Journal of Neuroscience Research 54:79–96 (1998)

Monoclonal Antibody 14F7, Which Recognizes a Stage-Specific Immature Oligodendrocyte Surface Molecule, Inhibits Oligodendrocyte Differentiation Mediated in Co-Culture With Astrocytes

Kazunori Yoshimura,[1,5] Yoko Sakurai,[1] Daisuke Nishimura,[1,2] Yoshihiro Tsuruo,[4] Masayuki Nomura,[5] Suguru Kawato,[2] Chika Seiwa,[1,3] Taisen Iguchi,[3] Kouichi Itoh,[6] Hiroaki Asou[1]*

[1]Department of Neuro-cell biology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo, Japan
[2]Department of Biophysics and Life science, University of Tokyo at Komaba, Meguro-ku, Tokyo, Japan
[3]Graduate School of Integrated Science, Yokohama City University, Yokohama, Japan
[4]Department of Anatomy, Tokushima University School of Medicine, Tokushima, Japan
[5]Department of Physiology, Saitama Medical School, Iruma-gun, Saitama, Japan
[6]The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Cells at an intermediate stage of oligodendrocyte lineage are not only well characterized by biochemical studies but also are likely to relate to the outcome of physiological events. To elucidate the molecular events leading to the development of oligodendrocyte lineage cells, we have raised monoclonal antibodies against stage-specific immature oligodendrocytes, which have previously been isolated by a novel oligodendrocyte-lineage cell culture technique (Sakurai et al.: J Neurosci Res 52:17–26, 1998).

We have isolated a mouse monoclonal antibody termed 14F7 which predominantly labels stage-specific immature oligodendrocytes and have found that the expression of 14F7 immunoreactivity in the developing neonatal rat forebrain is closely associated with cells expressing the oligodendrocyte progenitor marker A2B5 and to immature oligodendrocyte expressing O4 antigen. 14F7$^+$ cells were distributed in the ventricular and subventricular zone and the nearby forming corpus callosum as non-myelinating cells. In contrast to cell culture observations, 14F7$^+$ cells were seen only in oligodendrocyte lineage cells. For instance, dissociated cell culture studies indicated that 14F7 labels a cell surface molecule, and its cellular distribution is coincident with all of O4$^+$ cells and A2B5$^+$ cells, and even A2B5$^-$ cells. By contrast, 14F7-positive cells did not label astrocytes and, furthermore, did not label myelin basic protein (MBP)-positive oligodendrocytes. 14F7 recognized a 48-kDa protein on sodium dodecyl sulfate polyacrylamide gel electrophoresis. 14F7 immunoreactivity was detectable in rat brain as early as embryonic day 18. Furthermore, in these cells, the total time for differentiation was extended, and on maturation, these cells subsequently expressed an array of myelin-specific proteins, which normally occurs by direct contact with type-1 astrocytes. However, in the presence of 14F7, stage-specific oligodendrocytes co-cultured with astrocytes completely failed to express MBP. These data suggest that the 14F7 antigen is a novel cell surface molecule that is expressed in the intermediate stage of oligodendrocyte-lineage cells, and it is expected that it regulates the differentiation of oligodendrocyte throughout development. J. Neurosci. Res. 54:79–96, 1998.

Key words: oligodendrocyte-lineage cell; oligodendrocyte differentiation; oligodendrocyte progenitor; monoclonal antibodies 14F7

INTRODUCTION

Oligodendrocyte precursor cells are derived from mostly the subventricular zone (SVZ). For instance, in the rat, the precursor cells are generated primarily during the late embryonic and early postnatal stages (reviewed by Bayer and Altman, 1991). These cells give rise to

Contract grant sponsor: Ministry of Education, Science, Sports, and Culture, Japan.

Y. Tsuruo's present address is Department of Anatomy, Wakayama-Medical College, Wakayama, Japan.

*Correspondence to: Hiroaki Asou, Dept. of Neuro-cell Biology, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashi-ku, Tokyo 173, Japan. E-mail: asou@center.tmig.or.jp

Received 10 February 1998; Revised 11 June 1998; Accepted 12 June 1998

intermediates in the oligodendrocyte lineage that are small, process-bearing, motile, and rapidly proliferating (Raff et al., 1983a; Levine and Goldman, 1988; Hardy and Reynolds, 1991; Grinspan et al., 1993; Asou et al., 1995).

Previous studies demonstrated that oligodendrocyte precursor cells, O-2A cells, are recognized by binding of the A2B5 monoclonal antibody and by their lack of expression of glial filament or myelin component galactolipids (Eisenbrath et al., 1979; Raff et al., 1983b, Noble et al., 1988; Ginspan et al., 1990; Hardy and Reynolds, 1991). Under the proper conditions, these O-2A precursor cells, whose symmetrical cell divisions generate daughter cells that produce oligodendrocytes in a proliferative cell lineage, tend to share the same fate, either dividing again or differentiating (Raff et al., 1983a; Temple and Raff, 1986).

Differentiated oligodendrocytes may be recognized by surface binding of anti-O4/O1 antibodies (Sommer and Schachner, 1980), and mature oligodendrocytes (at the late stage of differentiation) may be recognized by antibodies against myelin structural proteins, such as myelin basic protein (MBP) and proleolipid protein (PLP) (Campagnoni and Macklin, 1988; Lemke, 1988; Trapp, 1990a).

In white matter of the developing central nervous system (CNS), the early oligodendrocytes have processes that contact, ensheath, and myelinate axons. The initial ensheathment of immature axons is preceded by formation of a plasma membrane of immature oligodendrocyte (Hildebrand et al., 1993). Although several members of the immunoglobulin superfamily have been identified at the contact zone between axons and myelinating oligodendrocytes, the molecular trigger for myelination is not known (Colman, 1991). Although our understanding of oligodendrocyte lineage is evolving, further characterization of the appearance, fate, and molecular events that occur during oligodendrocyte differentiation is needed.

Therefore, the important questions are, What is the developmental potential of the progenitor cells, and when/where do they become committed to become oligodendrocyte lineage cells? To elucidate the generation of oligodendrocyte-lineage cells and their differentiation in vivo and in vitro, we have generated monoclonal antibodies against stage-specific immature oligodendrocytes. Monoclonal antibodies from these clones, designated 4F2, 14F7, and 13E6, recognize stage-specific immature oligodendrocytes. In the present study, we have focused on one of these antibodies, 14F7, to characterize at cellular and biochemical levels using the developing rat brain.

MATERIALS AND METHODS

Animals

Pregnant and normal Wistar rats of both sexes at different ages (Japan SLC, Shizuoka) were used. The day of birth was defined as postnatal day zero (P0). The animals were maintained on a 12-hr light:12-hr dark cycle at a constant temperature of 20°C. The animals were fed ad libitum. Animals were anesthetized with pentobarbital and subjected to perfusion with ice-coated phosphate-buffered saline (PBS) (300 ml/rat) via the left cardiac ventricle to flush out the blood.

Preparation of Cultures

Cultures of stage-specific pure oligodendrocyte were prepared from primary mixed cell cultures of rat embryonic cerebral hemispheres with a slight modification as previously described (Sakurai et al., 1998). Briefly, the cerebral hemispheres from embryonic day 18 rat embryos were enzymatically dissociated in a solution of dispase II (0.3 mg/ml, Boehringer Mannheim, Mannheim, Germany) and 0.05% DNase (Boehringer Mannheim) in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY). After being washed with DMEM, the dissociated cells were sieved through 70-μm pore-sized nylon mesh (#2350, Becton Dickinson, Franklin, NJ) and seeded onto poly-L-lysine–coated culture dishes (1.4×10^7 cells/60-mm dish) (Greiner, Tokyo, Japan). After 7 days of culture, the cells were passaged with 0.25% trypsin in PBS, centrifuged for 10 min at 500*g* and 4°C, and seeded in tissue culture dishes at a density of 2×10^6 cells/dish in DMEM containing 10% fetal calf serum (FCS). At every 7 days of culture, the cells were dispersed with 0.25% trypsin in PBS and centrifuged for 10 min at 500*g* and 4°C. Then cells were resuspended in DMEM (as described above) for 2 days. On the 2nd day of the cultures, the medium was exchanged for a serum-free defined medium consisting of DMEM supplemented with glucose (5 mg/ml), insulin (5 μg/ml), sodium selenite (40 ng/ml), transferrin (100 μg/ml), progesterone (0.06 ng/ml), putrescine (16 μg/ml), thyroxine (40 ng/ml), triiodothyronine (30 ng/ml), and basic fibroblast growth factor (bFGF) (2 ng/ml). After an additional 7 days in serum-free condition, the cells were dispersed by treatment with 0.25% trypsin and seeded in culture dishes at a density of 1.7×10^6 cells/dish. These procedures were repeated a total of three times. The cells were cultured for more than 1 month for the immunocytochemistry and biochemical studies.

Production of Monoclonal Antibodies

To generate the desired immune response, we have developed a new technique for immunization as follows: Spleen cells (1×10^7/ml) from an unimmunized mouse (Balb/c) were co-cultured with stage-specific immature oligodendrocytes ($A2B5^-$, $O4^+$) and maintained in DMEM supplemented with 10% FCS for 2–3 weeks. After the number of lymphocytes increased significantly due to primary contact with surface antigens of stage-

specific immature oligodendrocytes, the lymphocytes were collected, rinsed with DMEM two times, and inoculated to the same strain of mouse via intravenous or intraperitoneal injection. A booster was performed two times at every 7 days after the primary immunization. Three days after the last inoculation, the animals were sacrificed, and their spleens were removed. The spleen cells were then fused with the NS1 myeloma cell line (American Type Culture Collection [ATCC], Rockville, MD) according to the established protocol (Asou et al., 1996). Hybridoma cells were tested for reactivity to the stage-specific immature oligodendrocytes by immunocytochemistry, especially with $A2B5^+$ and $O4^+$ oligodendrocytes, and those whose supernatants showed cell surface immunoreactivity to immature oligodendrocytes were subsequently cloned three times by limiting dilution and expanded and frozen in liquid N_2 as previously described (Yoshimura et al., 1996). Ascites fluid was generated by injecting 5×10^6/ml hybridoma cells into 2-month-old Balb/c mice that had been previously primed with pristane (2,6,10,14-tetramethyl-pentadecanoic acid) (Daiichi Kagaku, Tokyo, Japan).

