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Murine monoclonal antibodies cytotoxic to human glioma cells *in vitro*

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Summary. Six monoclonal antibodies (mABs) against human glioma cells (T2) were produced. T2 cells grown as solid tumors in nude mice, were dissociated and used to immunize Balb/c mice. After fusion of spleenocytes with myeloma cells, eight hybrids secreting mABs were selected according to their ability to react immunohistochemically with T2 cells, but not with normal adult human brain. Cytotoxicity of mABs was tested using (^{3}H)-thymidine incorporation assays *in vitro*. Four mABs showed complement-mediated cytotoxicity for T2 cells, other human glioma cells (T1), and a human melanoma cell line. Incubation with one antibody, mAb2A1, lowered (^{3}H)-thymidine incorporation in the T2 and T1 cells to ca. 10%, and in melanoma cells to ca. 35% of control levels. Another antibody, mAb3B2, displayed a similar cytotoxicity for T2 and T1 cells, but did not show measurable cytotoxicity for melanoma cells and rat primary astrocyte cultures. Moreover, this antibody did not crossreact with haematopoietic cells from patients bearing CNS tumors or normal subjects. MAb3B2, therefore, appears to recognize and epitope associated to human gliomas, will be a useful glioma tumor marker and may have some potential therapeutical value.

Key words: Monoclonal antibody, Glioma, Cytotoxicity, Tumor marker, Immunotherapy

Introduction

For many years, researchers have aimed at finding markers for tumors, and various groups have suggested the presence of specific antigens associated to neural malignant cells (Trouillas, 1971; Coakham, 1974; Wahlstroem et al., 1974). Production of monoclonal antibodies (mABs) using hybridoma technology has greatly facilitated the detection of such antigens in tumors derived from the central nervous system (Kennett and Gilbert, 1979; McComb and Bigner, 1985). Nevertheless, to our knowledge, truly brain tumor-specific antigens have not been documented. For example, mAbs to glioma crossreact with tumor cells of ectodermic origin including melanoma and neuroblastoma, and also with foetal cerebral tissue (Fischer et al., 1988).

Using a relatively new approach, we attempted to develop highly specific mAbs against human glioma cells (T2) established in our laboratory. Part of the results reported here have been published in abstract form (Sánchez-Gómez et al., 1989).

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Materials and methods

Origin and culture of tumor cells

Surgically-removed human tumoral masses were dissociated and seeded in culture dishes. Human glioma cells (T1 and T2) were isolated after several passes in culture and in nude mice. Morphological and immunohistochemical characterization of T2 is provided in the results section. Human lung carcinoma A549 was purchased from the American Tissue Culture Collection, and human melanoma Cal7 cell line was kindly provided by the Department of Cell Biology of the University of País Vasco. Murine myeloma P3U1 served both as a control for cytotoxic assays, and as a parental cell line to produce hybridomas secreting mAbs. All cells were grown in Iscove's modified Dulbeccos' medium supplemented with 10% foetal bovine serum (FBS), 500 U/ml penicillin, 5 mg/ml streptomycin and 2.5 µg/ml amphotericin B.

Primary astrocyte cultures

Rat astrocytes from neonatal brain were prepared according to McCarthy and de Vellis (1980). Briefly, cerebral cortex from 1-2 day old rats was gently dissociated with a pipette, and seeded on 75 cm^2 culture

flasks for 7 to 9 days. Most oligodendrocytes were removed by agitation, and the remaining cells were trypsinized and plated on 96-well plates at 2-4x10^5 cells/ml. Cultures were assayed with mAbs 3 to 5 days later.

Monoclonal antibody production

Solid tumors of T2 cells obtained in nude mice were gently dissociated in Hank's balanced salt solution with a pipette, and 3x10^6 cells injected intraperitoneally into a series of Balb/c mice. Animals were boosted two and four weeks later using the same procedures. Five days after the second injection, mice were bled and their sera tested. A mouse showing high titer of antibodies binding to cryostat sections of T2 solid tumor grown in nude mice (T2 sections) was further boosted, and four days later its spleen cells were hybridized with P3U1 cells using polyethylene glycol according to standard techniques (Galfré et al., 1977). Fusion products were seeded in 24-well plates and hybrids selected by adding 0.4 µM aminopterin to culture medium for 5 days. Large colonies of growing cells were observed within three weeks, and their conditioned media immunohistochemically tested on T2 sections. Selected hybrids were expanded, cloned twice by limiting dilution and frozen in FBS containing 10% dimethylsulphoxide with liquid nitrogen. Antibodies were isotyped with a mouse typer kit (Bio-Rad Laboratories, Richmond, CA) in immunohistochemical experiments with T2 sections.

