Antigenic and Functional Characterization of a Rat Central Nervous System-derived Cell Line Immortalized by a Retroviral Vector

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Abstract. We have immortalized rat central nervous system (CNS) cells of primary cultures of rat optic nerve with murine leukemia virus ψ-2,SV-40-6, which is defective in assembly and contains the SV-40 large T antigen and neomycin resistance genes, to produce a cell line that we named A7. After drug selection, >90% of the growing cells expressed nuclear SV-40 large T cells and a fraction of these contained the astrocyte-specific marker, glial fibrillary acidic protein. The majority of these cells also expressed surface marker A4 (specific for neural tube derivatives), Ran 2, p185 (the 185-kD phosphoprotein product of the *neu* oncogene), and fibronectin, but did not express the astrocyte enzymes glutamine synthetase and monoamine oxidase B. Surface markers characteristic of glial progenitors (A2B5) and oligodendrocytes (galactocerebroside) were not detected. After two rounds of cell cloning, subclone A7.6-3 expressed Ran 2, fibronectin, and the neural cell adhesion molecule (N-CAM) but not glial fibrillary acidic protein and A4. The A7 cell line and subclones also displayed certain functions of type 1 astrocytes: the conditioned medium of these cells had a potent mitogenic activity for glial progenitor cells which could be neutralized by anti-platelet-derived growth factor antibodies and monolayers of these cells supported the growth of embryonic hypothalamic neurons. We conclude that a retrovirus containing SV-40 large T antigen can immortalize rat CNS cells and that such immortalized glial cells retain at least two important functions of type 1 astrocytes: the ability to secrete platelet-derived growth factor and to support the growth of embryonic CNS neurons. Moreover, such stable immortalized clonal cell lines can be used to study gene regulation in glial cells.

THE usefulness of cell lines for the study of neurobiological problems has become increasingly apparent. Many cell lines, such as the C6 glioma (Benda et al., 1968), the C1300 neuroblastoma (Augusti-Tocco and Sato, 1969), and the PC12 pheochromocytoma (Tischler and Greene, 1978) have been derived by placing spontaneous or chemically induced tumors into cell culture. By continuous passage, subculturing, and selection, cell lines with specific phenotypes have been isolated and characterized, and they have been used to study these specialized properties. On the other hand, many of the subclones of these tumor-derived cell lines are not stable, and sometimes the phenotypic properties of the line are not expressed by all of the cells within any clone.

It would obviously be desirable to have glial cell lines which display cell-type specific properties, but whose origin and growth are more closely controlled. One way in which to accomplish this is to express the immortalizing protein product of an oncogene in otherwise normal cells. Under these circumstances, one might expect that at least some of the phenotypic properties of the parent cell would be retained and could be studied more intensively at both the molecular and functional level than would be possible with primary cells. Several glial and neuronal cell lines have already been produced by such methods, with varying degrees of preservation of phenotypic properties. In human astroglial cells that were transformed by SV-40 large T antigen (Major et al., 1985), glial fibrillary acidic protein (GFAP),[1] the intermediate filament protein of astrocytes, continued to be expressed in the transformed cells. A similar technique has been used to insert the SV-40 large T antigen into rat astrocytes where two clones expressing immunological and pharmacological properties of astrocytes were isolated (Neto et al., 1986).

1. *Abbreviations used in this paper*: CM, conditioned medium; CNS, central nervous system; GC, galactocerebroside; GFAP, glial fibrillary acidic protein; N-CAM, neural cell adhesion molecule; PDGF, platelet-derived growth factor.

Very recently, murine brain cells were transfected simultaneously with the polyoma virus large T and the Neo R genes (Evrard et al., 1986). This procedure resulted in immortalized glial cells that did not fully assume a transformed phenotype; although GFAP was not expressed in most of these cell lines, it was sometimes reinduced by procedures that promote differentiation (Evrard et al., 1986). Transformation of nerve cells has been obtained with temperature sensitive mutants of Rous sarcoma virus and SV-40 (Pessac et al., 1983; Mallat et al., 1986).

In the experiments described here, we used a retroviral vector containing the SV-40 large T antigen and the neomycin resistance gene (Jat et al., 1986) to immortalize a cultured rat glial cell, with the goal of producing a cell line that maintains glial-specific properties. We were especially interested in immortalizing the bipotential O-2A progenitor (Raff et al., 1983) or the type 1 astrocyte. The O-2A progenitor can differentiate into either an oligodendrocyte or a type 2 astrocyte, depending on culture conditions, whereas the type 1 astrocyte stimulates the mitosis of the O-2A progenitors (Noble and Murray, 1984). Characterization of the initial immortalized cell line after drug selection indicated an astrocytic, rather than O-2A lineage, origin. After subcloning, cells continued to display a phenotype resembling, but not identical with, type 1 astrocytes. However, they maintained two important functions of type 1 astrocytes: the ability to synthesize platelet-derived growth factor (PDGF) (Richardson et al., 1988) and to support the growth of central nervous system (CNS) neurons (Fallon, 1985; Ventimiglia and Geller, 1987).

Materials and Methods

Immortalization of Glial Cells

Purified type 1 astrocytes were prepared as described (Noble and Murray, 1984), seeded in 60-mm Petri dishes, grown to confluence in DME supplemented with 10% FCS and antibiotics, and then irradiated with 3,000 rads to eliminate mitosis. The medium was changed to a modified N2 medium (Bottenstein and Sato, 1979) supplemented with 0.5% FCS (N2/FCS) (Raff et al., 1983) 3 d before seeding optic nerve cells from newborn rats obtained by enzymatic dissociation (Raff et al., 1983). The latter were allowed to grow on the type 1 astrocyte monolayer for 1 d before viral infection.

A ψ-2 cell line that stably produces a defective recombinant retrovirus SV-40-6 containing the neomycin resistance and SV-40 large T genes (Jat et al., 1986) was kindly provided by C. Cepko, Harvard Medical School, Boston, MA (Cepko et al., 1984). 24-h conditioned N2/FCS medium from the ψ-2 cells was collected, filtered through a 0.45 μM filter (Millipore Corp., Bedford, MA), and added to the glial culture at 37°C in the presence of 4 μg/ml of Polybrene. After 4 h, the ψ-2 medium was removed and replaced by N2/FCS. 20 d later, a very large number of process-bearing oligodendrocyte-like cells could be identified on the type 1 astrocyte monolayer. The N2/FCS was then replaced with DME containing 10% FCS (DME/FCS) and 1 mg/ml G418 in order to select cells that had integrated the retrovirus construct DNA and were, therefore, resistant to this drug. The drug was left on for 10 d, then removed, and the remaining growing cells were expanded in plastic flasks in DME/FCS. Cells grew rapidly and were mostly flat (Fig. 1). They did not pile up and their doubling time was 2–3 d. After this astroglial cell line (A7) was characterized (see Results) and passed six times, we subcloned the cells by limiting dilution in 96-well plates. New clones, developed after 10 d to 2 wk, were expanded.