Immunocytochemistry

To determine the cellular specificity of 14F7 binding in vitro, dissociated cerebral cell cultures were labeled with 14F7 and either A2B5 antibody (Eisenbarth et al., 1979), anti-O1 antibody, anti-O4 antibody (Sommer and Schachner, 1981), or anti-MBP antibody (Hartman et al., 1982) for the identification of oligodendrocyte-lineage cells, or anti-glial fibrillary acidic protein (GFAP) antibody (Bignami et al., 1972) for the identification of astrocytes. For cell surface antigens, cultures were labeled prior to fixation, whereas for intracellular antigens, cultures were labeled after fixation with 5% acetic acid in 90% ethanol at −20°C for 10 min. All incubations were for 90 min in PBS containing 10% normal horse serum, 1% bovine serum albumin (BSA) with antibodies at the following dilution: 14F7 ascites (1:100), mouse O4 and O1 hybridoma (ATCC) supernatant (no dilution), mouse anti-MBP ascites (Boehringer Mannheim, 1:200), rabbit anti-GFAP antiserum (DAKO Japan Co. Ltd. Tokyo, Japan, 1:500). Binding of the antibodies was visualized with appropriate class-specific secondary antibodies such as rhodamine-conjugated goat anti-mouse IgG (ICN Pharmaceuticals, Inc., Tokyo, Japan, 1:200), fluorescein-conjugated goat anti-mouse IgM (ICN, 1:200), and rhodamine-conjugated goat anti-rabbit IgG (ICN, 1:400).

Tissue Section Preparation and Immunostaining

Under anesthesia by intraperitoneal injection of pentobarbital (50 mg/kg body weight), the animals were perfused through the ascending aorta with warmed (37°C) heparinized 0.1 M phosphate buffer (PB), pH 7.4, followed by a periodate-lysine-2% paraformaldehyde (PLP) fixative, and the tissues were immersed in the same fixative for 2–4 hr at 4°C. After fixation, the brain slices were successively soaked in 10%, 15%, and 20% sucrose in 0.01 M PBS, pH 7.4. These tissues were frozen and cut serially in 10-μm-thick frontal sections in a cryostat. Frozen sections were thaw-mounted on glass slides coated with chrome alum gelatin and air-dried for 2 hr.

Frozen sections were rinsed in 0.01 M PBS for 30 min, and endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide in distilled water for 30 min at room temperature. After incubation in PBS containing 1% normal goat serum (NGS), the sections were treated with avidin and biotin solution (Vectastain, Vector Laboratories, Burlingame, CA) successively in order to block the endogenous avidin-binding activity. The sections were incubated with mouse monoclonal antibody to mouse ascites A2B5 (1:200) or O4 (25 μg/ml) overnight at 4°C, treated with biotinylated goat anti-mouse IgM (1:100) (Vectastain) for 1 hr at 32°C, incubated in Elite ABC solution (mixing 50 μl of avidin and biotin-peroxidase solution in 5 ml PBS containing 1% NGS) for 1 hr at 32°C, and processed for the visualization of the reaction in 50 mM Tris-HCl buffer, pH 7.6, containing 0.01% 3, 3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. The sections were placed on a slide coated with a drop of 50% glycerin in PBS and covered with a coverslip and photographed.

Biochemical Analysis

To investigate the biochemical characteristics and distribution of 14F7 antigen, tissues from various rat CNS regions and other organs were examined. To investigate the developmental expression of this antigen, whole brain from rats of various ages were examined. For an immunoblotting analysis, cultured cells and tissues were homogenized with a Teflon-glass homogenizer in solubilizing buffer as previously described (Asou et al., 1996). Briefly, cells and tissues were homogenized on ice in 10 volumes (w/v) of cold solubilizing buffer (pH 7.4) containing 0.5% NP-40 and protease inhibitors (25 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 4.8 μg/ml leupeptin and 6.9 μg/ml pepstatin A). Samples were centrifuged at 100,000*g* to pellet insoluble material, and the supernatants were saved for further analysis. Protein concentration was determined by the Lowry method. Western blotting was performed as previously described (Asou et al., 1996). Briefly, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels and transferred to an Immobilon polyvinyl difluoride (PVDF) membrane (Millipore Lab., Tokyo, Japan). After blocking with 3% skim milk (Difco Lab., Rockville, MD) in Tris-buffered saline (TBS), pH 7.6, the blots were incubated with purified mouse IgM molecules from ascites of

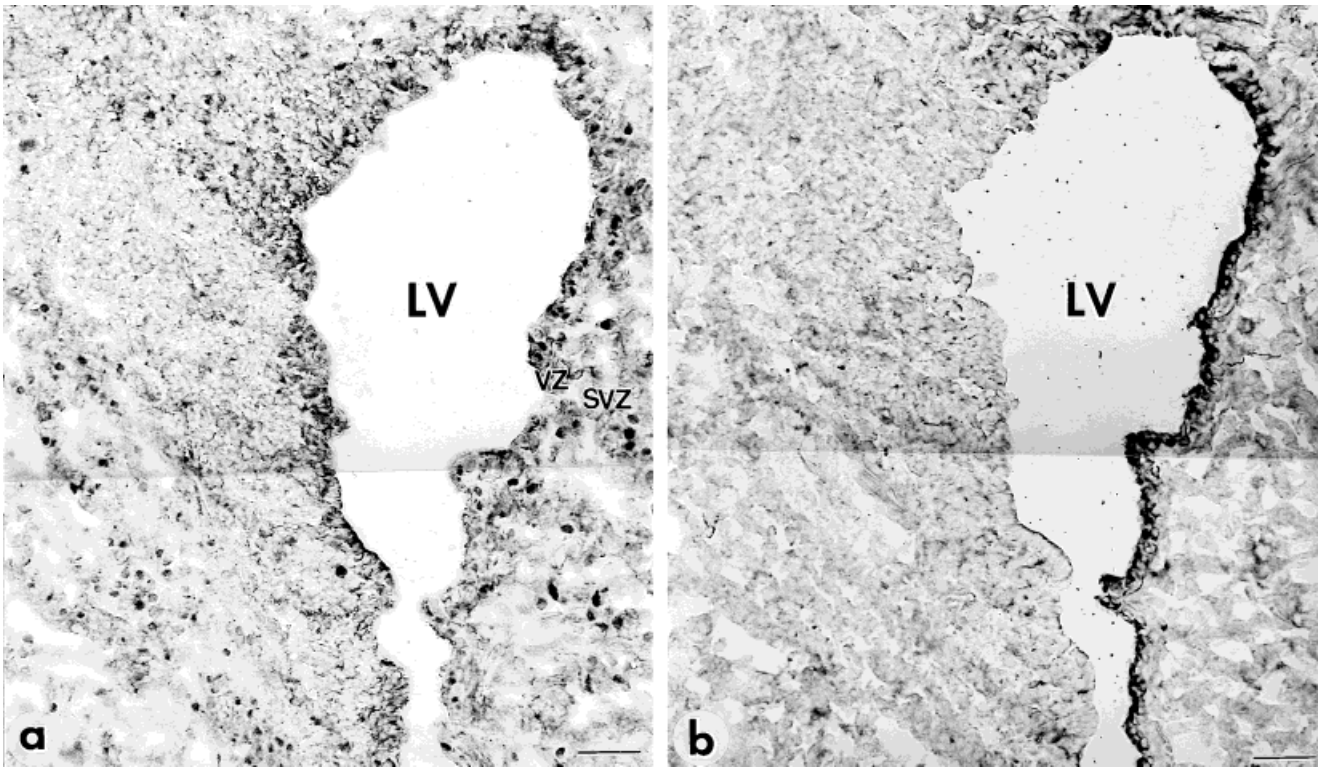

Fig. 1. Dorsal aspect of ventricular (VZ) and subventricular zone (SVZ) of the lateral ventricle (LV) at postnatal day 6 (P6). Semiadjacent sections in the frontal plane. Upper left corner of each figure is in the dorsolateral direction. **a:** A2B5 immunostaining. Both in the ventricular and subventricular zones, the A2B5 immunoreactivity is located on both lateral and medial sides of the LV. **b:** O4 immunostaining. In the VZ, the O4 immunoreactivity is intense and restricted to the medial side. O4 immunoreactivity is also found in the subventricular zone facing the dorsal aspect of the lateral ventricle. Scale bar = 50 μm.

14F7 for 2 hr at room temperature (RT). An immunoaffinity matrix for purification of IgM molecules was prepared by covalently coupling affinity-purified anti-mouse IgM μ-chain specific antibodies to agarose (Pierce, Rockford, IL). Blots were then rinsed three times with TBS, incubated with alkaline phosphatase–conjugated goat anti-mouse IgM (Sigma, St. Louis, MO) 1:100 dilution in TBS for 2 hr at RT, and developed with nitro-blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) solution (Sigma).

RESULTS

14F7 Antigen Specifically Bound to Oligodendrocyte-Lineage Cells in Sections of Developing Neonatal Brain

The monoclonal antibodies O4 and A2B5 have been used to define a distinct stage of oligodendrocyte-lineage cells in vivo. In the ventricular zone (VZ), the A2B5 immunoreaction was located both on the lateral and medial sides of the lateral ventricle and also in the dorsal aspect. In the subventricular zone, A2B5-immunoreactivity showed a patchy pattern of distribution (Fig. 1a). The A2B5-immunoreactive cells were located in the rostrocaudal extent of the SVZ (data not shown).

The O4 immunoreaction was located on the cell surfaces as in the case of A2B5. In the VZ, the O4 immunoreaction was located on both sides of the lateral ventricles, but it was much more intense on the medial aspect, and some of the processes were also positive (Fig. 1b). The O4 immunoreaction was seen in the whole extent of the SVZ, but the pattern of O4 staining was considerably different from that of A2B5. The intensity of the O4 immunoreactivity was pronounced in the medial side of the lateral ventricle. To examine the distribution of 14F7 antigen in vivo under the same conditions as described above, frozen sections obtained at the same time (P6) were also labeled with antibody. In all such sections, the pattern of 14F7 labeling was coincident with O4-positive cells in the medial side of the lateral ventricle, suggesting that 14F7 is expressed on premature oligodendrocytes (Fig. 2). In addition, 14F7 labeling was more distinctly associated with oligodendrocytes in VZ than in SVZ. The 14F7 immunoreactivity was present on the cellular surface and processes. Many immunoreactive processes were found in the medial SVZ and the nearby

