American Journal of Pathology, Vol. 136, No. 4, April 1990

Immunohistochemical Analysis of the A4 and AO10 (gp110) Cell-surface Antigens of Human Astrocytoma

Pilar Garin-Chesa, H. Richard Beresford, Sonia Walker, and Wolfgang J. Rettig

From the Department of Pathology and the Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, New York

The A4 and AO10 (110 kd glycoprotein) cell-surface antigens are biochemically distinct markers of cultured human astrocytomas that are expressed by only a limited number of other cultured cell types. To further characterize these two antigens, the authors used immunohistochemical methods to determine their expression in normal human tissues, astrocytomas, and over 100 tumors of other histologic types. They found that A4 is expressed 1) throughout the central (CNS), but not peripheral nervous system (PNS); 2) in smooth muscle and a small number of epithelial tissues; and 3) in reactive glia and in astrocytomas, but not in most tumors of other histologic types. In contrast, the AO10 antigen is expressed 1) in a small subset of CNS neurons, but not in astrocytes, PNS neurons, or other normal tissues; 2) in astrocytomas and reactive glia; and 3) in some additional neuroectodermal tumors, but not melanomas, carcinomas, or sarcomas. These findings show that A4 and AO10 are restricted markers for human astrocytomas in vivo. ***Furthermore, the antigens show distinct patterns of expression in normal human CNS but appear to be coordinately expressed in astrocytomas and astrocytoma-derived cell lines. (Am J Pathol 1990, 136:797–807)***

Serologic analysis of human neuroectodermal tumors (eg, melanomas, astrocytomas, and neuroblastomas) with monoclonal antibodies (MAbs) has identified several serologically and biochemically distinct cell-surface components, including glycoproteins, glycolipids, and proteoglycans.[1–8] These antigens provide markers for phenotypically distinct tumor subsets and for normal cells at distinct stages of neuroectodermal differentiation. In addition, cell-surface antigens that are expressed on tumor cells but have a restricted distribution in normal tissues may serve as targets for immunolocalization of tumors or for passive immunotherapy, using antibodies or antibody conjugates.[9] We have previously identified two cell-surface antigens of astrocytomas that show a highly restricted distribution when tested on a panel of cultured normal cells and cell lines derived from neuroectodermal, mesenchymal, epithelial, and hematopoietic tumors. One is a 110-kd cell-surface glycoprotein detected by MAbs AO10 and G184, designated as AO10 antigen or gp110.[1–3] The second is a cell-surface antigen originally defined on neurons of rat brain and detected by MAbs A4 and C5, designated as A4 antigen.[2,10] When characterized in rat neuronal cultures, A4 was found on central nervous system (CNS) neurons but not on neurons of the peripheral nervous system (PNS).[10] We subsequently found that A4 is also expressed in several human astrocytoma cell lines,[2] indicating that the A4 and C5 antigenic determinants are conserved among different species.[10] The general distribution of the human AO10 antigen or the rat or human A4 antigens in normal and tumor tissues have not previously been determined. Therefore, we undertook a detailed immunohistochemical comparison of AO10 and A4 expression in human tissues. We used the avidin-biotin immunoperoxidase procedure on frozen sections of normal tissues and a large panel of tumors of neural and non-neural origin to define the tissue specificity of the antigens. Our findings provide an essential framework for evaluating these cell-surface antigens as targets for localizing or cytotoxic antibody conjugates and as phenotypic markers for classifying glial tumors according to their lineage or stage of differentiation.

Materials And Methods

Cultured Cells

Tumor cell lines were obtained from the cell bank of the Human Cancer Immunology Laboratory at Sloan-Ketter-

Accepted for publication November 24, 1989.

Address reprint requests to Dr. Pilar Garin-Chesa, Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

Table 1. *Cell-surface Antigen Expression and Fibronectin Secretion in a Panel of Human Astrocytoma Cell Lines*

Cell line	Cell-surface antigen				Fibronectin secretion
	A4	AO10	Thy-1	S5	
SK-GS-1	+++	+++	+++	−	−
SK-MG-2	+++	+++	+++	−	−
U251MG	+++	+++	+++	−	−
U373MG	+++	+++	+++	−	+
SK-MG-10	−	−	+++	++	−
SK-MG-12	+	+++	+++	+++	+++
SK-MG-14	−	+++	+++	+++	+++
SK-MG-13	−	−	+++	+++	+++
SK-MG-3	−	−	+++	+++	++
A582	−	−	+++	++	++
SK-MG-17	−	−	+++	+++	+++

Cell-surface antigen expression was determined by MHA rosetting assays with serial dilutions of *nu/nu* sera or ascites fluid for MAbs A4 and C5 (A4 antigen), AO10, and G184 (AO10 antigen), K117 (Thy-1[12]); and S5. Cell-surface reactivity is indicated as follows: +++, strong reactivity (reciprocal titration endpoints > 10^4); ++, moderate (10^3 to 10^4); or +, weak (2×10^2 to 10^3); −, no reactivity at starting dilution of MAb (1:250). FN secretion was determined by solid-phase ELISA,[14] and results are indicated as strong (+++), moderate (++), and low reactivity (+) with antihuman FN MAb.

ing Institute. Cultured cells were maintained in Eagle's minimum essential medium or RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 to 15% fetal bovine serum.

Monoclonal Antibodies

Production and characterization of MAbs AO10 (IgG1) and A4 (IgM) have been previously described.[1,2,10] Monoclonal antibody C5 is an IgG1 antibody that recognizes the A4 antigen.[2] Monoclonal antibody G184 (IgG1) was derived from a (Balb/c × C57BL/6)F1 mouse immunized with cells of astrocytoma SK-GS-1.[11] Serologic reactivity of MAb G184 parallels that of MAb AO10, and both MAbs immunoprecipitate the same 110-kd cell-surface glycoprotein.

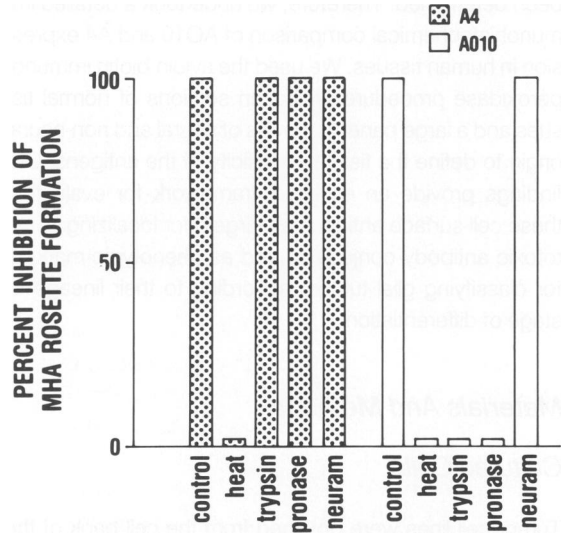

Figure 1. *Reactivity of MAbs AO10 and A4 with U251MG astrocytoma cells following treatment of target cells with heat or enzymes. U251MG cell pellets were treated with heat, neuraminidase, trypsin or pronase or left untreated (control), and incubated with appropriately diluted MAbs[13] to absorb out MAb AO10 or MAb A4. Residual antibody activity after absorption with test cells was assayed by MHA tests on untreated U251MG cells.*