The second cloning was done in a similar manner, starting from one selected clone (A7-6) which secreted the highest amount of mitogenic activity for O-2A progenitors (see below). One of the subclones (A7-6.3) has now been passed 14 times and displayed stable growth and mitogenic activity in its supernatant through these passages even after the cells had been frozen and thawed. After the fifteenth passage, however, cells tend to become confluent more rapidly. Therefore, large stocks of a fifth passage of A7-6.3 were frozen in DMSO, stored in liquid nitrogen, and thawed when needed.

Immunofluorescence

Various antibodies were used to identify the nature of the A7 cells and characterize them in double-labeling experiments. Cells were grown on 13-mm glass coverslips and used in log-phase growth. Staining for surface antigens was conducted on live cultures, or cultures fixed for 5 min in 4% paraformaldehyde in PBS. Staining for intracellular antigens was conducted on cultures fixed in 5% acetic acid in ethanol for 5 min at −20°C and then rinsed with serum containing medium. mAbs used included L19 directed against SV-40 large T antigen (kindly provided by Dr. D. Latchman, University College, London, UK); A4 (Cohen and Selvendran, 1981; Miller et al., 1984); Ran 2 (Bartlett et al., 1981); A2B5 (Eisenbarth et al., 1979); anti-galactocerebroside (GC; Ranscht et al., 1982); RT97 anti-neurofilament (Wood and Anderton, 1981); and anti-fibronectin kindly provided by Dr. Rhona Mirsky, University College, London. Rabbit polyclonal antibodies used included anti-GFAP (Bignami et al., 1972); anti-fibronectin (Price and Hynes, 1985); and anti-neural cell adhesion molecule (N-CAM) (Chuong et al., 1982). Each primary antibody was followed by an appropriate fluorescent conjugate purchased from either Cappel Laboratories (Malvern, PA) or Nordic Immunology (Capistrano Beach, CA). In double-label experiments, the surface antibody and its conjugate were initially applied; cultures were then fixed in acid alcohol and the intracellular antigen was decorated. For double-labeling with mAbs A2B5 and anti-GC, the cells were initially reacted with antibody A2B5, followed by rhodamine-conjugated goat anti-mouse IgM; the next incubation was with anti-GC followed by fluorescein-conjugated rabbit anti-mouse IgG3. Cultures were observed with a standard or universal microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence illumination.

For flow cytometric analysis of surface antigen expression, cells were grown in 75-cm^2 tissue culture flasks and harvested in log phase growth. Cells were suspended at a concentration of 3×10^6 cells/ml in balanced salt solution with 10% FCS containing mAb 7.16.4 directed against the 185-kD cell surface glycoprotein expressed by the *neu* oncogene (Drebin et al., 1984) at a concentration of 50 μg/ml for 30 min at 4°C. Cells were washed twice in balanced salt solution and resuspended in goat anti-mouse Ig-FITC (Cappel Laboratories) for a further 30 min at 4°C. Nonspecific binding was measured by omitting the primary antibody. Cells were also incubated with the vital dye propidium iodide to control for viability. After washing, cells were analyzed using a flow cytometer (Epics 5; Coulter Electronics Inc., Hialeah, FL).

Enzyme Assays

Glutamine synthetase and monoamine oxidase activities were kindly measured in the laboratory of Dr. W. Nicklas, Robert Wood Johnson Medical School (Piscataway, NJ). Glutamine synthetase was measured by a modification of the method of Patel et al. (1982). Monoamine oxidase activity was determined radioenzymatically using a modification of the method of White and Stine (1984). Protein was determined by the method of Lowry et al. (1951).

Test System for O-2A Progenitor Cell Mitosis

Optic nerves from 7- or 9-d-old rats were enzymatically dissociated and plated onto polylysine-coated glass coverslips at $2\text{–}5 \times 10^3$ cells/coverslip and grown in defined medium (Raff et al., 1983). A7 conditioned medium (CM) was obtained by growing A7 cells in DME with 2 or 10% FCS for 24–48 h. Test medium consisting of a 1:5 dilution A7 CM was added to the optic nerve cultures; control medium was supplemented with 0.5 to 2% FCS to match the final serum concentration with that of the test medium. Half of the test medium was renewed with fresh CM every day or at least once during the 3 d of culture. In some experiments, we compared the activity of these CMs with that of porcine PDGF, known to be devoid of transforming growth factor β activity (Bethesda Research Laboratories, Gaithersburg, MD; test kindly performed in the laboratory of Dr. A. Roberts, National Institutes of Health [NIH], Bethesda, MD).

After 3 d, cells were double-labeled with anti-GC and A2B5. Alternately, cells were labeled with A2B5 followed by GFAP as described above. Oligodendrocytes are identified as the GC^+ population, whereas O-2A progenitors are identified as the $A2B5^+$, GC^- population of cells (Raff et al., 1983). When [^{3}H]thymidine was used, cultures were labeled from 24 to 48 h with 0.05 μCi/ml of [^{3}H]thymidine (New England Nuclear, Boston,

MA; act 52 Ci/mmol) before being fixed and immunolabeled as above. NTB-2 emulsion was applied to the stained coverslips at 4°C for 3 d and autoradiograms were developed following standard procedures. When autoradiography was combined with double labeling, all O-2A lineage cells in 20–50 microscopic fields were counted in each experimental condition (using a 40× oil objective) and the percent of thymidine-labeled cells in each cell population (oligodendrocytes or progenitors) was calculated.

Neutralization of Mitogenic Activity of A7-6.3 Supernatants

The neutralization potency of purified IgG fractions of antibody to human PDGF (Collaborative Research, Inc., Waltham, MA) against A7-6.3 CM stimulation of progenitor cell mitosis was compared to that against porcine PDGF. Anti-PDGF antibody at a concentration of 40 μg/ml (known to neutralize most PDGF mitogenic activity on NIH 3T3 cells) was added to cultures of 9-d-old rat optic nerve along with serial dilutions of A7-6.3 CM and the mitosis assay was carried out as described. The cells were carefully washed of debris before being refed with antibody-containing medium at 2 d.