Immunohistochemistry

Cryostat sections (14 µm) were obtained from adult human cerebral cortex and T2 solid tumors. The sections were mounted on poly-L-lysine coated glass slides, treated with acetone at -20° C for 10 min, washed several times in phosphate-buffered saline (PBS, 145 mM NaCl, 10 mM sodium phosphate), and incubated with hybridoma-conditioned media overnight at 4° C. Further immunohistochemical processing was performed using the avidin-biotin-peroxidase system as described by the supplier (Vectastain kit, Vector Laboratories). Antibody binding was revealed by incubations of 3-10 min with 0.05% diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris-HCl pH 7.4. Haematopoietic cells from heparinized-blood from normal subjects and glioma-bearing patients were smeared onto poly-L-lysine-coated slides, incubated with 3% H_2O_2 in PBS for 10 min, and processed as described above for cryostat sections.

As negative controls for this immunocytochemical technique, we used P3U1-conditioned medium and irrelevant monoclonal antibodies, mAb3A12 to gamma-aminobutyric acid and mAb2D7 to glutamic acid (dilution 1:1000 in PBS), both conjugated to bovine serum albumin with glutaraldehyde (Matute and Streit, 1986; Matute et al., 1987; Liu et al., 1989).

Histochemical characterization of T2 cells in culture was performed by standard immunofluorescence techniques. In these experiments, cells were seeded on sterile poly-L-lysine-coated coverslips overnight. After washing thoroughly with PBS, cells were fixed with 95% ethanol and 5% acetic acid for 15 min at 4° C. Incubation with rabbit primary antibodies, at dilution 1:100, was for 2 hours, and was followed by several PBS washes and by FITC-anti-rabbit antibody, at dilution 1:250, for an additional hour. Coverslips were mounted on glass slides with PBS:glycerol. Antisera to glial fibrillary acidic protein (GFAP), vimentin and fibronectin were purchased from Dakopatts.

Cytotoxicity assays

Tumor cells were seeded in 24-well plates at a density of 2x10^4/ml. Forty-eight hours later, supernatants were removed and cells were incubated for one hour with 250 µl of hybridoma-conditioned media followed by 500 µl of fresh medium containing 2 µCi/ml of (^{3}H)-thymidine for 14-18 hours. After washing thoroughly with PBS, cells were removed from plates, mixed with scintillation liquid and (^{3}H)-thymidine incorporation measured in an LKB counter. As negative controls, we used P3U1-conditioned medium, and monoclonal antibodies mAb3A12 and mAb2D7. Effect of mAbs on cell growth was considered significant if (^{3}H)-thymidine incorporation was less than one-third of controls.

Results

Characterization of T2 cells

T2 cells used in this study were at passes 20 to 26 in nude mice. In culture, T2 cells showed a stable fibroblast-like or stellate appearance (Fig. 1A). At this stage, expression of glial fibrillary acid protein, as revealed by immunofluorescence with a specific antiserum, was present in approximately 5-15% of the cell population, without distinguishing one morphological type from another (Fig. 1B). In addition, all T2 cells were vimentin immunopositive (Fig. 1C and 1D) and did not express immunoreactivity to fibronectin antibodies.

Immunohistochemical screening

Out of 65 supernatants from wells containing large colonies of hybrids, 9 were strongly immunoreactive and 20 moderately positive with T2 sections. From all 29 selected antibodies, 8 did not bind to adult human brain sections from cerebral cortex. The pattern of immunoreactivity in T2 sections and human cerebral cortex with one of these antibodies (mAb3B2) is shown in Fig. 2. From these 8 antibodies, only 2B6 was immunoreactive with some haematopoietic cells which were weakly stained in their membranes (data not shown).