Serologic Procedures

Mixed hemadsorption (MHA) rosetting assays for detecting surface antigens on cultured cells have been described.[12] In summary, 200 to 300 cells per well were seeded into MicroWell plates (Nunc, Roskilde, Denmark) and cultured for 24 to 48 hours prior to testing. Cells were incubated with serial dilutions of MAbs for 1 hour, washed three times with phosphate-buffered saline (PBS) containing 1% fetal bovine serum, and incubated for 45 minutes with indicator red blood cells, prepared by coating type O human red blood cells with rabbit anti-mouse gamma G immunoglobulin (IgG) or goat anti-mouse gamma M immunoglobulin (IgM) (DAKO Corp., Santa Barbara, CA). Plates were washed and reactivity was scored microscopically by determining the percentage of target cells that showed rosetting with erythrocytes at each dilution of MAb. To determine heat stability of antigens, test cells were incubated for 5 minutes at 100°C prior to testing in MHA absorption assays.[13] Susceptibility of antigens to enzyme digestion was determined by incubation of test cells with trypsin (Sigma Co., St. Louis, MO; 0.5 mg/ml in Dulbecco's PBS, 30 minutes at 37°C), neuraminidase (Calbiochem, San Diego, CA, *Vibrio cholerae* neuraminidase, 0.1 IU/ml in Dulbecco's PBS, 30 minutes at 37°C), or pronase (Calbiochem, 0.5 mg/ml; 30 minutes at 37°C) prior to serologic analysis.[13]

Enzyme-linked Immunoadsorbant Assay

Confluent astrocytoma cultures were fed with fresh media, culture supernatants collected after 48 to 72 hours, and adsorbed onto MicroWell plates for 18 to 24 hours at 4°C.[14] Bound fibronectin (FN) was detected in a solid-phase enzyme-linked immunosorbant assay (ELISA)[14] with antihuman FN MAb (Calbiochem, starting dilution 1:200, five-fold dilution series).

Immunochemical Procedures

For immunoprecipitation assays, cells were metabolically labeled with [^{35}S]methionine or [^{3}H]glucosamine (New England Nuclear, Boston, MA) and extracted with 0.5% Nonidet NP-40 in TRIS buffer.[1] Lysates were incubated with

test and control MAbs, followed by rabbit anti-mouse Ig. Immune complexes were isolated with protein A-sepharose beads, extracted, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography as described.[1]

Immunohistochemistry

Normal and tumor tissues were obtained at autopsy or from surgical specimens in the Departments of Pathology at Memorial Hospital or North Shore University Hospital, New York, quick-frozen in isopentane precooled in liquid nitrogen, and stored at −70°C until used. Sections of 5 μm thickness were cut, mounted on gelatin-coated slides, air-dried for 30 minutes at room temperature (RT), and fixed by one of the following procedures: 1) cold acetone for 10 minutes at 4°C; 2) formalin (3.7% in PBS) for 10 minutes at RT; 3) methanol for 10 minutes at RT; or 4) 96% ethanol for 10 minutes at RT. The avidin-biotin immunoperoxidase procedure[15] was used as described.[16] Briefly, sections were treated with 0.3% H_2O_2 for 5 minutes at RT to block endogenous peroxidase, and incubated with normal horse or goat serum for 30 minutes at RT. Subsequently, sections were incubated with MAbs for 12 to 18 hours at 4°C. Monoclonal antibodies were used as ascites fluid (MAbs A4, C5, AO10; 1:500 to 1:1000 dilution) or hybridoma culture supernatant (MAb G184; 1:2 dilution), at a final Ig concentration of 10 to 20 μg/ml. Sections were incubated with biotinylated horse anti-mouse IgG or goat anti-mouse IgM for 30 minutes at RT, followed by avidin-biotin horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). The final reaction product was visualized with diaminobenzidine.

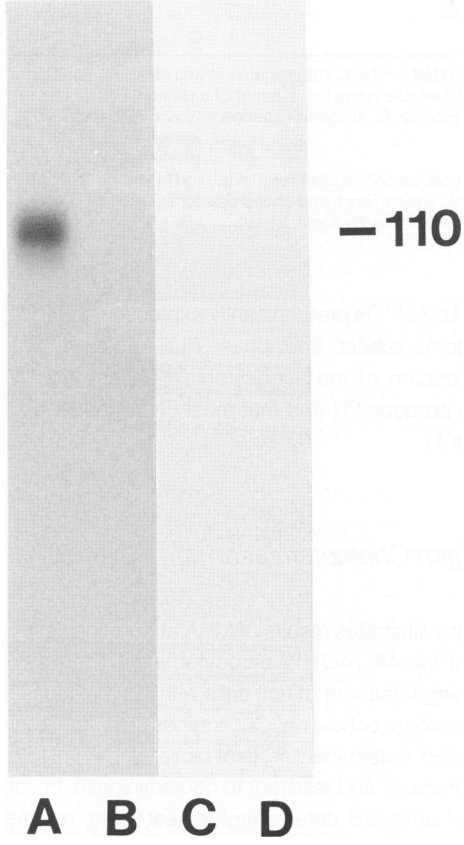

Figure 2. *Fluorogram of immunoprecipitates obtained with MAbs G184 (AO10 antigen), C5 and A4 (A4 antigen) from detergent extracts of radiolabeled U251MG astrocytoma cells and separated by SDS-polyacrylamide gel electrophoresis.* **A:** *MAb G184;* **B:** *MAb C5;* **C:** *MAb A4;* **D:** *negative control MAb.*

Table 2. *Distribution of Antigens AO10 and A4 in Normal Adult Human Tissues**

Tissue	Antigen-expressing cells or tissues	
	AO10 antigen	A4 antigen
Nervous system		
Cerebral cortex	Rare neurons	Gray > white matter
Globus pallidus	Neurons (subset)	Gray > white matter
Putamen	Neurons (subset)	Gray > white matter
Hypothalamus	Rare neurons	Gray > white matter
Mammillary body	Neurons (subset)	Gray > white matter
Substantia nigra	—	Gray > white matter
Pons	Neurons (subset)	Gray > white matter
Crus cerebri	—	Neuropil
Cerebellum	Granular layer	Gray > white matter
Spinal cord	Rare neurons	Gray > white matter
Peripheral nerves	—	—
Autonomic ganglia	—	—
Skin	—	Sweat glands, hair follicles
Mammary gland	—	Myoepithelial cells, duct epithelium
Gastrointestinal system		
Tongue	—	—
Parotid gland	—	Ducts, acini
Esophagus	—	Glands
Stomach, colon	—	—
Liver	—	Bile ducts
Pancreas	—	Ducts
Respiratory system		
Bronchus	—	Epithelium
Lung	—	—
Genitourinary system		
Kidney	—	Mesangium
Bladder	—	Urothelium
Testis, ovary	—	—
Prostate	—	—
Lymph node	—	—
Endocrine system		
Thyroid gland	—	—
Pancreas	—	—
Andrenal gland	—	Medullary cells (subset)
Muscle tissue		
Skeletal muscle	—	—
Cardiac muscle	—	—
Smooth muscle	—†	Visceral, vascular smooth muscle
Connective tissue	—	—

* Acetone-fixed frozen tissue sections were tested with MAbs AO10 and G184 for the AO10 antigen and with MAbs A4 and C5 for the A4 antigen, using the avidin-biotin immunoperoxidase procedure.