Glial Growth Factor Activity

CM from cell line A7-6.3 was tested for glial growth factor activity by Dr. J. Brockes at Kings College, London, England, as previously described (Brockes et al., 1980).

Neuronal Growth Assay

A7 cells were plated onto polylysine-coated coverslips and allowed to grow to near confluence. Embryonic day 17 hypothalamic neurons were then plated onto the monolayers, and the growth and attachment of the neurons was monitored using phase-contrast and modulation-contrast microscopy (Ventimiglia and Geller, 1987). At time periods of 1, 3, and 7 d, cultures were removed and labeled for neurofilament protein using mAb RT97.

Results

Initial Growth

No acute cytopathic effects were observed in the cultures after exposure to the virus. Over the initial several weeks after virus exposure, cultures grew normally; there were many phase-bright, round, process-bearing cells (presumptive O-2A glial progenitors and oligodendrocytes) on top of the astrocyte monolayer. The number of these phase-bright cells continued to increase with time in culture. After 3 wk in culture, an area of enhanced growth was detected by phase-contrast microscopic observation of the culture dish. The culture medium was then replaced with DME/FCS containing G418 (neomycin) at a concentration of 1 mg/ml for 10 d. All of the phase-bright cells were killed by the drug, while a population of drug-resistant flat cells survived and grew. These flat cells were then expanded in tissue culture flasks, and then refed twice a week. The growth of these cells was a function of the serum concentration in the medium: cells grown in DME/FCS grew more rapidly than those in N2/FCS. Cells were therefore grown in DME/FCS and passaged at confluence. These passaged cells (named A7 and characterized here) were then plated onto coverslips for immunocytochemical characterization.

The population of A7 cells, although polymorphic, were mostly flat (Fig. 1) and resembled cultured cortical astrocytes in serum-containing medium (McCarthy and de Vellis, 1980). Neither the A7 cell line nor the subclones showed foci formation or loss of contact inhibition. Cells had a rapid growth rate and doubled in 2–3 d. Although we did not test tumorigenicity of our cell lines, other SV-40 large T antigen–expressing cell lines obtained with the same murine retrovirus shuttle vector are not tumorigenic (Jat et al., 1986).

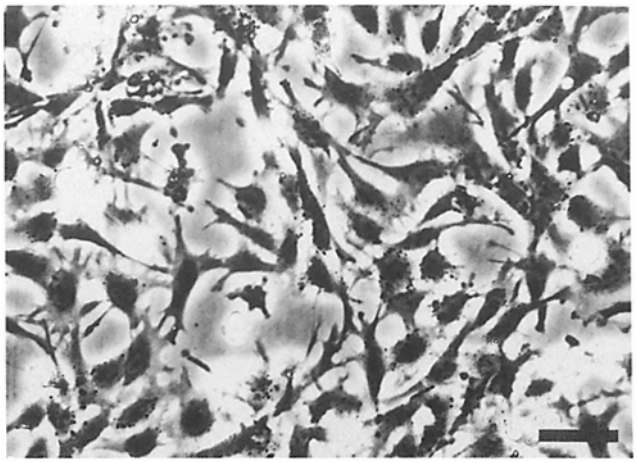

Figure 1. Morphology of A7 cells. Phase-contrast photomicrograph of living A7 cells grown in serum-containing medium in a 35-mm plastic tissue culture dish. Cells grow individually and are relatively flat, and demonstrate clear cytoplasm. Bar, 50 μm.

Antigenic Characterization

First passage A7 cells were stained for SV-40 large T antigen and 95–98% of the cells expressed this transforming gene in their nucleus (Fig. 2 *E*). These cells were also stained with other antibodies to detect specific markers for astrocytes, oligodendrocytes, and O-2A progenitors (Table I). The A7 cell line did not express the antigen recognized by mAb A2B5 nor GC, therefore excluding the possibility that these immortalized cells were O-2A progenitor cells, type 2 astrocytes, or oligodendrocytes. In contrast, the cells stained with antibodies to several other surface markers characteristic of type 1 astrocytes: A4, an mAb which reacts with neural tube derivatives (Cohen and Selvendran, 1981; Miller et al., 1984), and Ran 2 and fibronectin which are normally present on both type 1 astrocytes and meningeal cells (Bartlett et al., 1981; Price and Hynes, 1985). Unfixed cells labeled with anti-N-CAM showed a bright surface fluorescence. Fibronectin had a fibrillar rather than the punctate pattern described in normal type 1 astrocytes (Fig. 2).

A fraction of the A7 cells initially expressed GFAP, but the GFAP was diffuse in the cell cytoplasm in contrast to the filamentous pattern seen in normal type 1 astrocytes. This expression of GFAP was lost after several passages of the cells. To test the possibility that the cells expressed GFAP which did not polymerize correctly, we also performed immunoblots on extracts of A7 cells using the same anti-GFAP antibody. No GFAP expression was detected. In addition, we did not see an increase in GFAP expression when cells were treated with either dibutyryl cAMP or retinoic acid to enhance differentiation (data not shown).

We also examined this cell line for the production of glutamine synthetase and monoamine oxidase B, enzymes normally found in type 1 astrocytes (Patel et al., 1982; Levitt et al., 1982). The level of expression was below the limit of the assay, whereas levels in parallel cultures of type 1 astrocytes were found to be 1.5 μmol/mg protein per hour for