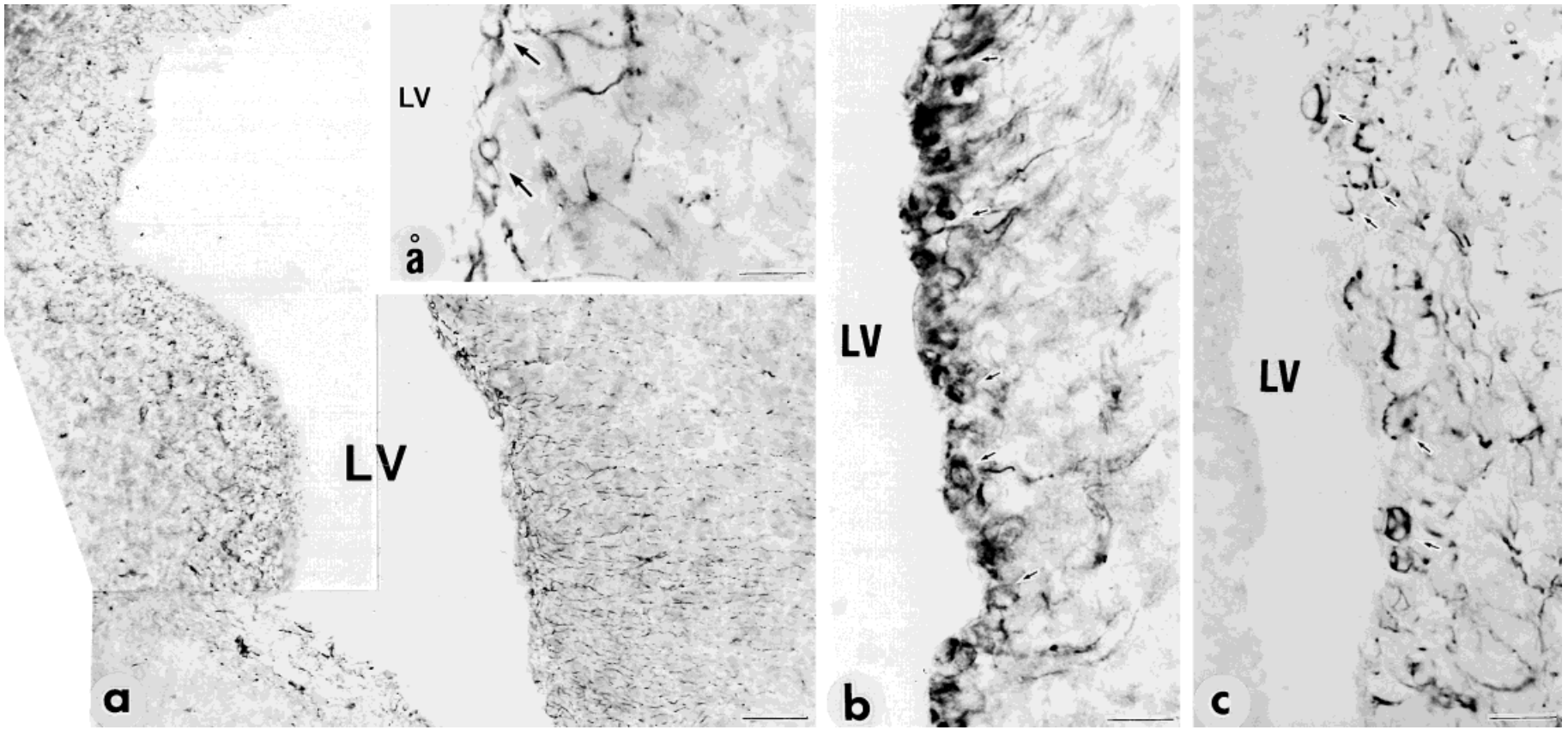

Fig. 2. Expression of 14F7 antigen in the frontal planes of developing rat brain at postnatal days 1 (P1), P3, and P6. Overview of the ventricular (VZ) and subventricular zone (SVZ) at P1 (**a**), and the medial side of lateral ventricle (LV) at P3 (**b**) and P6 (**c**). At P1, 14F7 immunoreaction is seen in the VZ and SVZ of the medial and lateral aspect of the LV. Many 14F7-immunoreactive processes are present in the hippocampal fimbria on the medial side. Some of the labeled cells and processes (arrows) in the hippocampal fimbria are shown in the **inset** at higher magnification (**å**). In the VZ, 14F7 immunoreactivity is seen on the cellular surfaces and processes predominantly on the medial side at P3. At P6, 14F7 immunoreactivity is exclusively located in the ventricular and subventricular zones facing the medial side of the LV. At the rostral forebrain, 14F7 immunoreactivity is located on the cellular surface and processes (arrows). Upper left corner of each figure is in the dorsolateral direction. Scale bars = 100 μm in a; 25 μm inset å, b, and c. LV, lateral ventricle.

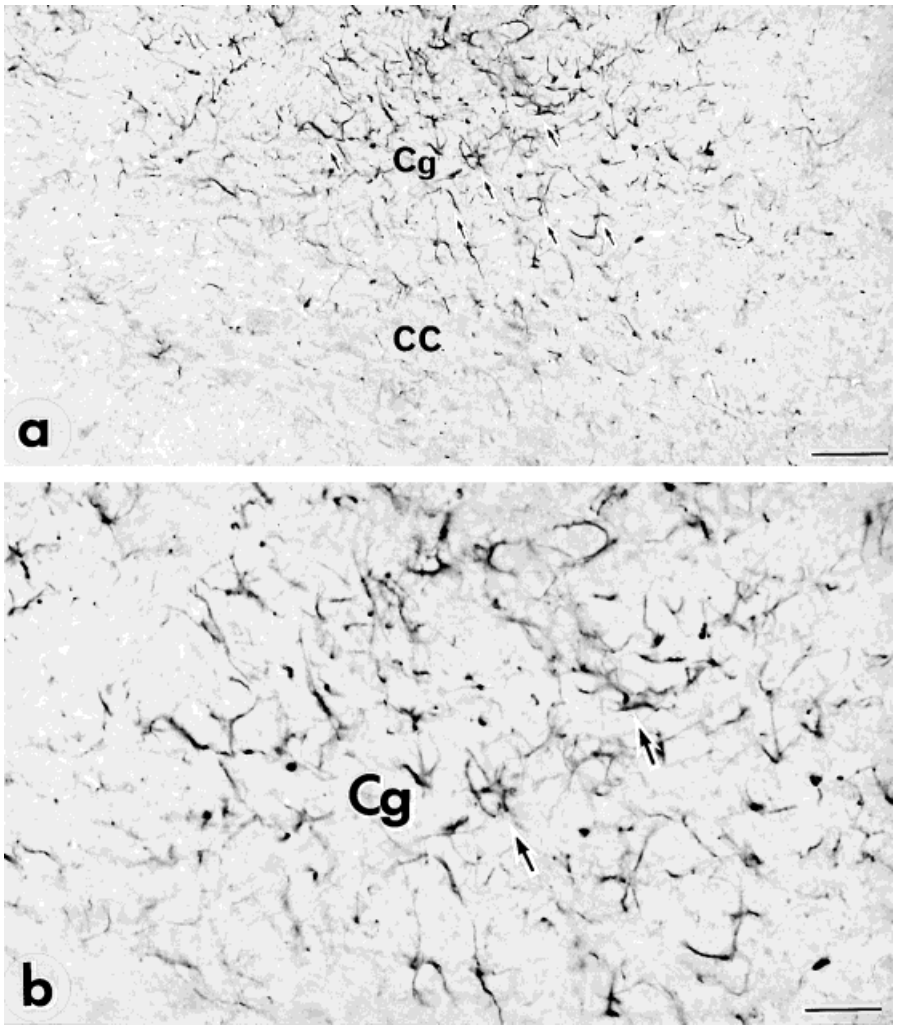

Fig. 3. Expression of 14F7 antigen in the frontal plane of the cingulum and the dorsal part of the corpus callosum at postnatal day 14. Many immunoreactive cells with several radially oriented processes (arrows) are present. A part of **a** is shown in **b** at higher magnification. Scale bars = 50 μm in a, 25 μm in b. Cg, cingulum; CC, corpus callosum. Upper left corner is in the dorsolateral direction.

white matter such as the hippocampal fimbria. In later stages of development, 14F7 immunoreactivity was located in the corpus callosum near the lateral ventricle as well as in the ependymal and subependymal cells surrounding the lateral ventricle. At P14, for example, there were many immunoreactive cells with several radially oriented processes in the cingulum and the dorsal part of the corpus callosum (Fig. 3).

14F7 Labels Stage-Specific Oligodendrocyte-Lineage Cells in Cultures

Primary dissociated cell cultures of cerebral hemispheres of 18-day-old rat embryos were double labeled with 14F7 and either anti-GFAP antibody to identify astrocytes (Bignami et al., 1972), anti-A2B5 antibody to identify oligodendrocyte precursor (O2A progenitor) (Raff et al., 1983a), and/or O4/O1 antibodies (Sommer and Schachner, 1981) to identify early and late differentiated oligodendrocytes or anti-MBP antibody to identify matured oligodendrocytes (Campagnoni and Macklin, 1988).

Fig. 4. Double-immunofluorescent labeling of 2-day-old primary cultured cells from embryonic day 18 (E18) with 14F7 (**a**) and GFAP (**b**) antibodies. Expression of 14F7 is clearly absent from the GFAP-positive cell (asterisk) and putative neurons (arrowheads) which are not stained by either GFAP (b) or 14F7 (a) antibodies. **c:** is the corresponding phase-contrast micrograph. Scale bar = 22 μm.