† Weak and inconsistent staining of visceral smooth muscle in some organs.

Table 3. *Expression of Antigens AO10 and A4 in Astrocytomas and Tumors of Other Histologic Types*

Tumor type	Antigen-expressing tumors	
	AO10 antigen	A4 antigen
Neuroectodermal tumors		
Astrocytoma	●●●●●●●●●●*	●●●●●●●◍◍◍
	●○○	○○
Medulloblastoma	●	●
Meningioma	○○	○○○
Neuroblastoma	◍◍◍	○○○
Ganglioneuroma	◍○	○○
Paraganglioma	●	○
Schwannoma	◍◍◍○○○	○○○○○○○
Melanoma	○○○○○○○	●○○○○○
PNET†	●	
Neuroendocrine tumors‡	●●●◒○○○○○○○	○○○○○○○○
Sarcomas		
Leiomyosarcoma	◍◍○○○○○○	●●○○○○○
Fibrosarcoma	●◍○	●◍○○○
Osteogenic sarcoma	○○	○○○○
Ewing's sarcoma	○	○○○○
ERMS†	○	○○○○
Other sarcomas§	○○○○○○○○○○○	◒○○○○○○○○○○
	○○	○○○○○
Lymphomas	○○○○○○	○○○○○○○○○○
Carcinomas		
Ovarian	○○○○○○○○○	◒◒⊖⊖⊖○○○○○○
Breast	○○○○○	●○○○○○○○○
Kidney	○○○○	○○○○○○
Bladder	○○○	◒◒⊖○○○
Lung	○○○○	◒◒◒⊖⊖⊖○○
Stomach	○○○	◒◒○○
Other carcinomas‖	○○○○○○○○○○○	○○○○◒○○◒⊖○○○
		○○○◒

* Acetone-fixed frozen sections of human tumors were tested with MAbs AO10 and G184 for the AO10 antigen and with MAbs A4 and C5 for the A4 antigen using the avidin-biotin immunoperoxidase procedure. Each circle in the body of the table refers to the tumor of a different patient and results are symbolized as follows: ●, antigen positive; ○, antigen negative; ◍, weak antigen expression; ◒, ⊖, antigenic heterogeneity with 20% to 50% or less than 20% of tumor cells, respectively, showing antigen expression.

† PNET, primitive neuroectodermal tumor; ERMS, embryonal rhabdomyosarcoma.

‡ Neuroendocrine tumors tested include carcinoids, thyroid medullary carcinoma, oat cell carcinoma, islet cell tumors, and Merkel cell tumor.

§ Malignant fibrous histiocytomas, synovial sarcomas, chondrosarcomas, liposarcoma, and mesenchymal chondrosarcomas.

‖ Testicular, colonic, prostate, esophageal, squamous cell, and basal cell carcinomas and a mesothelioma.

Sections were counterstained with Harris' hematoxylin. Unrelated negative control IgG and IgM antibodies obtained from Becton and Dickinson, Mountain View, CA, were included in all assays and did not produce any immunostaining.

Results

Expression of A4, AO10, and Fibronectin in Cultured Astrocytoma Cells

Mixed hemadsorption rosetting assays with serial dilutions of MAbs A4, C5, AO10, and G184 were used to type a panel of 11 human astrocytoma cell lines for surface antigen expression (Table 1). Antigens AO10 and A4 are coordinately expressed in this cell panel,[2] with five lines being $A4^+/AO10^+$, one $A4^-/AO10^+$, and five $A4^-/AO10^-$. In contrast, the Thy-1 cell-surface glycoprotein, recognized by MAb K117,[12] is expressed by all these cell lines, and the 120-kd cell-surface glycoprotein recognized by MAb S5[2,11] is predominantly expressed on the $A4^-$ astrocytoma subset. Solid-phase ELISA tests showed that a proportion of the astrocytoma cell lines used in this study produce FN and that most FN^+ lines are $A4^-/S5^+$ (Table 1).

Antigen Characterization

Figure 1 illustrates results of MHA absorption assays with one of the $A4^+/AO10^+$ astrocytoma cell lines, U251MG, following treatment of test cells with neuraminidase, trypsin, pronase, or heating. Our findings show that the AO10 antigenic determinant is heat-labile, sensitive to trypsin and pronase, and resistant to neuraminidase. In contrast, the A4 antigenic determinant is heat-labile, but resistant to neuraminidase, trypsin, and pronase. Figure 2 shows a comparative immunoprecipitation experiment with MAb G184, which detects gp110, and MAb C5, which, like MAb A4, does not precipitate any detectable compo-