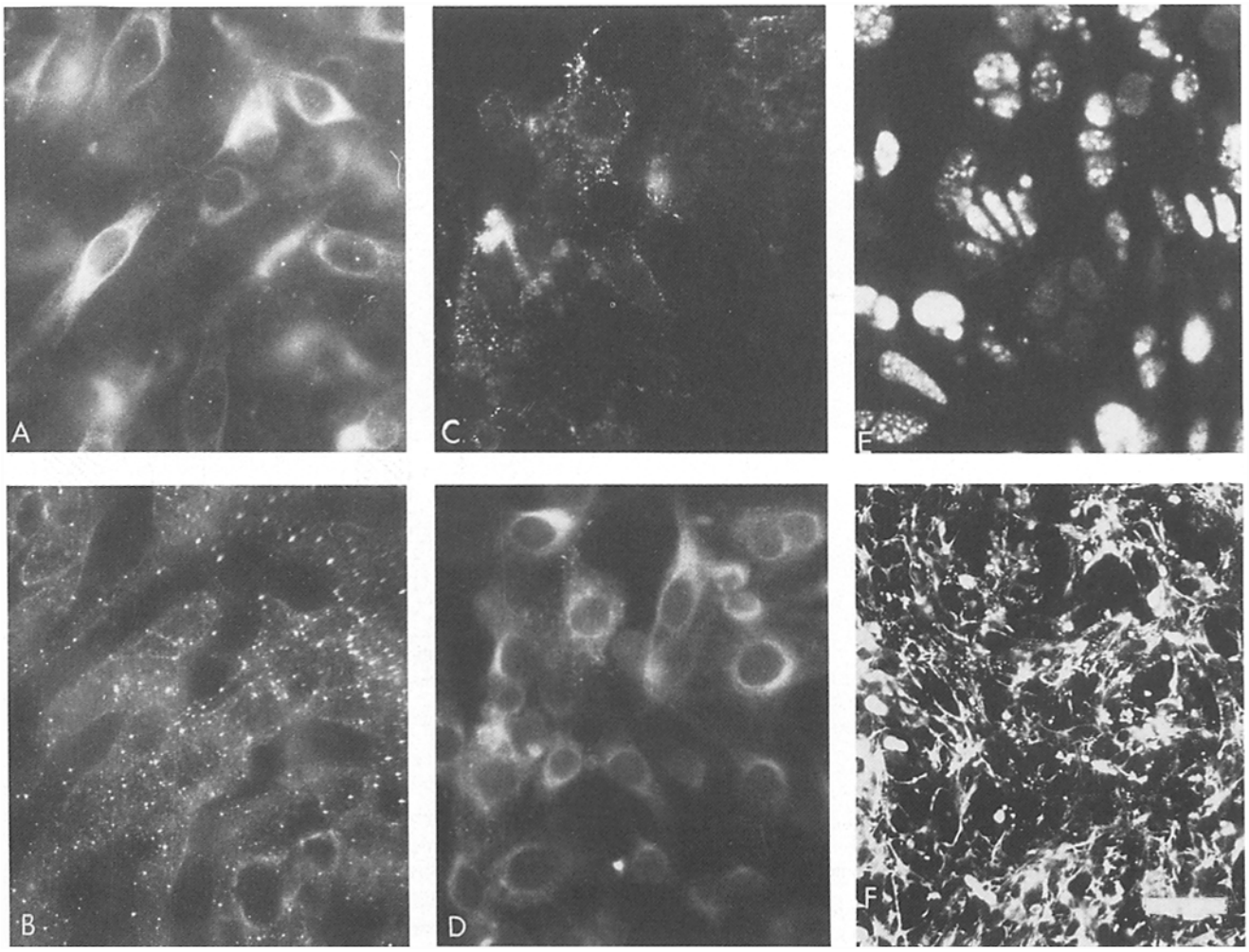

Figure 2. Antigenic characterization of the A7 cells. (*A* and *B*) Double immunocytochemistry illustrating the same field illuminated with rhodamine optics demonstrating A4 immunoreactivity (*B*) and fluorescein optics demonstrating the GFAP staining (*A*). (*C* and *D*) Double immunocytochemistry illustrating the same field stained for the surface marker Ran 2 under rhodamine optics and GFAP staining revealed with fluorescein. Note that some GFAP positive cells do not display Ran 2 reactivity. (*E*) Staining with mAb L19 for SV-40 large T antigen shows reactivity in the nuclei of most cells. (*F*) Staining with mAb to fibronectin illustrates the fibrillar organization of fibronectin on the cell surface. Bar, 50 μm.

glutamine synthetase and 100 pmol/mg protein per hour for monoamine oxidase B (Farinelli, S., and D. Panek, personal communication).

Flow cytometric analysis after immunolabeling with mAb 7.16.4 (Drebin et al., 1984) was used to examine the expression of the *neu* oncogene receptor phosphoprotein, p185 (Schechter et al., 1984), by the A7 cell line. For comparison, we also stained cultures of primary type 1 astrocytes derived form rat cerebral cortex and C6 glioma cells (Fig. 3). The analysis of these histograms demonstrated that 95% of A7 cells and C6 astrocytoma cells showed a specific reaction with antibody 7.16.4, while over 70% of the primary rat cortical astrocytes reacted with this antibody.

Because the A7 cell line appeared heterogenous, we cloned the cells twice successively. Subclones had lost the expression of GFAP and of the neural tube marker A4. These cloned cells were SV-40 large T, Ran 2, and fibronectin positive and also expressed N-CAM (Table I).

Table I. Characteristics of Immortalized Cells Derived from Rat CNS as Compared with Type 1 Astrocytes

Antigen/enzyme	A7 cell line	A7-6.3 cell line	Type 1 astrocytes
A2B5	−	−	−*
GC	−	−	−*
GFAP	±	−	+‡
Ran 2	+	+	+§
Fibronectin	+	+	+‖
A4	±	−	+¶
N-CAM	+	+	+**
p185	+	ND	+
Glutamine synthetase	−	ND	+‡‡
Monoamine oxidase B	−	ND	+§§
Supports neural outgrowth	+	ND	+‖‖

* Raff et al., 1983; ‡ Bignami et al., 1972; § Bartlett et al., 1981; ‖ Price and Hynes, 1985; ¶ Miller et al., 1984; ** Noble et al., 1985; ‡‡ Pishak and Phillips, 1980; §§ Levitt et al., 1982; ‖‖ Fallon, 1985.