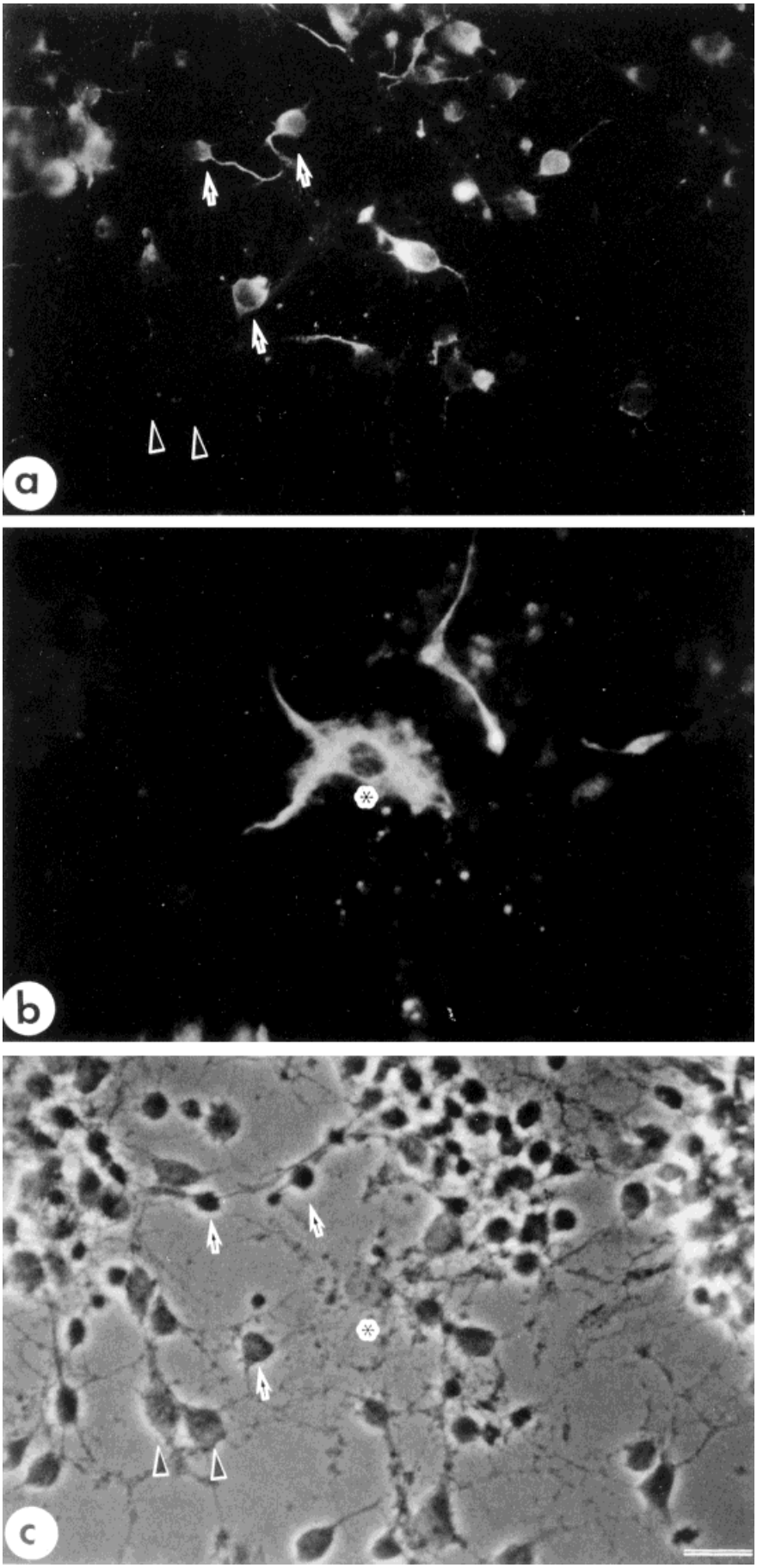

Figure 4.

When primary cultures from embryonic brain were maintained for 2 days in vitro, a significant number of the cultured cells (approximately 10 ± 3.1%) were 14F7 immunoreactive. The 14F7-positive cells had a relatively small cell body and were mainly process-bearing cells. 14F7 antigen should be expressed on the oligodendrocyte surface, since the antibody was treated prior to fixation and permeabilization (Fig. 4a).

In 14F7 and GFAP double-staining experiments, each antibody labeled a discrete population of cells in the same culture (Fig. 4). Some of the 14F7-positive cells were also positive for A2B5, because some cells from a 2-day-old primary culture of embryonic rat brain were double labeled with 14F7 and A2B5 antibodies (Fig. 5). However, the distribution of 14F7 binding cells was distinct from that of the A2B5 labeling pattern. Further, 14F7-labeled cells were present in small cells, bearing few cell processes (Fig. 5b), whereas A2B5-labeled cells tended to be more multi-process-bearing cells (Fig. 5a). The cultures contained more 14F7-positive cells than A2B5-positive cells, and all of the A2B5-positive cells expressed detectable 14F7 immunoreactivity, suggesting that not all progenitor cells had immatured sufficiently to express A2B5, since these cells became increasingly multipolar as they began to differentiate. They then acquired the ability to bind the mAb O4, resulting in good agreement with the expression of 14F7-positive cells (Fig. 6). On the other hand, in the double-labeling experiments (both the 14F7/O1 and 14F7/MBP experiments), 14F7-positive cells did not stain with anti-MBP antibody (Fig. 8A), but the cells intensely labeled by anti-O1-antibody showed faint 14F7-immunoreactivity (Fig. 7). Twenty-one-day-old after 3rd passaged cells in cultivation from rat cerebral hemispheres from embryonic day 18 contained a significant number of MBP-positive oligodendrocytes. However, in no case were these cells specifically labeled by the 14F7 antibody (Fig. 8B).

These observations suggest that oligodendrocyte precursor cells and early stage-specific immature oligodendrocytes, but neither astrocytes, O1-positive late differentiated oligodendrocytes, nor MBP-positive mature oligodendrocytes, express the 14F7 antigen in vitro (Figs. 5–8).

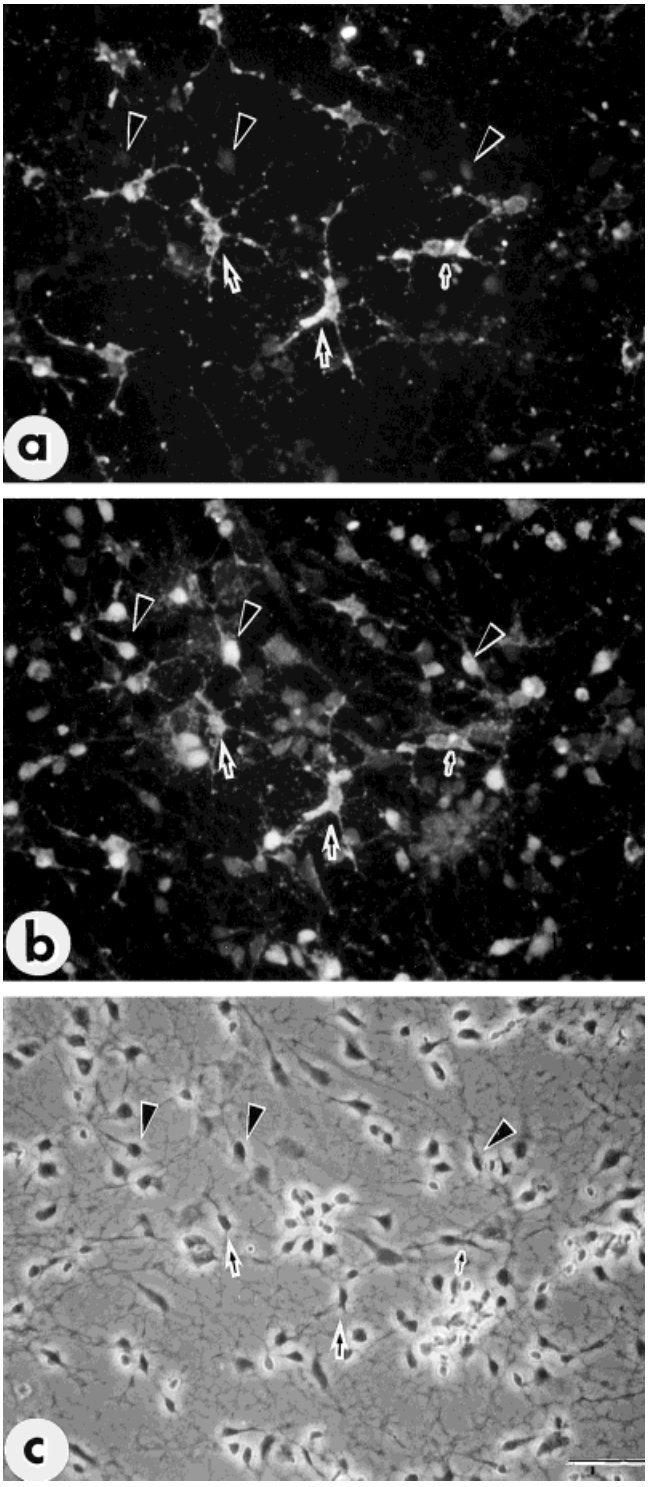

Fig. 5. Double-immunofluorescent labeling of 2-day-old primary cultured cells from embryonic day 18 (E18) with A2B5 (**a**) and 14F7 (**b**) antibodies. Co-localization of 14F7 and A2B5 (arrows) on oligodendroglial progenitors are visible, but some $14F7^{+}/A2B5^{-}$ cells are clearly visible (b, arrowheads). **c** is the corresponding phase-contrast micrograph. Scale bar = 33 μm.

In Vitro Perturbation of Oligodendrocyte Differentiation With Antibodies Against 14F7 Antigen

To test whether 14F7 is involved in oligodendrocyte differentiation when the oligodendrocytes are in contact with astrocytes, we used antibodies to perturb the differentiation of oligodendrocytes in culture. The perturbation of the differentiation of oligodendrocytes by the treatment of mAb 14F7 was determined by comparing the expression of $MBP^{+}/O4^{+}$ cells co-cultured with astrocytes (Fig. 9). Control cultures, which contained non-immune anti-

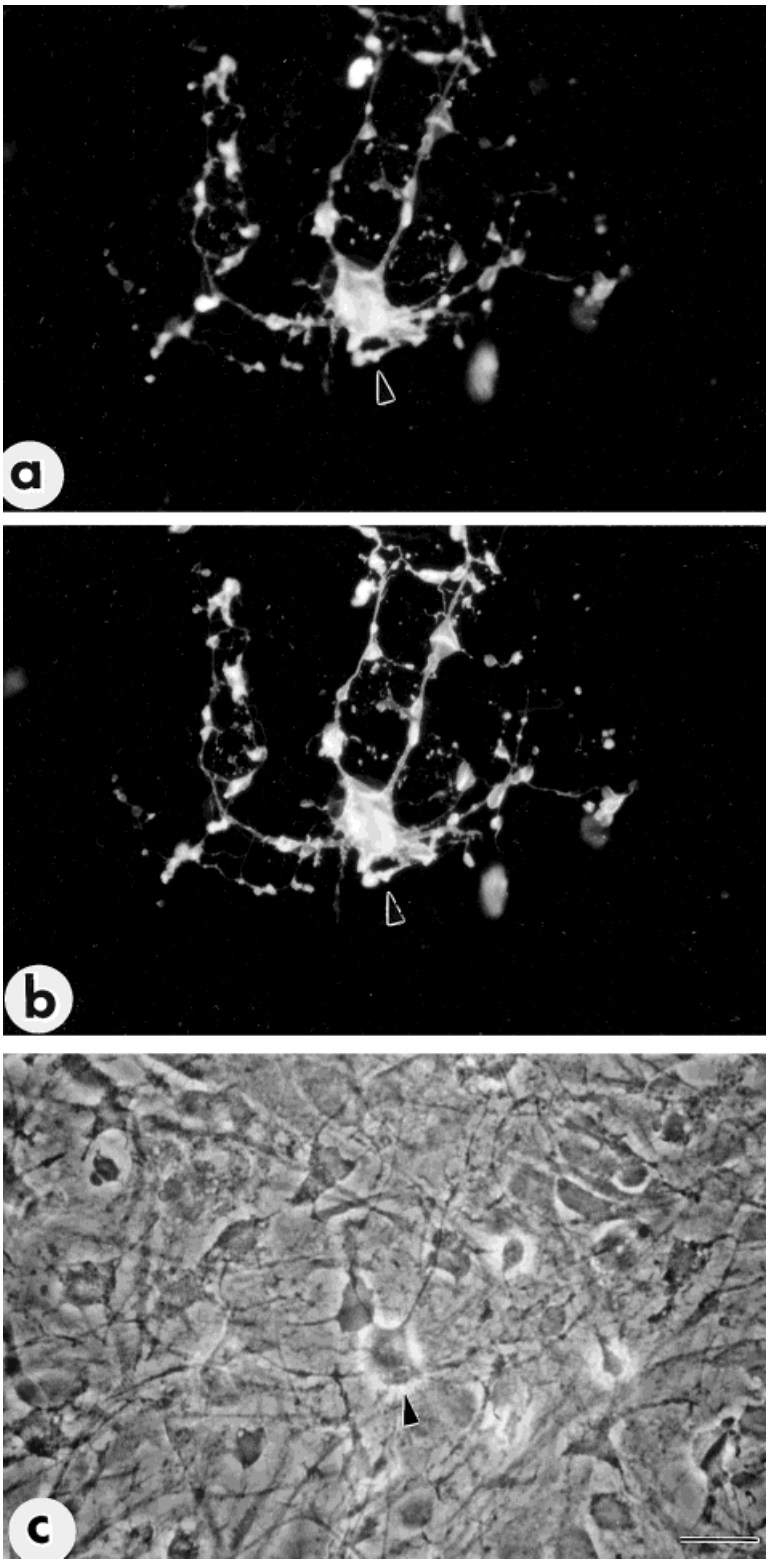

Fig. 6. Double-immunofluorescent labeling with O4 (**a**) and 14F7 (**b**) antibodies of first passaged cells of 21-day-old cell cultures derived from E18. O4-positive cells are coincident with 14F7-positive cells (arrowhead). **c** is the corresponding phase-contrast micrograph. Scale bar = 22 μm.