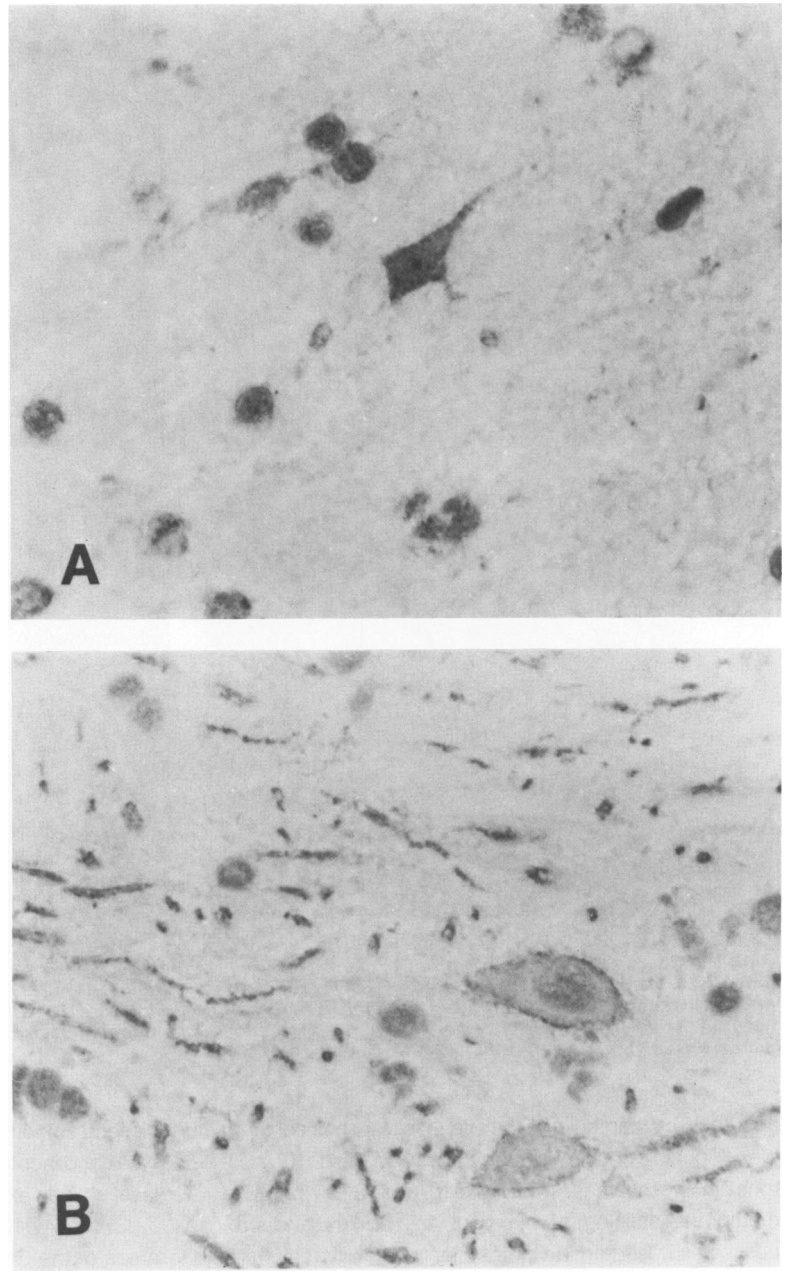

Figure 3. *Immunoperoxidase staining of normal brain tissues with mAb AO10.* **A:** *Cerebral cortex, showing strong AO10 immunostaining (cell membrane and cytoplasmic pattern) of a single neuron; the brown immunoperoxidase staining of the neuronal cell body is easily distinguished from the blue hematoxylin counterstaining seen in the nuclei of adjacent AO10-negative cells.* **B:** *Globus pallidus, immunostaining of neuronal cell bodies (cell membrane pattern) and multiple cell processes, some of which are shown in cross section. Acetone-fixed frozen sections were tested by the avidin-biotin immunoperoxidase procedure and counterstained with hematoxylin. Original magnification ×400* **(A),** *×200* **(B).**

nents from radiolabeled cell extracts of U251MG or SK-GS-1 astrocytoma cells.

Immunohistochemical Studies

The distribution of the AO10 and A4 antigens in a wide range of normal tissues (Table 2) and in over 100 tumors of different histologic types (Table 3) was determined by the avidin-biotin immunoperoxidase procedure. For these studies, both formalin-fixed and acetone-fixed frozen sections were used, since these fixatives were found in preliminary tests to give optimal preservation of immunoreactivity. In additional experiments, we tested selected normal tissues treated with ethanol or methanol to determine the effect of these fixatives on A4 and AO10 immunoreactivity.

Table 2 compares the distribution of AO10 and A4 in different areas of the normal adult CNS, the PNS, and in normal non-neural tissues. In the CNS, AO10 shows a highly restricted distribution, being expressed only in a small subset of neurons (Figure 3A, 3B, 4A). The large majority of CNS neurons as well as glial cells and meninges lack detectable AO10 expression. Although $AO10^+$ neurons were found in several distinct regions of the CNS, their number varied in different areas. For example, only rare neurons in the cerebral cortex show AO10 reactivity (Figure 3A), whereas other areas, such as globus pallidus

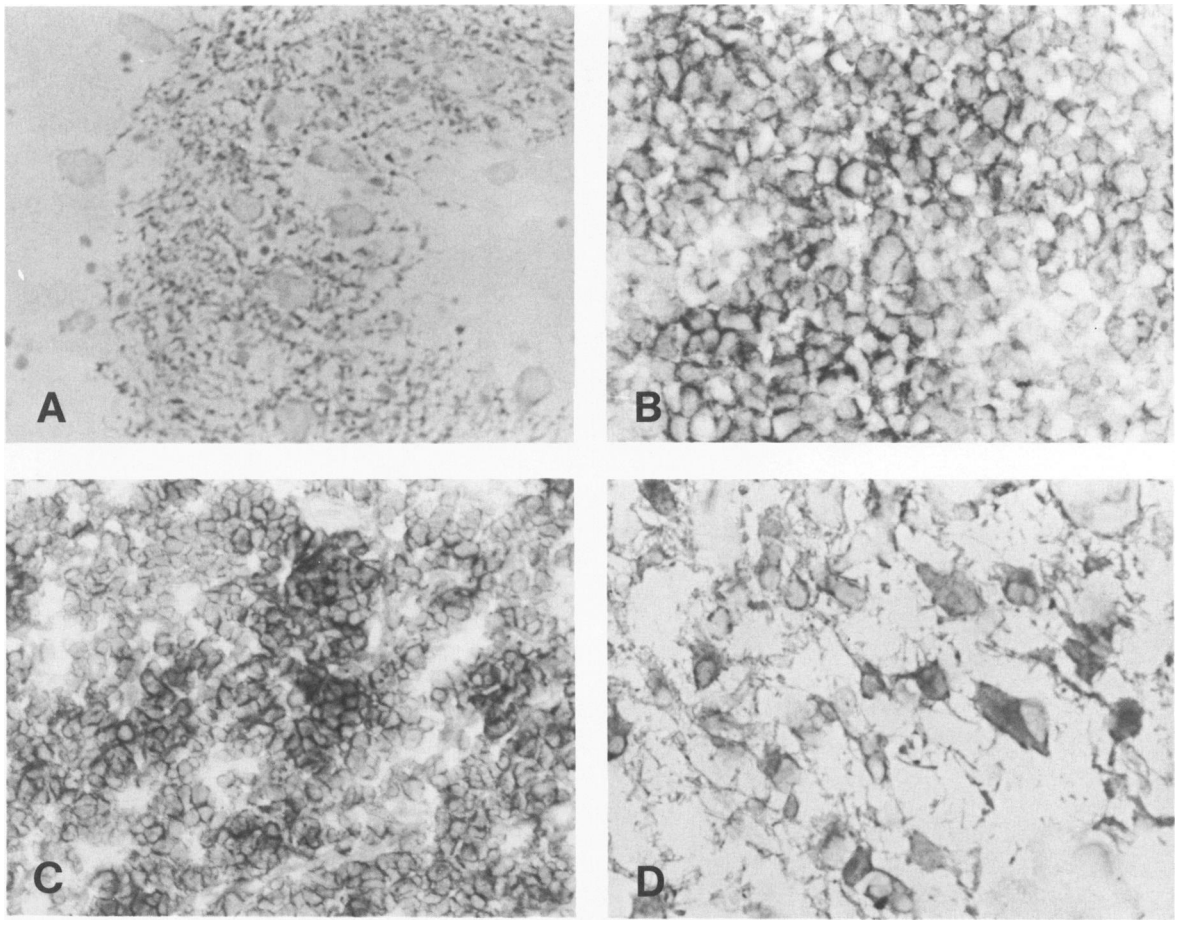

Figure 4. *Immunoperoxidase staining of normal tissues and tumors with MAb AO10.* **A:** *Normal adult brain (inferior olivary nuclear complex) showing AO10 immunostaining of neuronal cell bodies and processes.* **B:** *Neuroendocrine tumor of the duodenum (somatostatinoma) with strong AO10 expression.* **C:** *High-grade astrocytoma with strong AO10 expression.* **D:** *Reactive astrocytes adjacent to brain metastasis of a primary lung carcinoma; reactive astrocytes are AO10-positive while the tumor (not shown) was AO10-negative. Acetone-fixed frozen tissues were tested by the avidin-biotin immunoperoxidase procedure and counterstained with hematoxylin. Original magnification ×200* **(A–C),** *×400* **(D).**