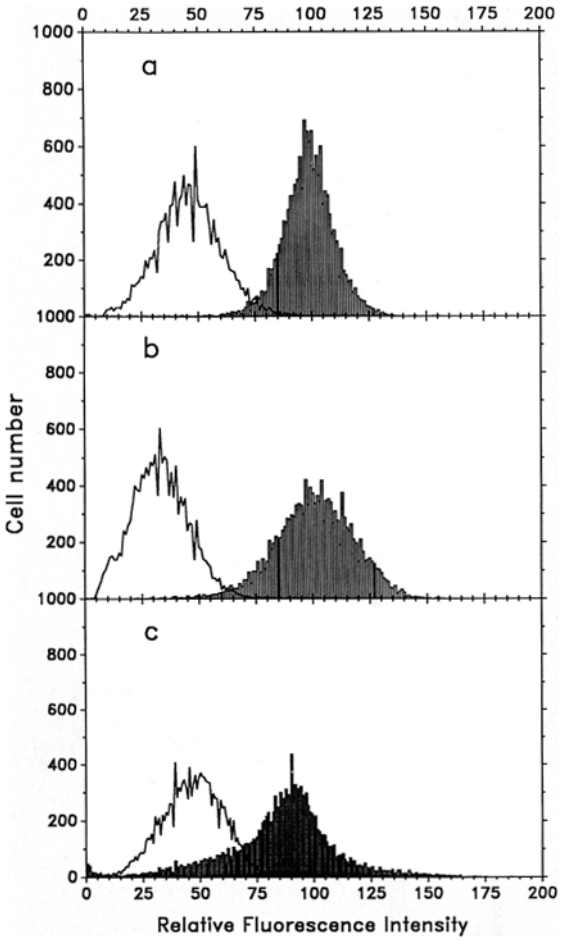

Figure 3. Flow cytometric analysis of mAb 7.16.4 binding to (*a*) A7 cells, (*b*) C6 glioma cells, or (*c*) primary type 1 astrocytes. Unhatched histograms represent background fluorescence of the fluorescent conjugate in the absence of primary antibody, while the hatched histograms represent fluorescence after treatment with the primary and secondary antibodies. Analysis of these histograms indicates that 95% of the A7 and C6 cells are positive for 7.16.4 binding, whereas 65% of the primary astrocytes are positive.

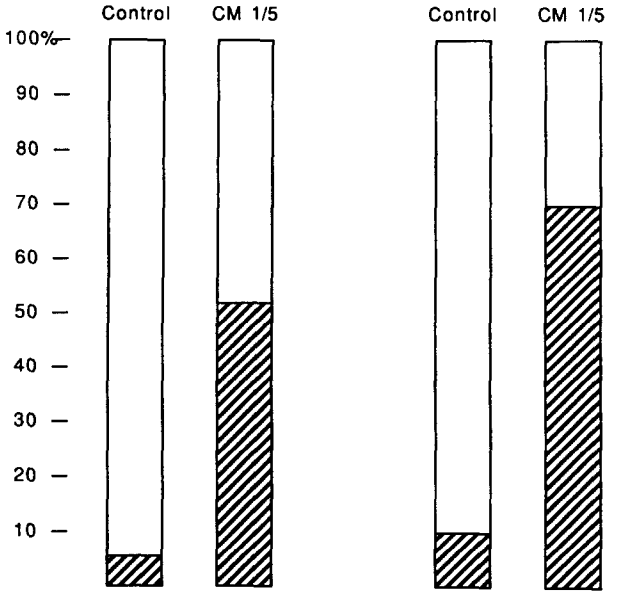

Figure 4. Ratio of oligodendrocytes to progenitors after 3 d of optic nerve culture in the presence or absence of conditioned medium from A7 and A7.6-3 cell lines. Progenitors and oligodendrocytes were counted in duplicate coverslips as described in Materials and Methods and their respective ratios were calculated. *Open boxes*, % oligodendrocytes (galactocerebroside$^+$); *hatched boxes*, % progenitor cells (A2B5$^+$, galactocerebroside$^-$).

Mitogenic Activity of the Supernatant

Since the A7 cell line expressed certain markers characteristic of type 1 astrocytes, we tested whether these cells expressed and secreted a mitogenic activity for O-2A progenitor cells from rat optic nerve (Raff et al., 1983). O-2A progenitor cells were obtained from 7-d rat optic nerve (Noble and Murray, 1984) and cultured in the presence or absence of 24-h A7 CM diluted 1:5 in defined medium. After 3 d, cultures were immunolabeled for A2B5 and GC. Total O-2A lineage cells (all cells expressing either A2B5 or GC) were counted on duplicate coverslips and the ratio of GC$^+$ oligodendrocytes to A2B5$^+$/GC$^-$ progenitor cells was calculated (Fig. 4). In defined CM, 95% of the cells were GC$^+$ oligodendrocytes whereas close to 50% of the O-2A lineage cells grown in CM were A2B5$^+$, GC$^-$ progenitors.

After two rounds of cell cloning, we obtained two cell lines with increased mitogenic activity in their supernatant. One of these clones (A7-6.3) is illustrated in Fig. 5. After 3 d in A7-6.3 CM (containing 10% FCS and diluted 1:5), 70% of total O-2A cells were progenitors; the remaining cells were oligodendrocytes indicating that differentiation could take place in the presence of this mitogen. CM containing 10% serum was found to be more mitogenic than CM with 2% FCS, and mitogenic activity was found in 24-h defined CM without serum, although in lesser amounts (data not shown). Since ~1/3 of the cells in a suspension of 7-d-old optic nerve are progenitors (Noble and Murray, 1984), we could estimate the number of progenitors per coverslip at the time of dissociation. Taking the ratio of the number of progenitors at 3 d to the initial number, we calculated that addition of A7 CM resulted in an approximately two doublings of the progenitor population in 3 d, whereas addition of A7-6.3 CM produced approximately four doublings in 3 d. Total cell population was also increased fourfold compared to untreated cells at 3 d.

Another clone (A7-6.1) had slightly different properties than A7-6.3. A7-6.1 CM also caused striking proliferation of progenitors; after 3 d, 67% of the total population were progenitors. However, only 15% of the remaining O-2A cells were oligodendrocytes (in contrast to 30% oligodendrocytes with A7-6.3 clone) while 18% of the cells were A2B5$^+$, GFAP$^+$ type 2 astrocytes. Thus, the A7-6.1 clone may synthesize not only mitogens for the O-2A progenitors but factors inductive of type 2 astrocyte differentiation.

