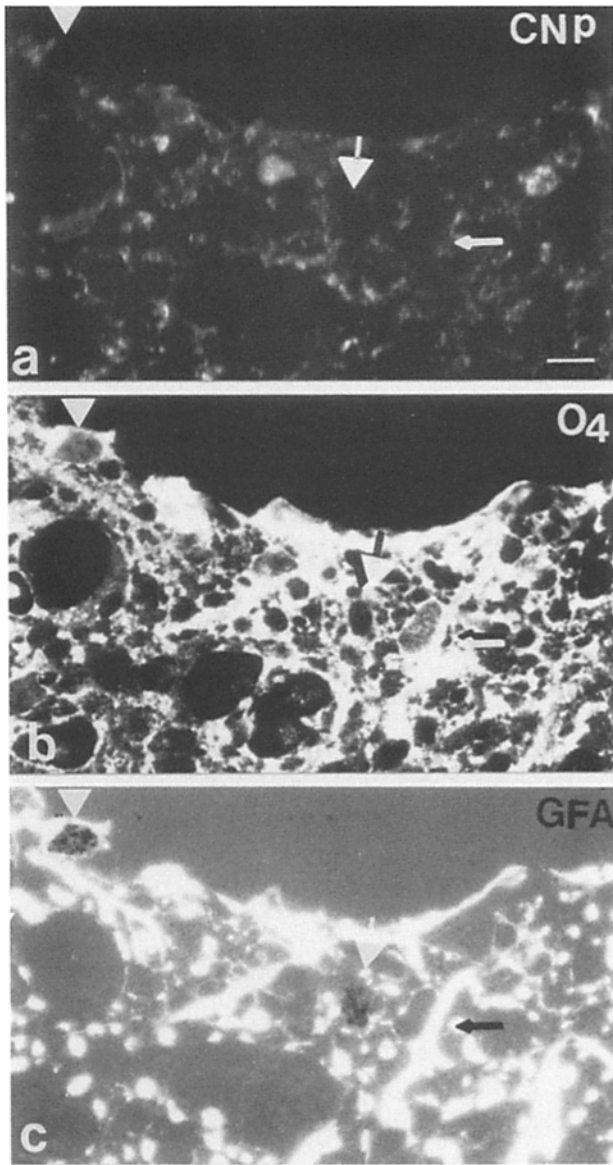
Figure 7. Dorsal funiculus of infected spinal cord, 3 WPI, [³H]thymidine injected 2 h before killing. The lower section is a double exposure showing both GFAP in immunofluorescence and silver grains (*bright field*). The white triangle in the left upper corner shows a ³H-labeled O₄+ GFAP+ CNP− cell. The triangle with a short tail in the middle points to an O₄+ only ³H-labeled cell and the thin arrow on the right side points to a large O₄+ GFAP+ cell without thymidine label. Bar, 10 μm.

tochemistry combined with thymidine autoradiography to characterize in vivo the glial cells reacting to a virus-induced demyelinating disease in mice. We identify some of these cells as members of the O-2A lineage based on their reactivity with the O₄ antibody which reacts with sulfatide and another putative glycolipid and which labels O-2A progenitors isolated from the adult rodent nervous system (Wolswijk and Noble, 1989). In our mice, O₄ antibody stained cells that were also GFAP+ or CNP+, and stained other cells which were negative for both of these differentiation antigens. While such O₄ only cells were rarely seen in normal mice, they were readily found throughout the spinal cord of viral inoculated mice at 1 WPI and were dividing. GFAP+ O₄− and GFAP+ O₄+ astrocytes were also more prominent throughout the spinal cord at that time. Later on, antigenic changes were mostly associated with the demyelinating lesions: while CNP reactivity disappeared, cells with O₄ reactivity increased in the lesions and both putative O-2A progenitors and type 2 astrocytes were found to incorporate tritiated thymidine after a 2-h pulse. Remyelination followed and CNP staining returned to control levels.