(Figure 3B), putamen, and the inferior olivary nuclear complex (Figure 4A), show a large proportion of $AO10^+$ neurons. In these areas, MAbs AO10 and G184 produce membrane staining of neuronal cell bodies and processes as illustrated in Figures 3 and 4. A different staining pattern was observed in the cerebellum, where AO10 reactivity is restricted to the granular layer. Here, the antigen appears to be localized to discrete granular structures that lie among granule cells (Figure 5). This characteristic staining pattern does not appear to be due to immunohistochemical artifacts in the frozen sections, since the same specimens tested with MAbs against Thy-1, Le^X, NeuAc2→3LNT, and $p21^{ras}$ produced the expected labeling of neuronal and glial cell bodies and processes.[17–20] Despite an extensive survey of brain sections obtained from six different individuals (ages 2 to 65 years), we did not find any evidence for AO10 expression in normal glial cells. Furthermore, AO10 is not expressed in any of the normal PNS tissues tested, and non-neural tissues are generally $AO10^-$ (Table 2). One possible exception is visceral smooth muscle, which, in some organs, showed weak and inconsistent immunostaining that suggests low-level antigen expression.

Unlike AO10, A4 is widely expressed throughout all areas of the CNS (Table 2). Its immunoreactivity in CNS tissues is strongest and most uniform in areas of gray matter, where it appears to be present in neuronal cell bodies as well as neuropil (Figure 6A). However, A4 immunoreactivity is also found in areas of white matter, showing a fibrillary pattern (Figure 6A). Findings in the rat have suggested that both neurons and glial cells in the CNS are $A4^+$.[21] Our findings suggest a similar distribution for A4 in the human CNS, but do not establish whether astrocytes in general or specific subsets of astrocytes express the antigen. As in the rat, A4 was not detected in any of the human PNS structures tested. However, we did find strong A4 staining in a number of non-neural tissues for which no information is available in the rat (Table 2). For example, A4 is expressed in myoepithelial cells and ductal epithelium of the breast (Figure 6B), in bile ducts, coe-

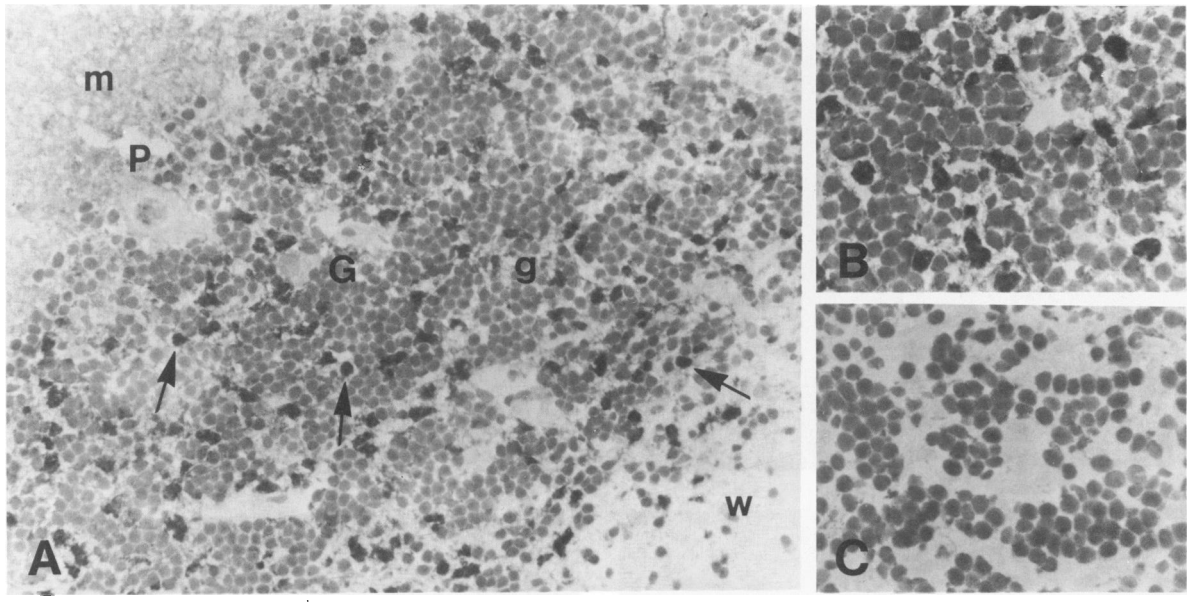

Figure 5. *Immunoperoxidase staining of normal cerebellum with MAb AO10.* **A:** *Low-power view; arrows indicate the sites of AO10 staining (brown) among the granule cell nuclei (blue counterstaining) in the granular layer (g). No AO10 immunostaining of cells or processes was seen in the molecular layer (m), Purkinje cells (P), or white matter (w).* **B:** *Higher power view of area shown in panel A; note AO10-immunoreactive cerebellar "glomeruli" among the granule cell nuclei.* **C:** *Parallel section to section shown in panels A and B, but tested with an unrelated negative control MAb of IgG1 subclass; no immunoreactivity. Acetone-fixed frozen tissues were tested by the avidin-biotin immunoperoxidase procedure and counterstained with hematoxylin. Original magnification* ×*250* **(A),** ×*400* **(B, C).**

lomic epithelium of the ovary (Figure 6C), and vascular and visceral smooth muscle cells (Figure 6D).

A panel of over 100 neural and non-neural tumors was tested by the avidin-biotin immunoperoxidase procedure for AO10 and A4 expression. AO10 was detected on 11 of 14 astrocytomas (grades III and IV) tested, in neuroblastomas, a subset of Schwannomas, and some other neural and neuroendocrine tumors (Table 3). In contrast, melanomas, most sarcomas, and epithelial tumors are $AO10^-$. Figure 4 illustrates AO10 staining for an astrocytoma, a duodenal somatostatinoma, and the antigen-positive reactive glia adjacent to an $AO10^-$ brain metastasis of a primary lung cancer.

The A4 antigen was found in 13 of 15 astrocytomas, but not in other neuroectodermal or neuroendocrine tumors (Table 3), except for one melanoma. A large proportion of sarcomas and epithelial tumors was found to be $A4^-$. However, some sarcomas are $A4^+$ (Figure 7D), as are subsets of breast (Figure 7C), ovarian, bladder, lung, and other carcinomas (Figure 7F).