The mitogenic effect of A7-6.3 CM was further demonstrated by [^{3}H]thymidine incorporation combined with double immunolabeling. CM activity was compared to that of PDGF, which displays strong mitogenic activity for O-2A progenitors (Richardson et al., 1988). The results of this experiment are presented in Table II. The mitogenic activity of 2% serum CM diluted 1:5 (final serum concentration 0.4%) was similar to the activity of 25 ng/ml porcine PDGF in 0.5% serum medium; ~50% of progenitor cells had incorporated

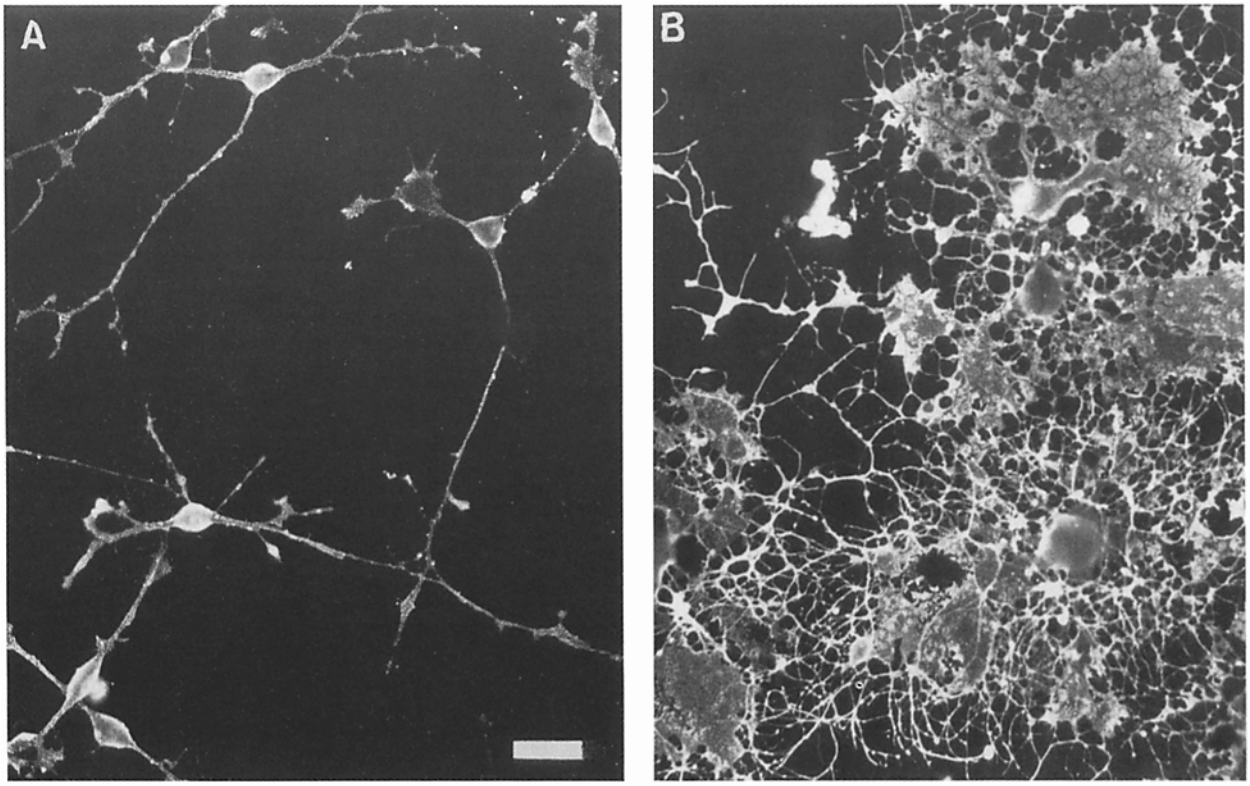

Figure 5. Representative fields of optic nerve cells cultured 3 d in the presence (*A*) or absence (*B*) of A7.6-3 CM. In the presence of CM, the majority of the cultured cells are bipolar progenitors as revealed with antibody A2B5 (*A*), while most cells are oligodendrocytes when reacted with anti-GC antibody (*B*) with no added CM. These results are quantitated in Fig. 4. Bar, 20 μm.

[^{3}H]thymidine in the period immediately before fixation. Rarely were oligodendrocytes observed to undergo mitosis, and those were only immature oligodendrocytes (cells labeled both with anti-GC and A2B5).

To determine if the mitogenic activity of A7-6.3 CM was due to PDGF-like molecules, we tested the ability of a goat polyclonal anti-human PDGF antibody to inhibit progenitor cell mitosis. We compared it to the ability of the same antibody to neutralize O-2A progenitor mitosis triggered by porcine PDGF (Table III). At a dilution of 1:20, 95% of the mitogenic activity of A7-6.3 CM was neutralized; this was progressively diminished with increasing concentration of A7-6.3 CM. Porcine PDGF activity was also substantially neutralized by our antibody. These results suggest that PDGF is the major factor responsible for progenitor cell mitosis induced by A7-6.3 CM.

None of our clones were found to secrete glial growth factor activity as tested on Schwann cells (Brockes, 1980).

Growth of Neurons on A7 Cells

One characteristic of type 1 astrocytes is the ability to support neuronal adhesion and neuritic outgrowth. Hypothalamic neurons dissociated from embryonic rats are strongly dependent on the astrocyte monolayer for their survival and growth (Ventimiglia and Geller, 1987), and we therefore used a similar protocol to assess the ability of the immortalized cells to support neuronal growth. Neurons plated onto a monolayer of A7 cells remained as isolated neurons and showed little evidence of clumping. Neuritic outgrowth from these neurons was apparent as early as 1 d after plating, and by 7 d the neurons demonstrated significant neurite outgrowth as visualized by anti-neurofilament antibody (Fig. 6). The neurites showed little evidence of fasciculation and generally rambled on the surface of the A7 monolayer as isolated processes. The neurites tended to be of smaller caliber than neurites which grew on parallel cultures plated onto type 1 astrocytes. Neuronal survival on A7 cells was somewhat lower than neurons plated onto primary type 1 astrocytes. This lower survival appeared to be due to the high metabolic rate of the A7 monolayer which necessitated frequent media exchange; our previous results indicate that hypothalamic neurons do not benefit from frequent feeding (Ventimiglia and Geller, 1987).

Table II. Tritiated Thymidine Incorporation by O-2A Progenitors

	[^{3}H]thymidine-labeled nuclei (%)	
Addition to culture medium	Progenitor cells	Oligodendrocytes
None	3	1.25
Porcine PDGF (25 ng/ml)	47.5	2
A7-6.3 CM	45.5	0.5

A 1:5 dilution of A7-6.3 CM was added to cultures of 7-d-old optic nerve, and the numbers of cells incorporating [^{3}H]thymidine were counted. Values given are means of two experiments.

Table III. Neutralization of Mitogenic Activity in A7-6.3 CM by Anti-PDGF Ig

Addition	Concentration	Number of progenitors/coverslip		Neutralization (%)
		Without antibodies	With antibodies	
None		1	ND	
Porcine PDGF	12.5 ng/ml	29	4.5	87
A7-6.3 CM diluted	1:5	155	155	0
	1:10	150	62	59
	1:20	175	10	95

Porcine PDGF or A7-6.3 CM with or without anti-PDGF antibodies were added to cultures of 9-d-old rat optic nerve and the numbers of progenitors were counted on day 3. Anti-PDGF antibodies were added at the time of dissociation and on day 2.