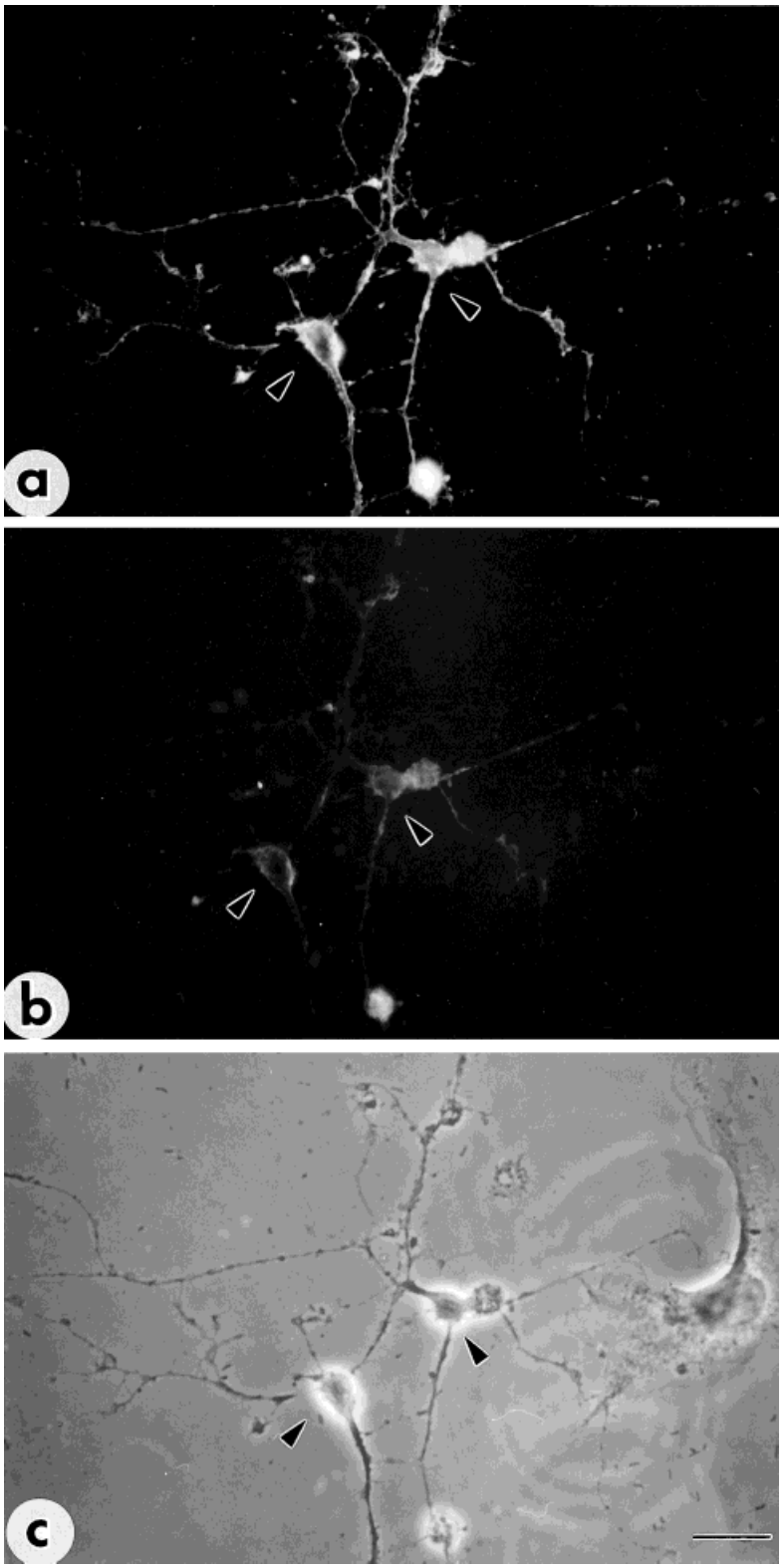

Fig. 7. Double-immunofluorescent labeling with O1 (**a**) and 14F7 (**b**) antibodies of second-passaged cells of 21-day-old cell cultures derived from E18. The cells indicated by arrowheads show intense O1-immunoreactivity but faint 14F7-immunoreactivity. **c** is the corresponding phase-contrast micrograph. Scale bar = 22 μm.

body (mouse IgM fractions), did not change $O4^+/MBP^+$ oligodendrocytes (Fig. 9B). MBP expression is dependent on the contact with astrocytes as shown in Figure 9C. However, in the presence of anti-14F7 antibodies (mouse ascites IgM fraction 20 μg/ml), all of the stage-specific oligodendrocytes co-cultured with astrocytes were labeled with only O4 antibodies, but not MBP antibodies (Fig. 9A).

Biochemical Characterization of the 14F7 Antigen

Cellular specificity of the 14F7 antigen. To determine whether 14F7 is specific for stage-specific immature oligodendrocytes, the cell lysates from different types of cell cultures of neurons, astrocytes, and oligodendrocyte lineage cells were used for a Western blot analysis (Fig. 10A). The Western blot analysis of NP-40–solubilized oligodendrocytes were electrophoresed on a (SDS)-polacrylamide gel. A Western blot using anti-14F7 antibody revealed an immunoreactive 48-kDa band that was absent from non-immunoreactive neurons and the astrocyte membrane.

Developmental expression of the 14F7 antigen. To examine the expression of 14F7 antigen during development, rat brain samples of different ages were assayed by Western blot analysis (Fig. 10B). 14F7 immunoreactivity was detected as early as embryonic day 18 (E18) and faded throughout early development, but once again it was detected on postnatal day 14 (P14). There was no major modification in the apparent molecular weight of the antigen during development. At the late stage of development to adulthood, the band appeared stronger than in the embryonic days. The relative abundance of the 14F7 antigen appeared to significantly decrease between neonatal and postnatal day 7 (P7) and again appeared strongly at P14. The increase in relative 14F7 expression may be correlated with the stage before the onset of active myelination (Schwab and Schnell, 1989; Morell et al., 1990), suggesting that 14F7 is related to the immature oligodendrocyte-associated molecules, and also suggesting the existence of adult oligodendrocyte progenitors (Reynolds and Hardy, 1997).

Tissue distribution of the 14F7 antigen. To determine where the 14F7 antigen was expressed, a variety of different tissues were assayed by Western blot analysis. 14F7 immunoreactivity was detected in some regions of the nervous tissues examined, including the sciatic nerve and spinal cord (Fig. 10C). In this case, 14F7 antibody identified a single band with an apparent molecular weight 48 kDa, suggesting the 14F7 antigen is similar throughout the nervous tissues. In the same analysis, 14F7 immunoreactivity was not detectable in extracts from kidney, lung, thymus, spleen, or muscle (Fig. 10C).

Taken together, these data indicate that in the adult rat, expression of 14F7 antigen is restricted to tissues of the nervous system.

DISCUSSION

The main aims of the present study were to generate markers of the stage-specific oligodendrocyte-lineage cells that could be used as an additional marker for $A2B5^+/O4^-$ progenitor cells and $O4^+/O1^-$ immature oligodendrocyte (Singh and Pfeiffer, 1985; Bansal et al., 1989) and to biochemically characterize these antigens during brain development.

In tissue sections of developing rat brains, the 14F7 antigen is expressed in the ventricular zone facing the medial side of the lateral ventricles. A similar pattern of O4-immunopositive cells was observed in the lateral ventricle (Figs. 1, 2). An in vitro analysis of the immunostaining pattern indicated that 14F7 binds to the surface of oligodendrocyte progenitors and early stage-specific immature oligodendrocytes but not to MBP-positive oligodendrocytes and astrocytes or to neurons (Fig. 4–8). Interestingly, in the cerebrum of a 14-day-old rat, the 14F7 antibody labeling pattern in the corpus callosum did not correlate with the labeling pattern of myelinated axons by the Rip antibody which identifies mature oligodendrocytes and their processes (Ranscht et al., 1982; Friedman et al., 1989) as well as antibodies to other mature oligodendrocyte-associated molecules such as MBP, MAG, Tpo1 MOG, and 2B10 antigen (Linington et al., 1984; Lemke, 1988; Zhou et al., 1995; Krueger, 1997). Taken together, these results indicate that the 14F7 antigen may be localized in immature oligodendrocytes. Similarly, in cultures, the 14F7 labeling pattern reflects the pattern of pre-myelinating cells, since MBP^+ mature oligodendrocytes did not stain with 14F7 antibodies (Fig. 8B). Several additional pieces of evidence support the belief that the 14F7 antigen is not associated with myelinating cells. For example, the 14F7 antigen is detectable in embryonic rat brain prior to myelin formation (Fig. 10B). In addition, during postnatal development, the relative amount of 14F7 antigen in cerebral hemisphere extracts appears to decrease rather than increase during the period of the peak of myelin sheath formation (data not shown). SDS-PAGE results also suggest that the 14F7 antigen is an approximately 48-kDa protein, which appears in the early stage of development and has a very restricted appearance at postnatal day 14 (P14). 14F7 immunoreactivity is detectable in peripheral nerve tissues or the spinal cord, but is not an integral component of the myelin sheath and to our knowledge is the first identified protein for stage-specific immature oligodendrocytes. This suggests that the 14F7 antigen may be a pre-myelinating oligodendrocyte surface mol-