Discussion

In cultured cells, the A4 and AO10 cell-surface antigens exemplify restricted serologic markers for human astrocytoma.[1,2] While the analysis of cultured cells is invaluable in comparing different cell-surface antigenic systems and in defining the biochemical nature and genetic control of MAb-defined cell-surface components, it does not provide a full view of their tissue specificity.[22] As one limitation, most normal human cell types, including astrocytes and neurons, and a majority of malignant tumors cannot be maintained and tested in tissue culture. Furthermore, tissue culture conditions may induce complex surface antigenic changes in both normal and malignant cells and thus perturb the *in vivo* phenotype.[11,22] The present study was designed to establish the tissue specificity of the A4 and AO10 cell-surface antigens, using immunohistochemical analysis of a large number of normal tissues and a panel of over 100 malignant tumors.

The A4 antigen was originally described by Cohen and Selvendran[10] as a cell-surface antigen of rat neurons that distinguishes CNS ($A4^+$) and PNS neurons ($A4^-$). Subsequently, A4 was also detected on rat glial cells[21] and on a proportion of human astrocytoma cell lines,[2] suggesting that it was a marker for neural tube derivatives rather than being unique to CNS neurons. Here we show that A4 expression in human nervous system tissues follows the pattern described for the rat. The only other cell-surface antigen that is known to be expressed throughout the human CNS and to be absent from the PNS is the Le^x carbohydrate structure (lacto-N-fucopentaose III[19,23]). Since the biochemical nature of the rat and human A4 molecules is unknown, they cannot be compared directly with Le^x. However, in addition to CNS tissues, Le^x is expressed by epithelia and myelomonocytic cells that lack the A4 antigen.[19,23] Conversely, $A4^+$ astrocytoma cell lines are unre-

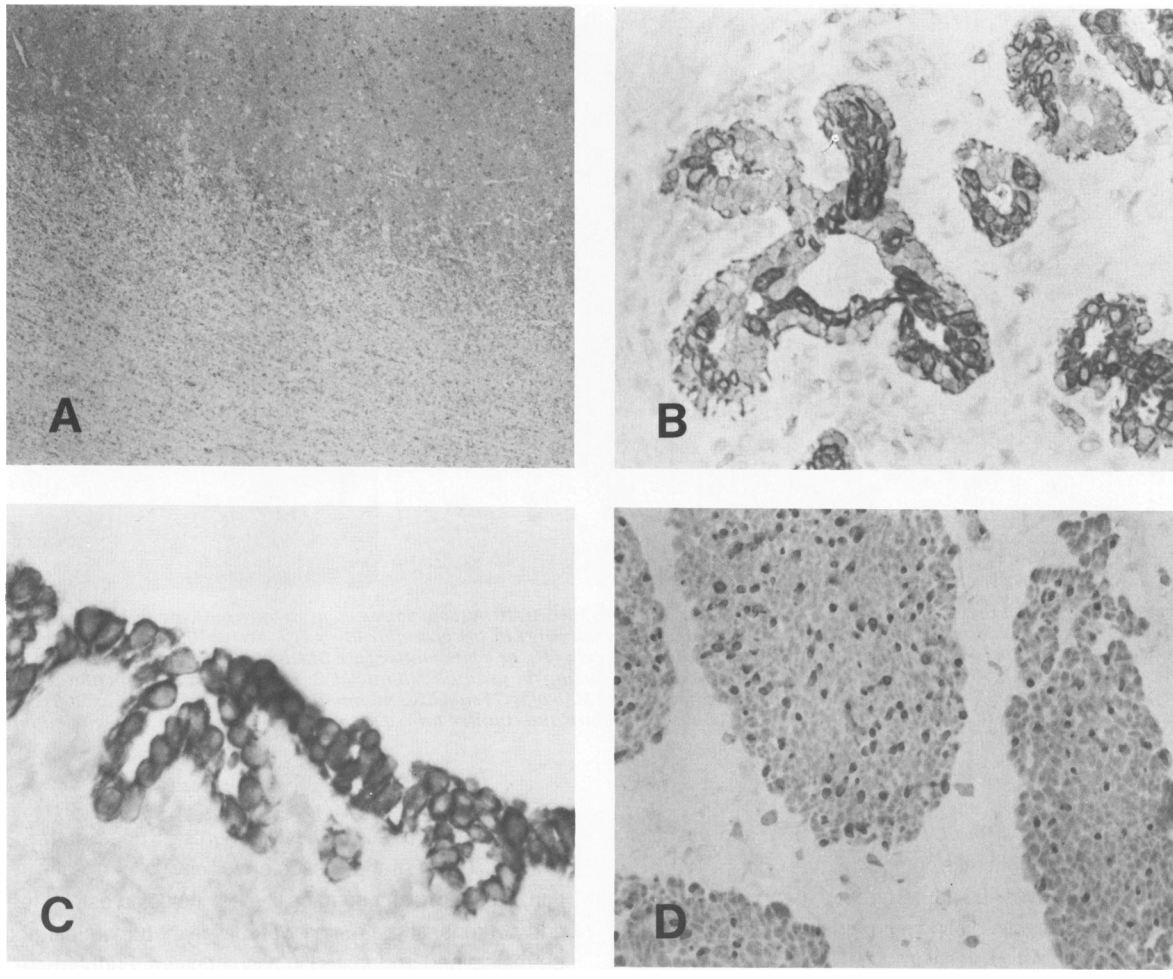

Figure 6. *Immunoperoxidase staining of normal human tissues with MAb A4.* **A:** *Normal adult brain (globus pallidus), showing strong immunostaining in areas of gray (top) and white matter (bottom).* **B:** *Normal mammary gland, showing A4 immunostaining of myoepithelial cells and ductal epithelial cells but not the surrounding stroma.* **C:** *Normal ovary with A4 immunostaining in the coelomic surface epithelium.* **D:** *Normal urinary bladder showing A4 immunoreactivity in the visceral smooth muscle. Acetone-fixed frozen sections were tested by the avidin-biotin immunoperoxidase procedure and counterstained with hematoxylin. Original magnification* ×*200* **(A, B, D),** ×*400* **(C).**

active with the anti-Le^x MAb P12.[13] Thus, A4 and Le^x are clearly distinct antigenic systems with widely different tissue specificities outside the nervous system. A final, immunohistochemical distinction is provided by the fact that Le^x, which is a heat-stable carbohydrate determinant carried by plasma membrane lipids and proteins,[13] is detected equally well in frozen sections of CNS tissues left unfixed or fixed with formalin, acetone, or ethanol, and even in routine paraffin-embedded material.[19] In contrast, we show here that A4 immunoreactivity is lost following fixation of frozen sections with ethanol or methanol, consistent with the idea that A4 may be a glycolipid antigen rather than a glycoprotein.[10] Based on our serologic analysis of cultured cells and tissues and biochemical evidence, the AO10 antigen can be distinguished from both A4 and Le^x and from several other astrocytoma cell-surface glycoproteins described by us and other investigators.[1,2,24]