Discussion

We describe here the antigenic and biological properties of a cell line derived from rat CNS cells after infection with a murine recombinant retrovirus containing the SV-40 large T transforming gene (Jat et al., 1986). Our goal was to immortalize glial cells in order to allow an approach to the control of expression of cell-type specific properties of glial cells. We expected that, since our primary culture was comprised of type 1 astrocytes and O-2A progenitors, the immortal cell would resemble one phenotype or another. In fact, the A7 cell line that we have obtained, as well as its subclones, ex-

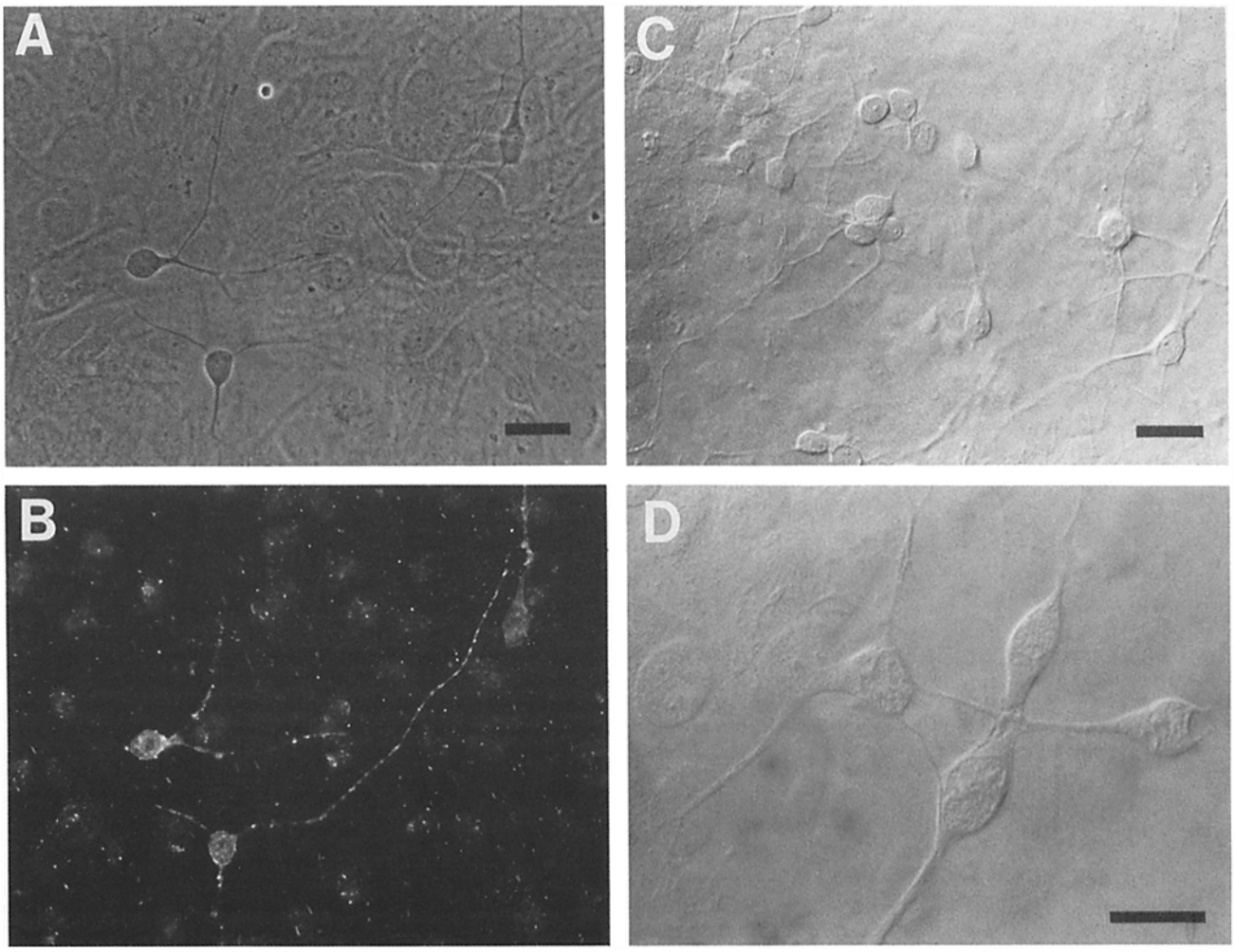

Figure 6. Growth of embryonic hypothalamic neurons on A7 cells. (*A*) Phase-contrast and (*B*) fluorescence photomicrographs after decoration with anti-neurofilament antibody of several neurons on a monolayer of A7 cells 24 h after plating, showing isolated cell bodies and processes. (*C*) Modulation-contrast photomicrograph of living neurons 3 d after plating on a monolayer of A7 cells, showing thick processes rambling over the monolayer. (*D*) High-power differential interference-contrast photomicrograph of several neurons 7 d after plating on the A7 monolayer. Bars: (*A–C*) 50 μm; (*D*) 25 μm.

presses properties reminiscent of type 1 astrocytes (Table I). Similarly to type 1 astrocytes, both the A7 cell line and several subclones support the growth of dissociated embryonic neurons and secrete PDGF. The A7 line also expresses high levels of the p185 phosphoprotein product of the *neu* oncogene, which is expressed by primary type 1 astrocytes in culture, and is a characteristic of many neuroblastoma and glioblastoma cell lines (Schechter et al., 1984). In addition, the expression of GFAP, the major intermediate filament protein of astrocytes, and the neural tube marker A4, were detected in early passage cells, but this expression was lost with passage. No properties of O-2A lineage cells are expressed by A7 cells. The reason why no O-2A lineage cell lines were established is obscure: O-2A progenitors may not have been present in sufficient numbers to be affected by the virus, or may not have been receptive to the insertion of the virus.