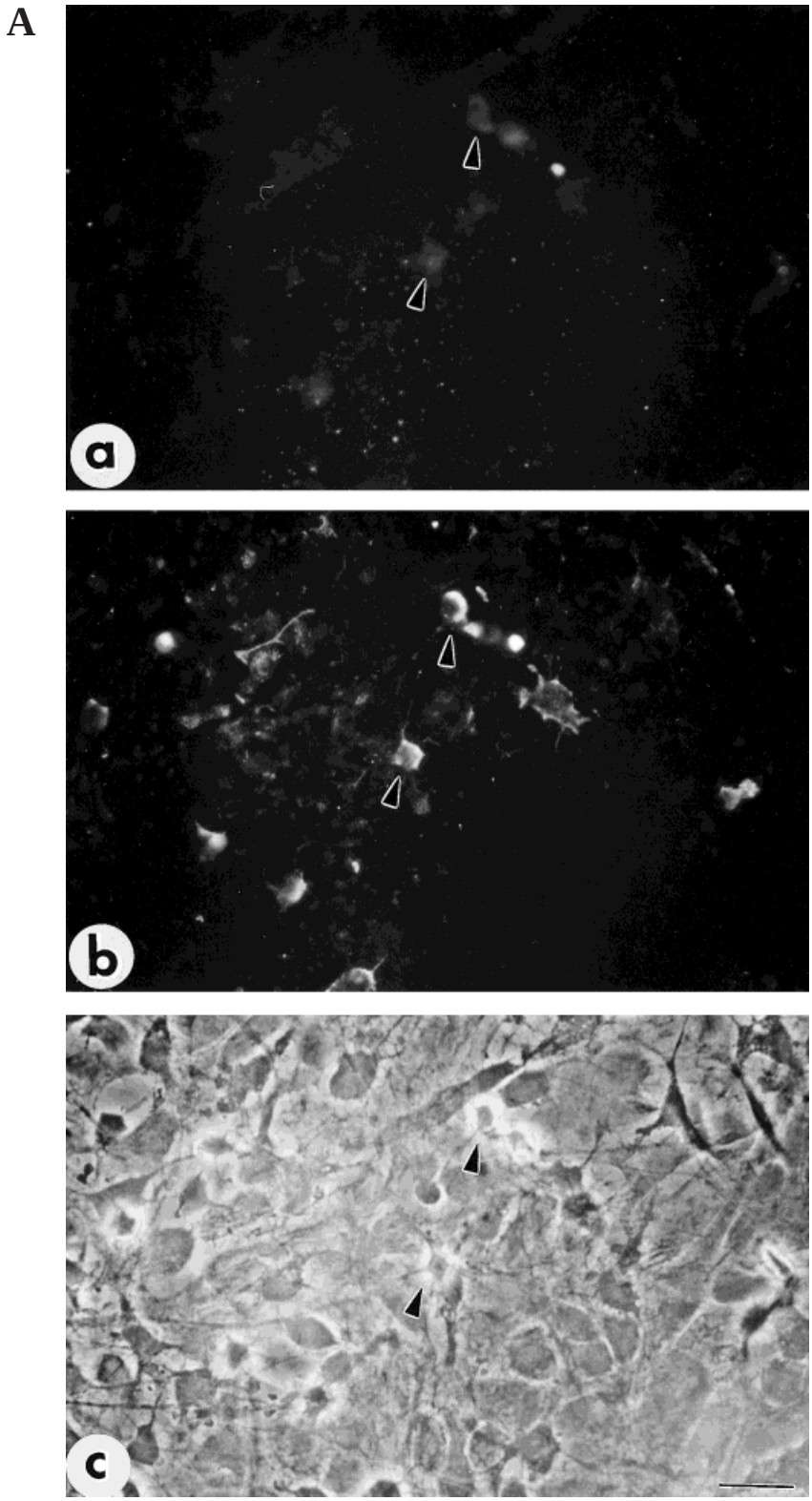

Fig. 8. **A:** Double-immunofluorescent labeling with MBP (**a**) and 14F7 (**b**) antibodies of first passaged cells of 21-day-old cell cultures derived from E18. 14F7-positive cells could not be stained with anti-MBP antibody (arrowheads). **c** is the corresponding phase-contrast micrograph. Scale bar = 22 μm. **B:** Double-immunofluorescent labeling with MBP (**a**) and 14F7 (**b**) antibodies of third passaged cells of 21-day-old cell cultures derived from E18. MBP-positive oligodendrocytes (a) did not stain with 14F7 antibodies (b). **c** is the corresponding phase-contrast micrograph. Scale bar = 22 μm.

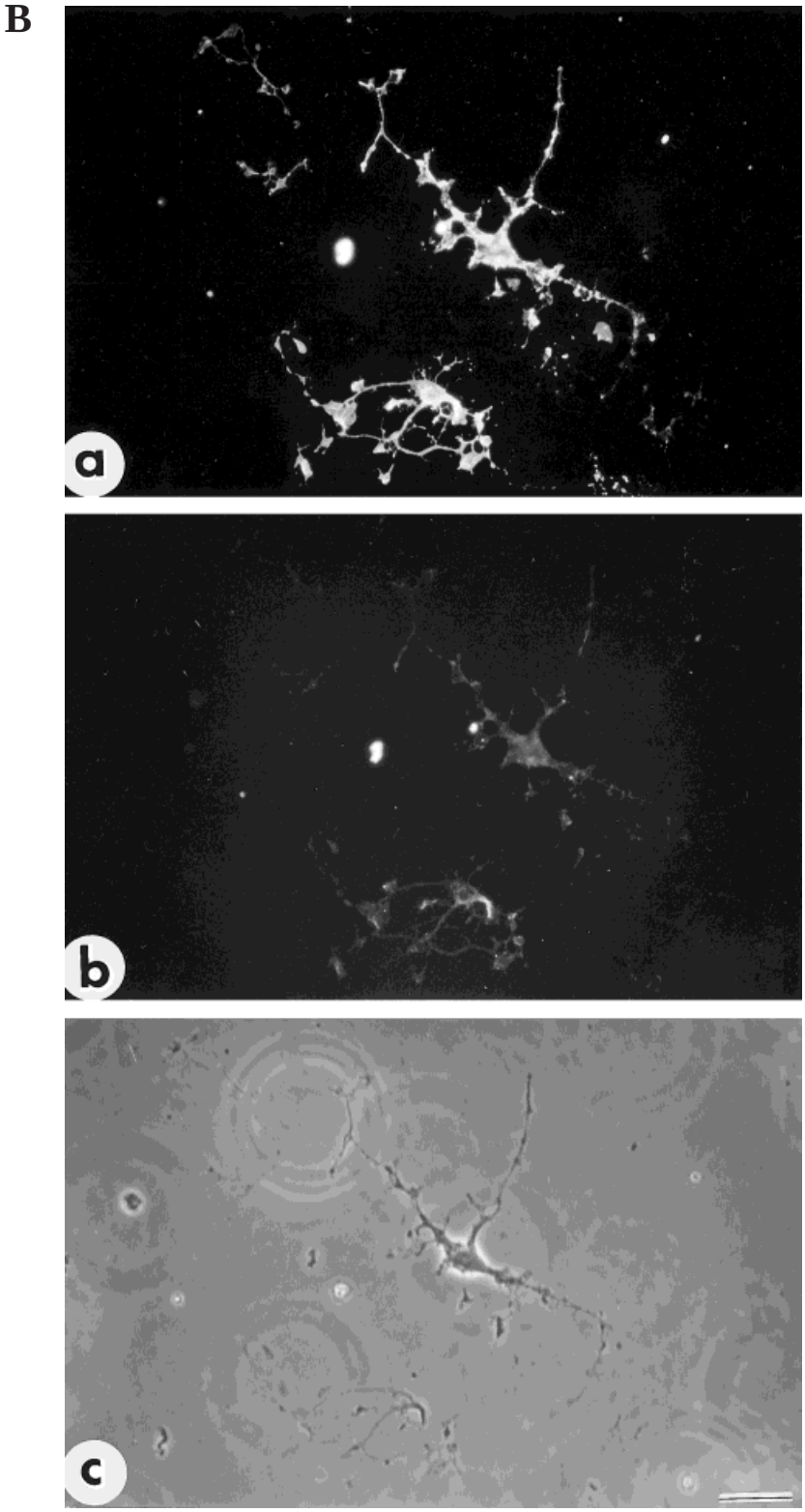

Figure 8. (Continued.)