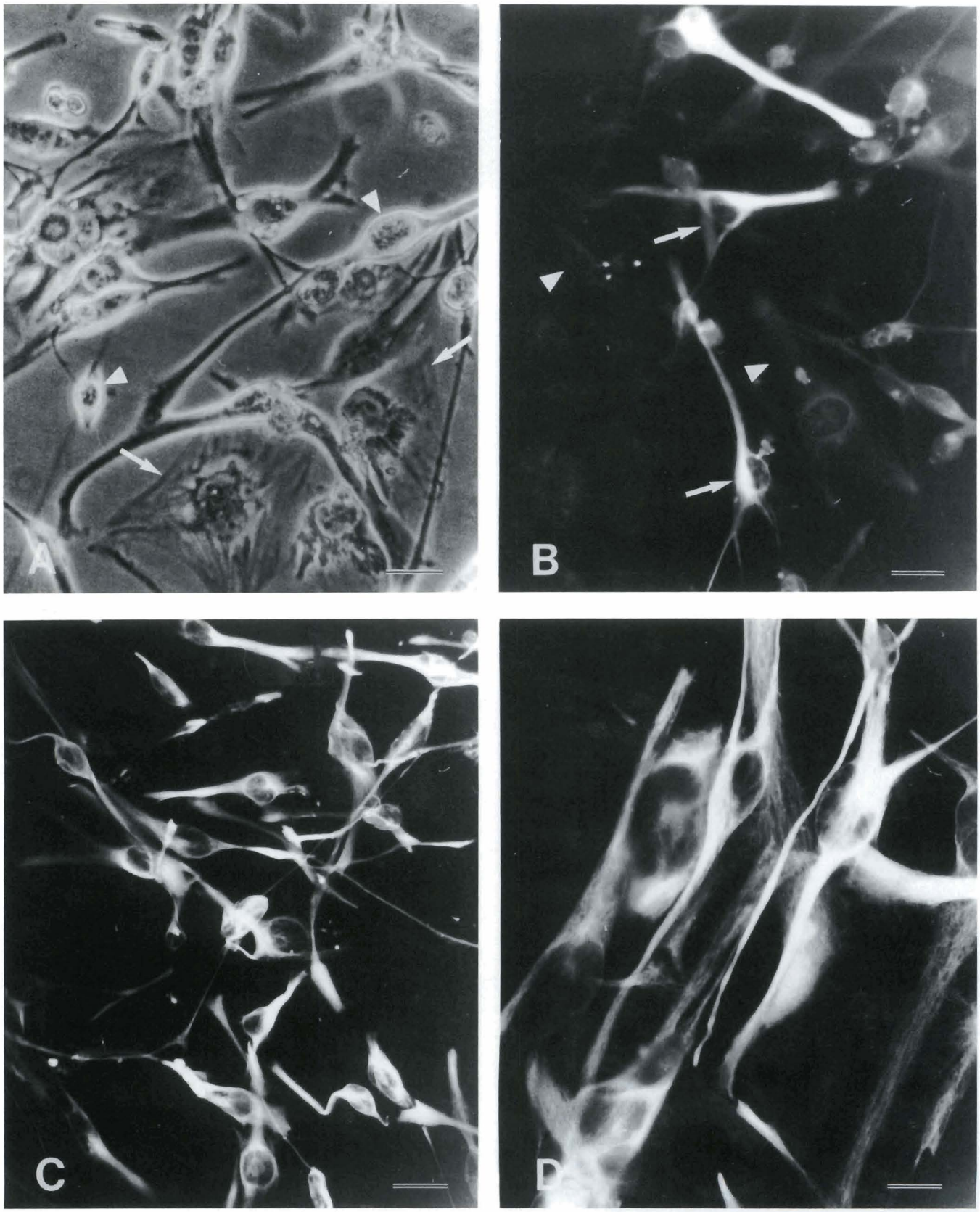

Fig. 1. Morphological and immunocytochemical appearance of T2 cells. **A.** Phase contrast microphotograph of T2 cells in culture. Note two distinct populations of cells (arrows and arrowheads). **B.** Immunofluorescence antibody staining for GFAP. Three cells are intensly labelled (arrows) while many others show background staining (arrowheads). **C and D.** Immunofluorescence antibody staining for vimentin. Abbreviation: GFAP, glial fibrillary acidic protein. Calibration bars: A: 10 μm; B and C: 15 μm; D. 7 μm