The reasons why GFAP expression and the neural tube marker A4 were lost in our immortalized cell line are unclear. It is possible that the cell we have immortalized is not an astroglial cell; this seems unlikely based on the initial expression of GFAP and A4. The other possibility is that the immortalization resulted in the loss of astrocyte-specific gene expression in those type 1 astrocytes having the highest growth potential. Other virus-transformed glial cell lines have demonstrated variable levels of GFAP expression, from very low (Neto et al., 1986) to high (Alliot and Pessac, 1981); however, the level of GFAP expression was not correlated with doubling time in these earlier experiments. The C6 astrocytoma cell line expresses low levels of GFAP under normal growth conditions, but, similarly to normal type 1 astrocytes (Goldman and Chiu, 1984), GFAP expression is dramatically increased in C6 cells by exposure to dibutyryl cAMP (Raju et al., 1980). Neither dibutyryl cAMP nor retinoic acid increased GFAP expression by A7 cells. An inverse correlation between growth potential and cell-type specific gene expression would also explain the absence of detectable glutamine synthetase and monoamine oxidase B activity in A7 cells. Glutamine synthetase activity in low passage C6 cells is relatively low as compared to primary astrocytes, and can be increased by dibutyryl cAMP (Browning and Nicklas, 1982) and glucocorticoids (Pishak and Phillips, 1980). Thus, the A7 cell line resembles the C6 cell in basal conditions, but does not respond to hormonal stimulation.

The expression of the neural tube marker, A4, by these cells is also interesting. A4 was originally described as having a distribution limited to CNS neurons in culture (Cohen and Selvandran, 1981). Later experiments demonstrated that A4 was also expressed by all astrocytes in vivo and both type 1 and type 2 astrocytes in culture, although the expression on type 1 astrocytes was low after several days in culture (Miller et al., 1984). Human astrocytomas have recently been shown to express A4, with a high degree of correlation of expression of GFAP and A4 (Rettig et al., 1986). Few astrocytomas which expressed only A4 or GFAP were identified, leading to the conclusion that A4 is a marker for differentiated tumor cells. Our results are consistent with this observation, since the expression of A4 and GFAP by the A7 cell line and its progeny appear to be coordinated, in that the early passage cells expressed both markers while later passage cells express neither. One other characteristic of A7 cells is that they have fibrillar fibronectin on their surface. The dual or alternate expression of GFAP and fibronectin has been described in glioma cells (Bigner et al., 1981). Fibronectin is usually synthesized in a nonfibrillar form by type 1 astrocytes (Price and Hynes, 1985).

Both A7 cells and C6 cells expressed high levels of the p185 phosphoprotein product of the *neu* oncogene; primary cultured astrocytes were also positive, but at a lower level of expression. The *neu* oncogene, also known as c-erbB-2 (Yamamoto et al., 1986) and HER2 (Coussens et al., 1985), was identified by transfection of DNA extracted from chemically induced neural and glial tumors into NIH 3T3 cells (Shih et al., 1981). mAb 7.16.4 was developed by immunizing mice with one of these transfected cell lines (Schechter et al., 1984); this antibody was found to react specifically with p185 (Drebin et al., 1985). p185 is structurally related to the epidermal growth factor receptor, and also possesses tyrosine kinase activity (Stern et al., 1986). Recent studies have shown that *neu* expression is elevated in a variety of normal embryonic tissues, including the nervous system, connective tissue, and secretory epithelium; this expression is not apparent in adult tissue (Kokai et al., 1987). This suggests that c-*neu* may be involved with normal growth control in embryonic development. That both A7 and C6 cell lines strongly express p185, coupled with the fact that there is a high level of expression by cultured primary rat cortical astrocytes, would support the role of the p185 phosphoprotein in growth control. Further support for the correlation between c-*neu* expression and mitosis is that cultured primary astrocytes, which continue to be mitotically active, express p185, whereas adult astrocytes in situ do not (Kokai et al., 1987).

The potent mitogenic activity for O-2A progenitors secreted by A7 and A7-6.3 cells closely resembles that secreted by type 1 astrocytes (Noble and Murray, 1984). The type 1 astrocyte-derived mitogenic factor comigrates with human PDGF on a size-exclusion column and, when reduced, yields the A and B chain of PDGF (Richardson et al., 1988). Both type 1 astrocytes and A7-6.3 cells synthesize the major PDGF A-chain transcript as well as low levels of PDGF B-chain transcripts (Richardson et al., 1988). PDGF transcripts can be detected in rat brain during gliogenesis. These studies would thus support a role for PDGF secreted by type 1 astrocytes in the stimulation of the division of O-2A progenitors during CNS gliogenesis.

The fact that A7 cells support the growth of embryonic hypothalamic neurons would lend further support to the astrocyte character of this cell line. Hypothalamic neurons appear to require an astrocyte monolayer for attachment and neuritic outgrowth; very poor survival was observed when neurons were plated onto other substrates, such as fibronectin, polylysine, or laminin (Ventimiglia and Geller, 1987). It is thus unlikely that the fibronectin associated with the A7 cell surface would support the extent of neuronal growth observed here. The majority of neurons plated on a monolayer of A7 cells exhibited visible neurites by 24 h in culture, and continued to grow for several weeks after plating. Furthermore, neurons plated on the A7 substrate, as on astrocytes, generally remained as single units, with little evidence of neurite fasciculation. This property has been attributed to the presence of N-CAM on the surface of astrocytes (Noble et al., 1985); A7 cells and the A7-6.3 subclone express immunologically identified N-CAM on their surface which

would assist in the adhesion of neurons. Thus, these immortalized cells may serve as a ready source of feeder cells for studies of dissociated neurons in culture.

In summary, retroviral infection of primary rat glial cells by a virus containing SV-40 large T antigen has produced an immortal cell line with a mixed astrocytic phenotype. This mixed phenotype is likely to be a characteristic of incompletely differentiated cells, thus suggesting that the insertion of an immortalizing oncogene may disrupt terminal differentiation while promoting cellular division. On the other hand, some important functional properties of type 1 astrocytes, such as the synthesis of PDGF and the ability to support neuronal survival (Noble et al., 1984), continue to be expressed in these immortalized cells. Likewise, the expression of the p185 phosphoprotein is enhanced as compared to type 1 astrocytes. Very recent studies indicate that A7-6.3 cells and type 1 astrocytes also synthesize tranforming growth factor β (E. Van Obberghen-Schilling, T. Behar, M. B. Sporn, and M. Dubois-Dalcq, unpublished observations). Thus, A7-6.3 immortalized cells may be a useful cell line to study various factors normally synthesized by type 1 astrocytes and/or the developing brain. We expect that the A7 cell line and other stable immortalized cell lines are likely to be useful to study regulation of cell-type specific gene expression in glial cells.

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