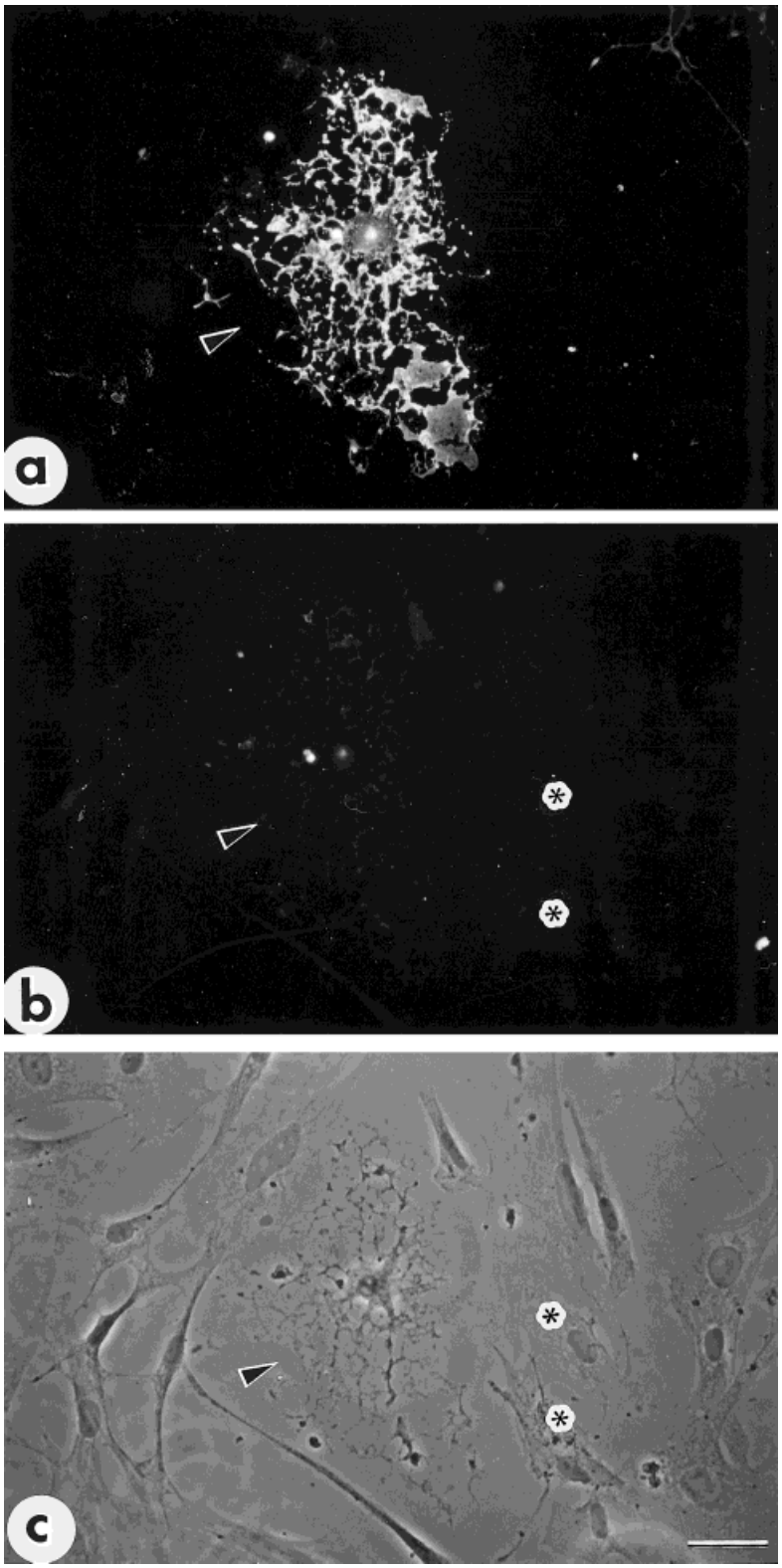

Fig. 9. Double-immunofluorescence staining by anti-O4 and MBP antibodies. **A:** Stage-specific immature oligodendrocyte + co-culture with type-1 astrocytes (asterisk) in the presence of anti-14F7 antibody (20 µg/ml as IgM fractions). **B:** Control of A (absence of anti-14F7 antibody). These are the same microscopic fields of 4-day-old cultures labeled with anti-O4 (**a,** fluorescein optics, arrowhead) and anti-MBP (**b,** rhodamine optics) antibodies and phase contrast (**c**). MBP immunoreactivity is accelerated in the absence of anti-14F7 antibody. **C:** (See page 94.) Stage-specific immature oligodendrocyte alone. These are the same microscopic fields of 4-day-old cultures labeled with anti-O4 (**a,** fluorescein optics, arrowhead) and anti-MBP (**b,** rhodamine optics) antibodies and phase contrast (**c**). Scale bar = 22 µm.

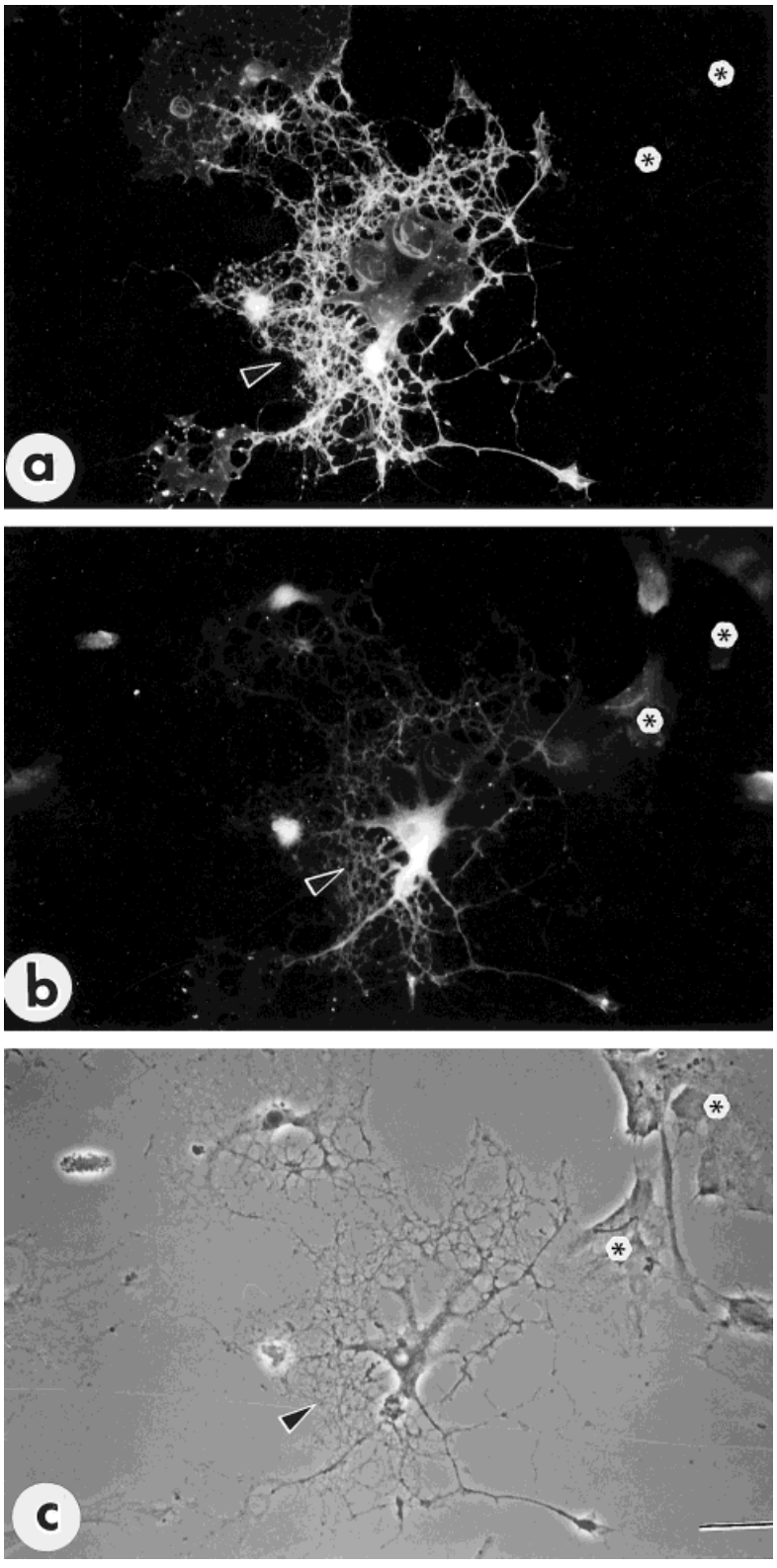

Figure 9. (Continued.)

C

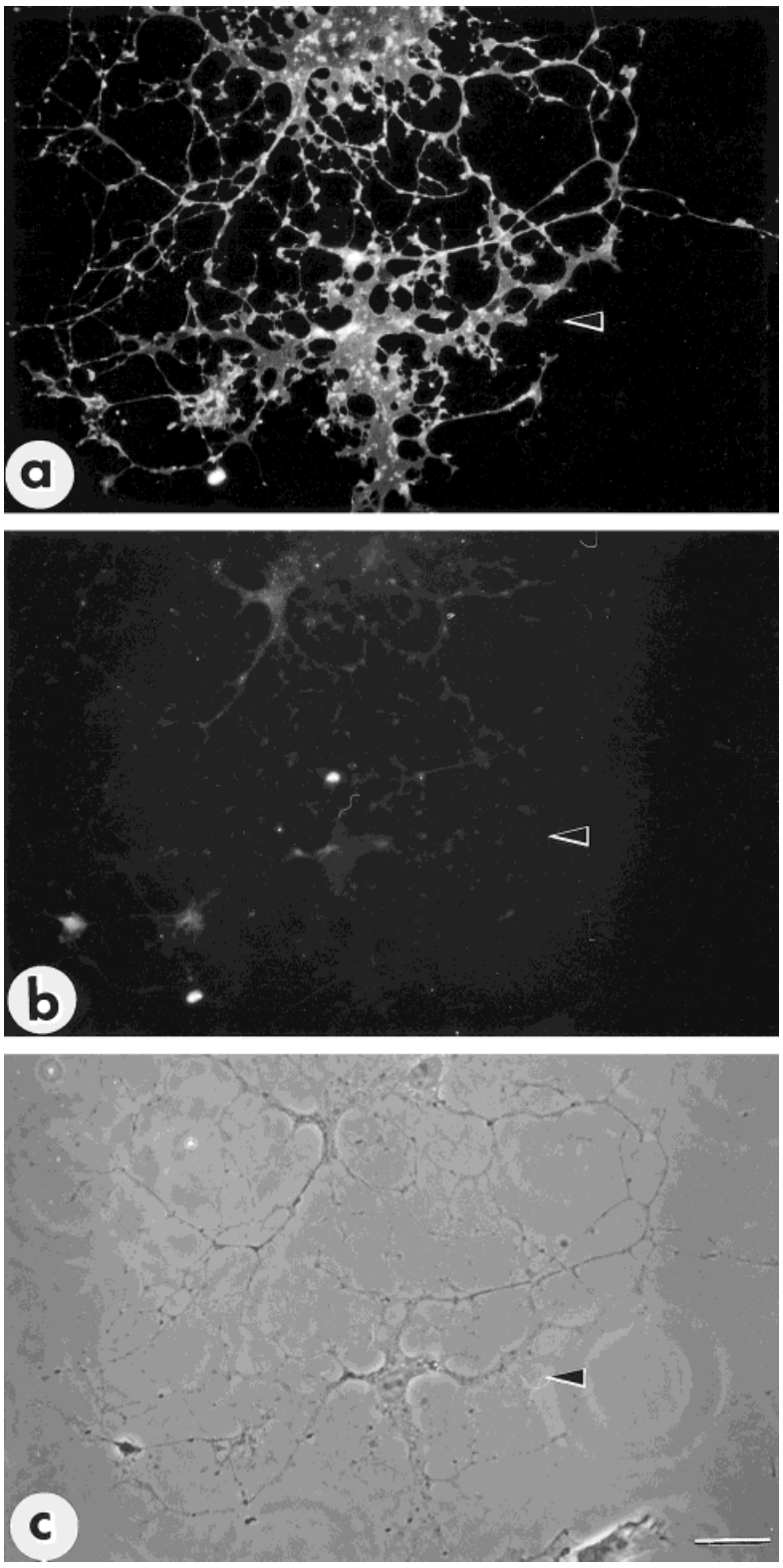

Figure 9. (Continued. Legend appears on page 92.)