In vitro cytotoxicity of mAbs

The 8 antibodies which recognized T2 antigens, and were not reactive with human cerebral cortex, were then assayed to test their cytotoxicity with several tumor cells *in vitro* based on (^{3}H)-thymidine - incorporation measurements. Six mAbs reduced DNA synthesis by T2 cells to about 10% of controls (Fig. 3). In addition, these antibodies were cytotoxic to T1 glioma cells. However, rat primary astrocyte cultures were not significantly affected by two antibodies in the panel (mAb2A1 and mAb3B2) (Fig. 3). The antibody cytotoxic action is complement-mediated, since it was completely abolished after heat inactivation of FBS used to supplement culture media. All antibodies in the panel were of the IgM(k) class, an isotype known to efficiently fix the complement (Dillman, 1987; Fogler et al., 1989).

To check the specificity of mAbs, we tested tumor cells from other tissues. Figure 4 summarizes our findings in these experiments. MAb2A1 was cytotoxic to Cal7 human melanoma cells but not to mouse myeloma P3U1 cells (Fig. 4). On the other hand, mAb3B2 had largely no effect on Cal7 and P3U1 cells, but some toxicity to human lung carcinoma A549.

Discussion

Antibodies raised to glioma cells often crossreact with adult brain (Wikstrand and Bigner, 1979; Schnegg et al., 1981; Cairncross et al., 1982), foetal tissue or tumors from neuroectodermic origin (Wikstrand and Bigner, 1979; Fischer et al., 1988). The goal of this study was to obtain mAbs to antigens associated with human glioma cells which were not present in normal central nervous tissue, and therefore might be glioma-specific cytotoxic agents. Accordingly, antibodies were selected following two criteria. Firstly, that they should react immunohistochemically with T2 cells grown in nude mice as subcutaneous tumors and be negative with normal adult human brain. Secondly, that these mAbs should be cytotoxic to T2 cells *in vitro*. Following this paradigm, we obtained six mAbs, which were further characterized to determine their specificity.

The cells used as an immunogen, T2, do not seem to constitute a clonal cell line, since some morphological and immunohistochemical diversity was found in culture. More than 80% of the T2 cell population did not express GFAP, a fact that is common to most cell lines derived from human gliomas (Bigner et al., 1981), though some recently established cell lines show this