The function of a serologically defined cell-surface component cannot generally be deduced from its tissue distribution[22] and nothing is presently known about the function of A4 or the AO10 glycoprotein, gp110. However, in view of the differential AO10 expression on subsets of CNS neurons, it may be of interest to examine its possible role as a cell-surface receptor. Structural analysis of gp110 and characterization of the gp110-encoding gene may help address this question. Further analysis also is needed to determine whether AO10 expression on specific subsets of CNS neurons changes during CNS development or correlates with structural or functional properties of CNS neurons. So far, no such correlation has emerged from our immunohistochemical studies. In addition, we cannot explain the distinctive granular pattern of AO10 immunostaining observed in the cerebellum, which contrasts with the cell membrane staining of neuronal cell bodies seen in other CNS regions. Conceivably,

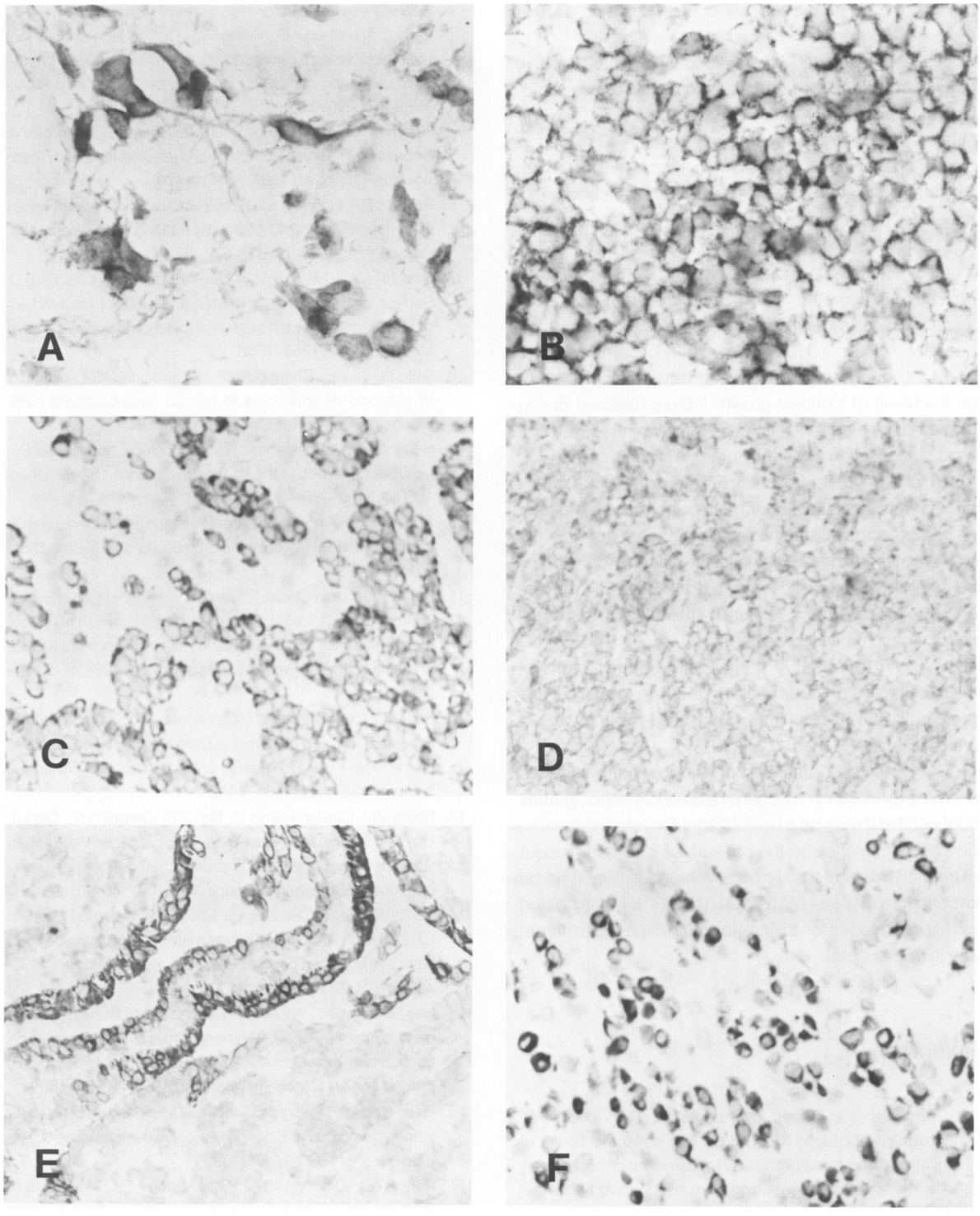

Figure 7. *Immunoperoxidase staining of reactive astrocytes and tumor tissues with MAb A4.* **A:** *Reactive astrocytes adjacent to brain metastasis of an A4-negative primary lung cancer.* **B:** *High-grade astrocytoma.* **C:** *Infiltrating ductal carcinoma of the breast.* **D:** *Leiomyosarcoma.* **E:** *Endometrial carcinoma.* **F:** *Pleural mesothelioma, showing marked antigenic heterogeneity for A4. Acetone-fixed frozen sections were tested by the avidin-biotin immunoperoxidase procedure and counterstained with hematoxylin. Original magnification* ×*400* **(A),** ×*200* **(B–F).**

in the granular layer of the cerebellum, AO10 is not associated with individual neuronal cell bodies or processes. Instead, it may be restricted to the specialized synaptic complexes that lie in the "cerebellar islands" among the granule cell bodies, which are referred to as "cerebellar glomeruli."[25] Immunoelectronmicroscopy may provide di-

rect information on the subcellular localization of AO10 in the cerebellum and other regions of the CNS.

A shared characteristic of A4 and AO10 noted in previous studies is their reciprocal, although partially overlapping expression with glial fibrillary acidic protein (GFAP) in subsets of cultured astrocytoma cell lines.[1,2] These coordinate phenotypic patterns of cultured astrocytomas, which also include S5 (gp120) and FN expression,[2,26-28] may reflect normal glial differentiation programs that persist after malignant transformation. The fact that AO10 is found in reactive glia and astrocytoma cells *in vivo*, but is not coexpressed with A4 and GFAP in normal adult brain astrocytes, may indicate that resting astrocytes are induced to express AO10 only when they become proliferatively active, either following malignant transformation or as the result of extrinsic growth factors released during tissue injury or repair. An unresolved question is the histotypic classification of astrocytoma cell lines that lack A4, AO10, and GFAP, but express FN, an extracellular matrix protein not generally found in either normal CNS parenchyma or astrocytoma specimens.[26-28] One possible explanation is that these cell lines arise from tumors that express glial characteristics *in vivo* but assume a "mesenchymal" phenotype in culture. Alternatively, individual astrocytomas may contain both $A4^+/AO10^+/S5^-/FN^-$ and $A4^-/AO10^-/S5^+/FN^+$ tumor cells, and either one of these cell types (or additional intermediate forms) survive preferentially in tissue culture, giving rise to phenotypically uniform tumor cell lines.