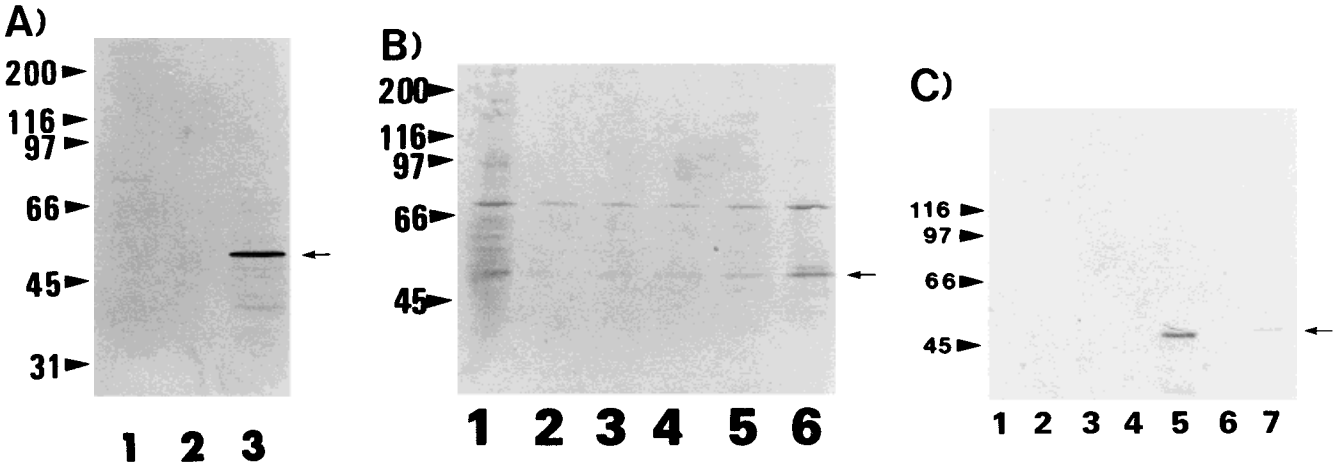

Fig. 10. **A:** Western blot showing the presence of 14F7 antigen in stage-specific immature oligodendrocytes. Note that 14F7 antigen is detected in the oligodendrocyte fraction (**lane 3**) but not in neurons (**lane 1**), or astrocytes (**lane 2**). Proteins were separated by 7% SDS-PAGE, and each lane was loaded with 200 μg total protein. **B:** Developmental expression of 14F7 antigen in rat brain examined by Western blot analysis. Note that the antigen was detectable on embryonic day 18 (E18) and decreased gradually after birth, but the antigen reappeared after postnatal day 14 (P14). 14F7-immunoreactivity is visible as an approximately 48-kDa band (arrow). Non-specific bands also appeared in the reaction with mouse IgM second antibody. Proteins were separated by 7% SDS-PAGE, and each lane was loaded with 200 μg total protein. **Lane 1:** P14; **lane 2:** P7; **lane 3:** P2; **lane 4:** P1; **lane 5:** P0; **lane 6:** E18. **C:** Western blot analysis of 14F7 antigen distribution in tissue extracts. Note that the 14F7 antigen is detectable in extracts of spinal cord (**lane 5**) and sciatic nerve (**lane 7**), but is not detectable in extracts from thymus (**lane 1**), lung (**lane 2**), spleen (**lane 3**), kidney (**lane 4**) or muscle (**lane 6**). Equal amounts of protein (200 μg) were loaded in each lane and were separated by a 10% SDS-PAGE.

ecule, since developmentally, the amount of the 14F7 antigen in whole brain decreases prior to active myelination, which peaks at around 2 weeks later (Morell et al., 1990) (Fig. 10B).

It may be that there is a developmentally regulated decrease in the expression of the 14F7 antigen. Such reduction may indicate that the antigen regulates proliferation or differentiation, such as the differentiation that occurs after oligodendrocyte maturation. The developmentally regulated expression of the 14F7 antigen is an indication that this molecule is functionally important in myelinogenesis. It is important to note that the proliferative phase of oligodendrocyte development is a critical determinant of myelin sheath formation in the CNS (Wood and Bunge, 1984). Many oligodendrocyte-specific molecules are known to be important in maintaining oligodendrocyte functions including myelin sheath formation. For instance, MAG appears to play a role in the initiation of myelination and the maintenance of myelination of the myelin sheath (Trapp, 1990b; Umemori et al., 1994). It seems likely that the 14F7 antigen, although not expressed in myelin-forming cells, is very important in oligodendrocyte differentiation.

Our previous studies demonstrated that a stage-specific oligodendrocyte is controlled by interaction with astrocytes in vitro resulting in the expression of MBP (isoform) (Sakurai et al., 1998). When the stage-specific immature oligodendrocytes were co-cultured with astrocytes in the presence of antibody against 14F7 antigen, the expression of MBP in oligodendrocytes, as shown by immunocytochemistry, disappeared in oligodendrocytes (Fig. 9A). Thus, although it has not yet been sequenced, the 14F7 antigen may be a novel molecule associated with oligodendrocyte differentiation and may have a crucial function in oligodendrocyte maturation. The molecular properties of the 14F7 antigen and its functional roles in pre-myelinating oligodendrocytes are currently being examined.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan to H.A. We greatly thank Dr. J. Raymond for editing the manuscript.

REFERENCES

Asou H, Hamada K, Sakota T, Miyazaki T, Takeda Y, Uyemura K, Itoh K (1995): CNS myelinogenesis in vitro: time course and pattern of rat oligodendrocyte development. J Neurosci Res 40:519–534.

Asou H, Ono I, Umemura I, Sugawa M, Uyemura K (1996): Axonal growth-related cell surface molecule, Neurin-1, involved in neuron-glia interaction. J Neurosci Res 45:571-587.

Bansal R, Warrington AE, Gard AL, Ranscht B, Pfeiffer SE (1989): Multiple and novel specificities of monoclonal antibodies O1, O4, and R-mAb used in the analysis of oligodendrocyte development. J Neurosci Res 24:548–557.

Bayer SA, Altman J (1991): ''Neocortical Development.'' New York: Raven Press.

Bignami A, Eng LF, Dahl D, Uyeda CT (1972): Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. Brain Res 43:429–435.

Campagnoni AT, Macklin WB (1988): Cellular and molecular aspects of myelin protein gene expression. Mol Neurobiol 2:41–89.

Colman DR (1991): Functional properties of adhesion molecules in myelin formation. Curr Opin Neurobiol 1:377–381.

Eisenbarth GS, Walsh FS, Nirenberg M (1979): Monoclonal antibody to a plasma membrane antigen of neurons. Proc Natl Acad Sci USA 76:4913–4917.

Friedman B, Hockfield S, Black JA, Woodruff KA, Waxman SG (1989): In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody. Rip. Glia 2:380–390.

Grinspan J, Stern J, Pustilnik S, Pleasure D (1990): Cerebral white matter contains PDGF-responsive precursors to 02A cells. J Neurosci 10:1866–1873.

Grinspan J, Kamholz J, Wrabetz L (1993): Oligodendrocyte maturation and myelin gene expression in PDGF-treated cultures from rat cerebral white matter. J Neurocytol 22:322–333.

Hardy R, Reynolds R (1991): Proliferation and differentiation potential of rat forebrain oligodendroglial progenitors both in vitro and in vivo. Development 111:1061–1080.

Hildebrand C, Remahl S, Persson H, Bjartmar C (1993): Myelinated nerve fibers in the CNS. Prog Neurobiol 40:319–384.

Krueger WHH, Gonye GE, Madison DL, Murray KE, Kumar M, Spoerel N, Pfeiffer SE (1997): Tpo1, a member of a novel protein family, is developmentally regulated in cultured oligodendrocytes. J Neurochem 69:1343–1355.

Lemke G (1988): Unwrapping the genes of myelin. Neuron 1:535–543.

Levine SM, Goldman JE (1988): Spatial and temporal patterns of oligodendrocyte differentiation in rat cerebrum and cerebellum. J Comp Neurol 277:441–455.

Linington C, Webb M, Woodhams PL (1984): A novel myelin-associated glycoprotein defined by a mouse monoclonal antibody. J Neuroimmunol 6:387–396.

Morrell P, Roberson M, Meissner G, Toews AD (1990): Myelin: From electrical insulator to ion channels. In Hashim GA, Moscaarello M (eds): ''Dynamic Interactions of Myelin Proteins.'' New York: Wiley-Liss, pp 1–24.

Noble M, Murray K, Stoobant P, Waterfield MD, Riddle P (1988): Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type 2-astrocyte progenitor cell. Nature 333:560–562.

Raff MC, Miller RH, Noble M (1983a): A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on the culture medium. Nature 303:389–396.

Raff MC, Abney ER, Cohen J, Lindsay R, Noble M (1983b): Two types of astrocytes in culture of developing rat white matter. Differences in morphology, surface gangliosides, and growth characteristics. J Neurosci 3:1289–1300.

Ranscht B, Clapshaw PA, Price J, Noble M, Seifert W (1982): Development of oligodendrocytes and schwann cells studied with a monoclonal antibody against galactocerebroside. Proc Natl Acad Sci USA 79:2709–2713.

Reynolds R, Hardy R (1997): Oligodendroglial progenitors labeled with the O4 antibody persist in the adult rat cerebral cortex in vivo. J Neurosci Res 47:455–470.

Sakurai Y, Nishimura D, Yoshimura K, Tsuruo Y, Seiwa C, Asou H (1998): Differentiation of oligodendrocyte occurs in contact with astrocyte. J Neurosci Res 52:17–26.

Schwab ME, Schnell L (1989): Region-specific appearance of myelin constituents in the developing rat spinal cord. J Neurocytol 18:161–169.

Singh H, Pfeiffer SE (1985): Myelin-associated galactolipids in primary cultures from dissociated fetal rat brain: biosynthesis, accumulation, and cell surface expression. J Neurochem 45: 1371–1381.

Sommer I, Shachner M (1981): Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. Dev Biol 83:311–327.

Temple S, Raff MC (1986): Clonal analysis of oligodendrocyte development in culture: evidence for a development clock that counts cell divisions. Cell 44:773–779.

Trapp BD (1990a): Distribution of myelin protein gene products in actively myelinating oligodendrocytes. In Quarles RH, Trapp BD (eds): ''Cellular and Molecular Biology of Myelination.'' Berlin: Springer-Verlag, pp 59–79.

Trapp BD (1990b): Myelin-associated glycoprotein: location and potential functions. Ann NY Acad Sci 605:29–43.

Umemori H, Sato S, Yagi T, Aizawa S, Yamamoto T (1994): Initial events of myelination involve fyn tyrosine kinase signaling. Nature 367:572–576.

Wood P, Bunge RP (1984): The biology of oligodendrocyte. In Norton WT (ed): ''Oligodendroglia.'' London: Plenum Press, pp 1–46.

Yoshimura K, Negishi T, Kaneko A, Sakamoto Y, Kitamura K, Hosokawa T, Hamaguchi K, Nomura M (1996): Monoclonal antibodies specific to the integral membrane protein P0 of bovine peripheral nerve myelin. Neurosci Res 25:41–49.

Zhou L, Szigeti V, Miller H (1995): Characterization of a novel oligodendrocyte cell surface molecule. J Neurosci Res 42:504–515.