The O₄ antibody stains oligodendrocytes, type 2 astrocytes and oligodendrocyte precursors cultured from perinatal rat and mouse as well as a majority of O-2A progenitors cultured from 7-d-old rat optic nerve (Sommer and Schachner, 1981; Schachner et al., 1981; Sommer, I., and M. Noble, unpublished observations; Trotter and Schachner, 1989). Clones derived from single O-2A progenitor cells from neonatal optic nerve or brain often contain multipolar O₄+ GC− cells capable of proliferation and differentiation (Dubois-Dalcq, 1987; McMorris and Dubois-Dalcq, 1988). Similar O₄+ cells have also been isolated from adult rat optic nerve. These cells proliferate slowly and give rise in vitro to oligodendrocytes and type 2 astrocytes; they have been termed O-2A adult progenitor cells (Wolswijk and Noble, 1989). Cells with similar phenotype (O₄+ GFAP− GC−) have also been isolated from adult mouse spinal cord. Precursor cells of oligodendrocytes have been identified in vivo in the developing white matter using antibody to GD3 gangliosides (Levine and Goldman, 1988; Reynolds and Wilkin, 1988) but until now O₄+ precursor of oligodendrocytes had not been visualized in adult CNS in vivo.

Using 1-μm frozen sections and triple immunofluorescence, we have found cells with the O-2A adult progenitor antigenic phenotype in normal and virus infected mice. These cells were increased in number during viral infection and, since they incorporated tritiated thymidine, were actively
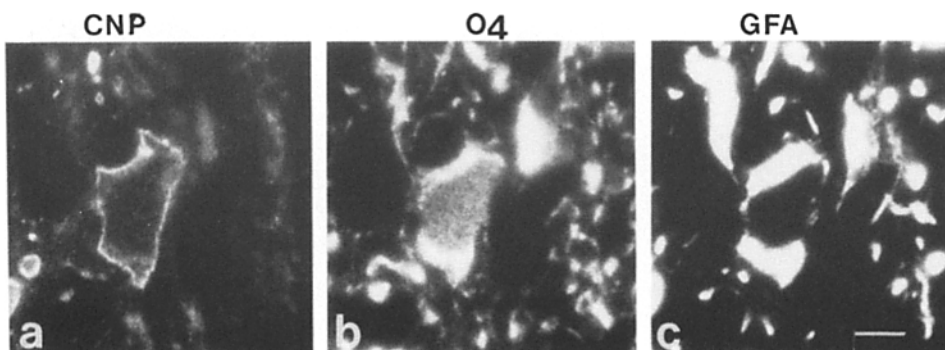
Figure 8. Example of a cell with a mixed phenotype. A CNP+ O₄+ GFAP+ cell in the spinal cord of an infected mouse, 2 WPI. Such cells were found infrequently in spinal cord of infected mice but never in controls. They were most abundant at 2 WPI and were never labeled by [³H]thymidine injected shortly before killing. Bar, 10 μm.