The restricted distribution of AO10 in normal tissues and its expression in a large proportion of astrocytomas suggest that it may be a useful target for immunotherapy, despite its presence on the cell-surface of a small subset of normal CNS neurons. To pursue this possibility, it will be important to evaluate MAbs AO10 and G184, conjugated with radioisotopes or toxins, for their capacity to target and kill tumor cells.

References

1. Cairncross JG, Mattes MJ, Beresford HR, Albino AP, Houghton AN, Lloyd KO, Old LJ. Cell surface antigens of human astrocytoma defined by mouse monoclonal antibodies: Identification of astrocytoma subsets. Proc Natl Acad Sci USA 1982, 79:5641–5645
2. Rettig WJ, Garin-Chesa PG, Beresford HR, Feickert H-J, Jennings MT, Cohen J, Oettgen HF, Old LJ. Differential expression of cell surface antigens and glial fibrillary acidic protein in human astrocytoma subsets. Cancer Res 1986, 46:6406–6412
3. Rettig WJ, Spengler BA, Garin-Chesa P, Old LJ, Biedler JL: Coordinate changes in neuronal phenotype and surface antigen expression in human neuroblastoma cell variants. Cancer Res 1987, 47:1383–1389
4. Garin-Chesa P, Beresford HR, Carrato-Mena A, Oettgen HF, Old LJ, Melamed MR, Rettig WJ: Cell surface antigens of human melanoma: Immunohistochemical analysis of the gp57, G_{D3}, and mel-CSPG antigenic systems. Am J Pathol 1989, 134:295–303
5. Herlyn M, Clark WH, Rodeck U, Mancianti ML, Jambrosic J, Koprowski H: Biology of tumor progression in human melanocytes. Lab Invest 1987, 56:461–474
6. Reisfeld RA, Cheresh SA: Human tumor antigens, Advances in Immunology. Vol. 40. Edited by FJ Dixon. New York, Academic Press, 1987, pp 323–377
7. Houghton AN, Eisinger M, Albino AP, Cairncross JG, Old LJ: Surface antigens of melanocytes and melanomas: Markers of melanocyte differentiation and melanoma subsets. J Exp Med 1982, 156:1755–1766
8. Saito N, Yu RK, Cheung NKV: Ganglioside GD2 specificity of monoclonal antibodies to human neuroblastoma cells. Biochem Biophys Res Commun 1985, 127:1–7
9. Houghton AN, Scheinberg DA: Monoclonal antibodies: Potential applications to the treatment of cancer. Semin Oncol 1986, 13:165–179
10. Cohen J, Selvandran SY: A neuronal cell surface antigen is found in the CNS but not in peripheral neurons. Nature 1981, 291:421–423
11. Rettig WJ, Garin-Chesa P, Beresford HR, Oettgen HF, Melamed M, Old LJ. Cell-surface glycoproteins of human sarcomas: Differential expression in normal and malignant tissues and cultured cells. Proc Natl Acad Sci USA 1988, 85:3110–3114
12. Rettig WJ, Nishimura H, Yenamandra AK, Seki T, Obata F, Beresford HR, Old LJ, Silver J: Differential expression of the human Thy-1 gene in rodent-human somatic cell hybrids. J Immunol 1987, 138:4484–4489
13. Rettig WJ, Cordon-Cardo C, Ng JSC, Oettgen HF, Old LJ, Lloyd KO: High-molecular-weight glycoproteins of human teratocarcinoma defined by monoclonal antibodies to carbohydrate determinants. Cancer Res 1985, 45:815–821
14. Rettig WJ, Garin-Chesa P, Beresford HR, Melamed MR, Old LJ: Definition of an extracellular matrix protein in rostral portions of the human central nervous system. Brain Res 1988, 438:315–322
15. Hsu SM, Raine L, Fanger H. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 1982, 29:577–580
16. Garin-Chesa P, Rettig WJ, Melamed MR: Expression of cytokeratins in normal and neoplastic colonic epithelial cells. Am J Surg Pathol 1986, 10:829–835
17. Rettig WJ, Dracopoli NC, Garin-Chesa P, Spengler BA, Beresford HR, Davies P, Biedler JL, Old LJ. Role of human chromosome 11 in determining surface antigenic phenotype of normal and malignant cells. Somatic cell genetic analysis of eight antigens, including putative human Thy-1. J Exp Med 1985, 162:1603–1619
18. Gordon JW, Garin-Chesa P, Nishimura H, Rettig WJ, Maccari JE, Endo T, Seravalli E, Seki T, Silver J: Regulation of Thy-1 gene expression in transgenic mice. Cell 1987, 50:445–452
19. Garin-Chesa P, Rettig WJ: Immunohistochemical analysis of LNT, NeuAc2→3LNT and Le^x carbohydrate antigens in hu-

man tumors and normal tissues. Am J Pathol 1989, 134: 1315–1327
20. Garin-Chesa P, Rettig WJ, Melamed MR, Old LJ, Niman HL: Expression of $p21^{ras}$ in normal and malignant human tissues: Lack of association with proliferation and malignancy. Proc Natl Acad Sci USA 1987, 84:3234–3238
21. Miller RH, William BP, Cohen J, Raff MC. A4: An antigenic marker of neural tube-derived cells. J Neurocytol 1984, 13: 329–338
22. Rettig WJ, Old LJ: Immunogenetics of human cell surface differentiation. Ann Rev Immunol 1989, 5:481–511
23. Fox N, Damjanov I, Knowles BB, Solter D: Immunohistochemical localization of the mouse stage-specific embryonic antigen 1 in human tissues and tumors. Cancer Res 1983, 43:669–678
24. Lloyd KO: Human tumor antigens: Detection and characterization with monoclonal antibodies, Basic and Clinical Tumor Immunology. Edited by RB Herberman. Boston, Nijhoff Publishers, 1983, pp 159–214
25. Eccles JC, Ito M, Szentagothai J: The Cerebellum as a Neuronal Machine. New York, Springer Verlag, 1967.
26. McComb RD, Moul JM, Bigner DD. Distribution of type VI collagen in human gliomas: Comparison with fibronectin and glioma-mesenchymal matrix glycoprotein. J Neuropathol Exp Neurol 1987, 46:623–633
27. Jones TR, Ruoslahti E, Schold SC, Bigner DD: Fibronectin and glial fibrillary acidic protein expression in normal human brain and anaplastic human gliomas. Cancer Res 1982, 42: 168–177
28. McKeever PE, Smith BH, Taren JA, Wahl RL, Kornblith PL, Chronwall BM. Products of cells cultured from gliomas: VI. Immunofluorescent, morphometric and ultrastructural characterization of two different cell types growing from explants of human gliomas. Am J Pathol 1987, 127:358–372

Acknowledgments

The authors thank Drs. L. J. Old and M. R. Melamed for their generous support, Dr. P. Farmer for valuable contributions, Dr. J. Cohen for providing monoclonal antibodies, and K. C. Kong for excellent photographic work.