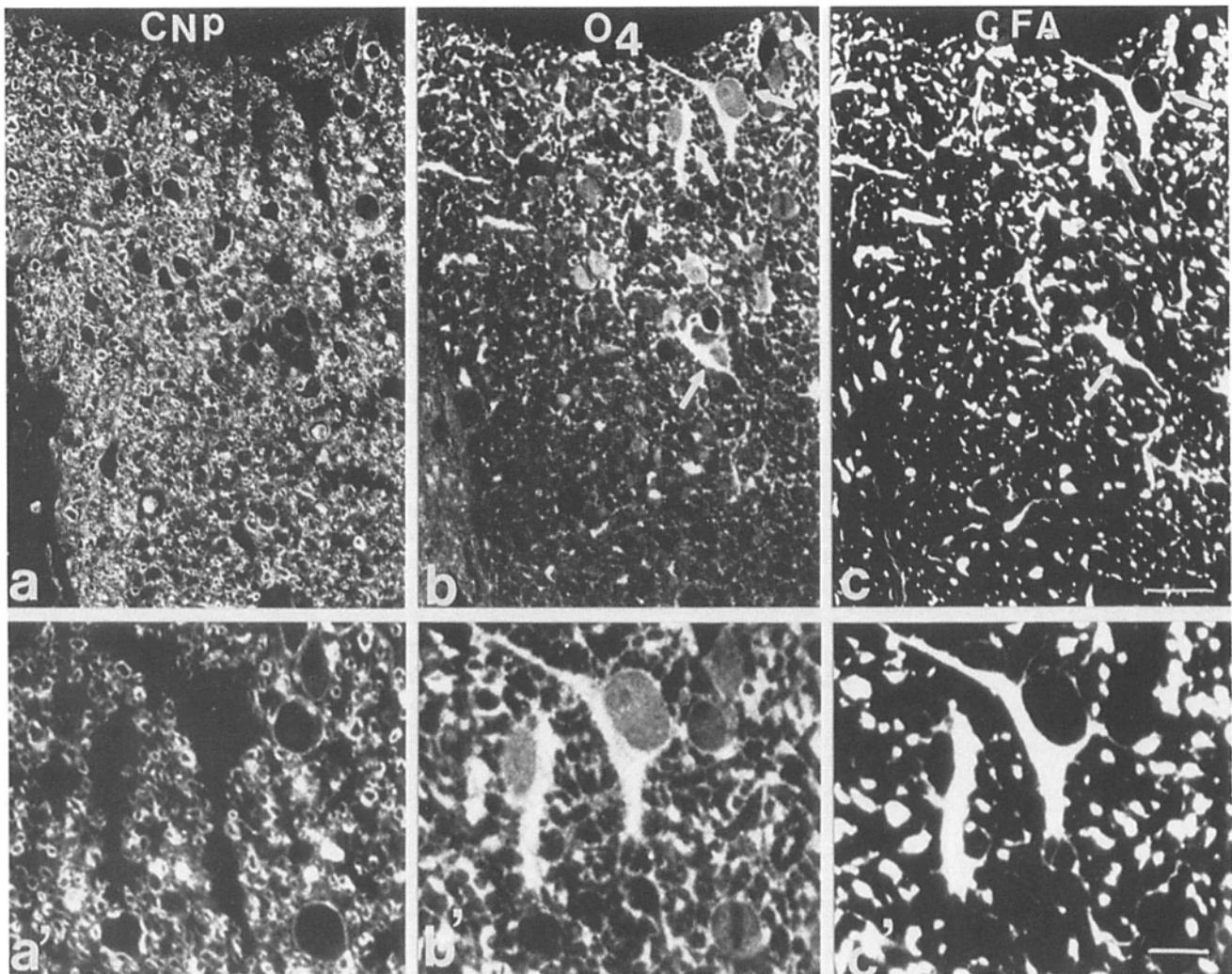*Table I. Incorporation of [³H]Thymidine by Glial Cells after Viral Infection*

	Infected animals		Control animals
Phenotype	Labeled cells per section	Percentage	Labeled cells per section
O₄+	9 ± 2	71	0
O₄+ CNP+	0.3 ± 0.4	2	0
O₄+ GFAP+	3 ± 2	24	0
GFAP+	0.3 ± 0.4	2	0

* Mean ± SEM of immunoreactive, ³H-labeled cells per whole spinal cord transverse section. Data from duplicate counts from 2 WPI tissue labeled with [³H]thymidine 2 h before sacrifice.

dividing. While not definitive, the evidence at hand suggests that these O₄+ GFAP− CNP− cells, seen in situ for the first time, might give rise to oligodendrocytes formed in these adult animals during recovery from viral infection. Similarly, induction of oligodendrocyte proliferation and remyeli-

nation after chronic demyelination in guinea pigs and multiple sclerosis has been described (Raine et al., 1988).

In addition to the O₄+ only O-2A adult progenitor cell, we found increased numbers of O₄+ GFAP+ cells in infected mice. Many of these cells also incorporated tritiated thymidine, as was seen previously in developing optic nerve (Miller et al., 1985). Type 2 astrocytes are normally formed during developmental myelination, where they extend processes to the nodes of Ranvier (ffrench-Constant and Raff, 1986); the O₄+ GFAP+ cells which proliferate in the infected animals might play the same role during remyelination. In addition, we found occasional cells with the mixed phenotype O₄+ GFAP+ CNP+. The existence of this intermediate form raises the possibility that transdifferentiation of type 2 (O₄+ GFAP+) astrocytes might also contribute to the generation of oligodendrocytes. Interestingly, the expression of GFAP in immature oligodendroglia has been described before in the developing human spinal cord (Choi and Kim, 1984) and coexistence of the oligodendrocyte marker carbonic anhydrase and GFAP have been reported in radial

Figure 9. Dorsal funiculus of infected spinal cord, 7 WPI. *a′, b′, c′* are close up of the upper region of *a, b,* and *c.* CNP immunofluorescence shows nearly normal intensity and is distributed as rings around and within myelin sheaths reformed during remyelination (*a* and *a′*). O₄ immunofluorescence is still greater than normal though somewhat reduced as compared to lesions at earlier stages; however, several large O₄+ GFAP+ cells are scattered in the remyelinated area (*white arrowheads in b and c*). Such cells were never found in control specimens. Bars: (*a–c*) 50 μm; (*a′–c′*) 20 μm.

fibers of developing spinal cord (Hirano and Goldman, 1988). Similarly cultures of spinal cord from our remyelinating animals yield a substantial number of cells expressing both oligodendrocyte and astrocyte markers (Armstrong, R., V. Friedrich, K. V. Holmes, and M. Dubois-Dalcq, manuscript in preparation).

The mechanisms by which CNS tissue reacts to a demyelinating virus and reconstructs myelinated and functionally ensheathed axons after a dramatic tissue destruction are completely unknown. It is likely that polypeptide factors can trigger mitosis, migration, and differentiation in vivo during remyelination just as they do in vitro. Interleukin 2 has been shown to induce proliferation and differentiation of oligodendroglial cells (Benveniste and Merrill, 1986) and insulin-like growth factor 1 has an effect on proliferation and differentiation of O_4 precursors in oligodendrocytes in the developing rat (McMorris and Dubois-Dalcq, 1988). Platelet derived growth factor is a potent mitogen for the O-2A neonatal progenitor (Richardson et al., 1988; Noble et al., 1988) but it is not known whether it also influences the growth of O-2A adult progenitors. In vitro studies have shown that O-2A neonatal and adult progenitors are migratory cells (Small et al., 1987; Wolswijk and Noble, 1989) and that adult optic nerve glia can migrate $\geqslant 0.6$ mm to myelinate naked axons (Wolf et al., 1986). Ciliary neurotrophic factor which is synthesized by type 1 astrocytes in vitro can induce transient GFAP expression in O-2A progenitor cells in developing optic nerve and brain cultures (Hughes and Raff, 1987; Lillien et al., 1988). Ciliary neurotrophic factor molecules are released locally in CNS tissue after a lesion (Nieto-Sampedro et al., 1983). Such factors could also be released in the spinal cord of our infected mice.

Whatever the polypeptide factors involved may be, the end result of the glial cell reaction to mouse hepatitis virus infection is the birth of new oligodendrocytes (see also Herndon, 1977) and efficient remyelination. We cannot exclude that some oligodendrocytes might be able to dedifferentiate, and turn off the expression of myelin protein genes but the presence of a small number of O-2A progenitors in the normal mice suggests that this pool of precursors might be amplified in the course of this demyelinating disease. CNP+ oligodendrocytes persisted throughout the course of the infection in many areas not demyelinated, but we rarely saw them labeled after 2 h exposure to tritiated thymidine. Thus, in contrast to previous observations in other models in rat (Aranella and Herndon, 1984; Ludwin and Bakker, 1988), we found no evidence for proliferation of differentiated oligodendrocytes as a substantial component of response to injury. Instead, our results indicate that newly formed oligodendrocytes arise from mitotic O_4+ CNP− GFAP− precursors. In vitro analysis of these cells may lead to a more detailed understanding of their behavior and the identification of signal factors which control gliogenesis during remyelination.

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