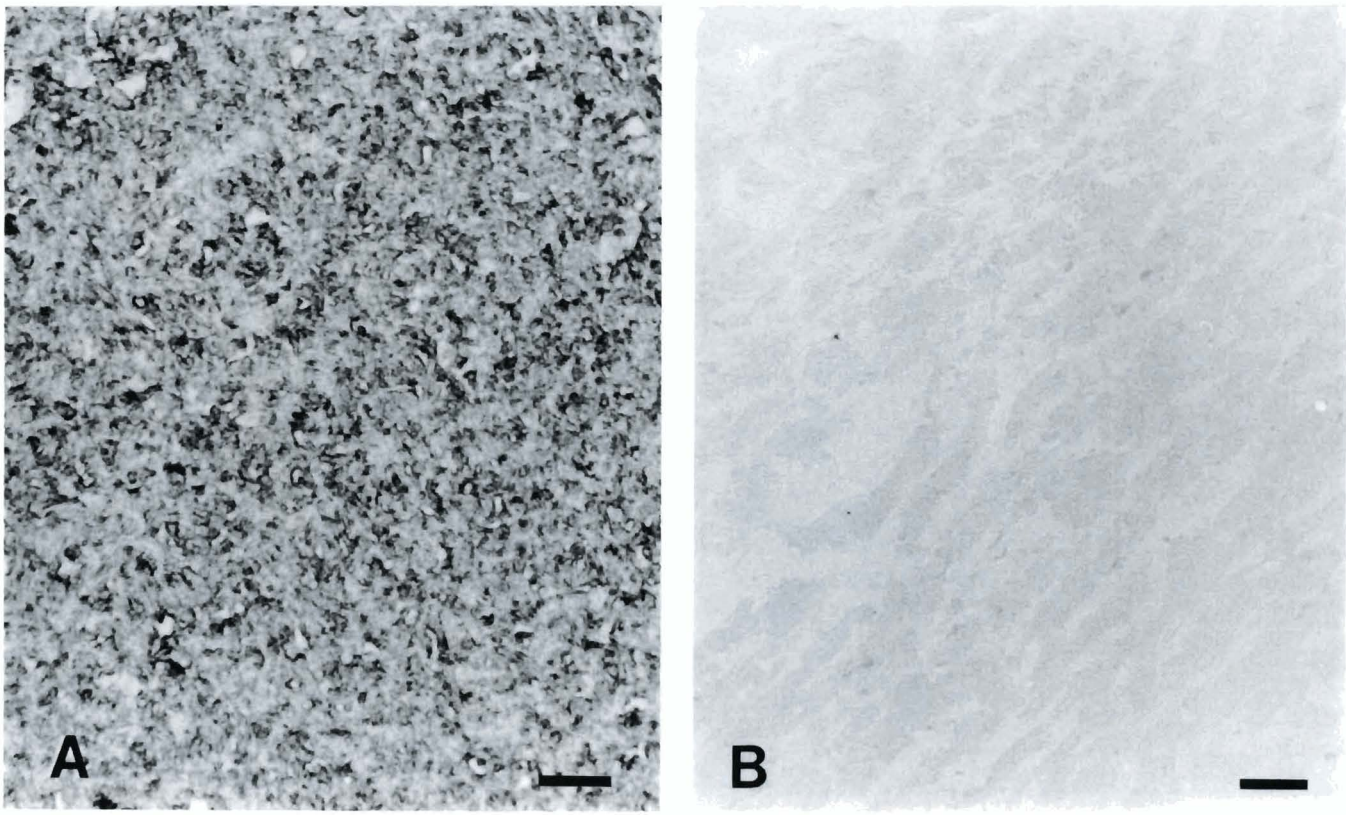

Fig. 2. Immunohistochemical labelling with mAb3B2 of cryostat sections from a T2 tumor grown subcutaneously in nude mice **(A)** and human adult normal brain **(B)**. Note the lack of stain in human brain. Calibration bar: 100 μm

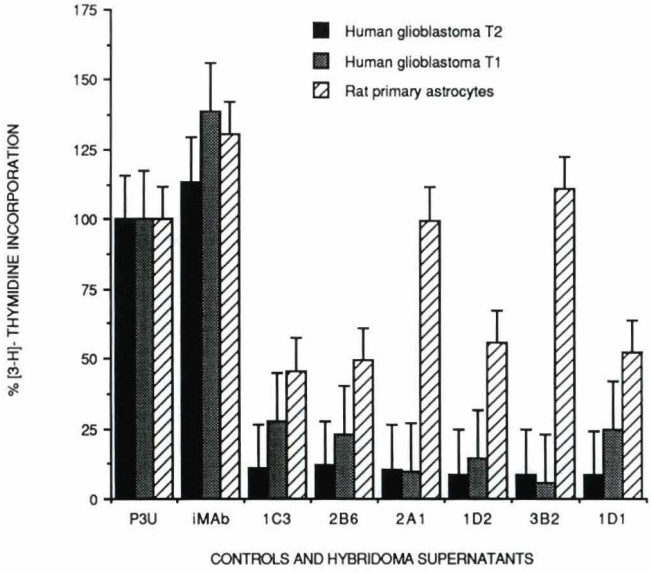

Fig. 3. Incorporation of (^{3}H)-thymidine to human glioblastoma cell lines and rat primary astrocytes, after a one-hour exposure to mAbs. Values are the average of triplicates in two separate experiments (mean ± SD) and are referred as percentage of control using conditioned supernatants by myeloma line P3U1. Abbreviations: H, human; iMAb, irrelevant monoclonal antibody.

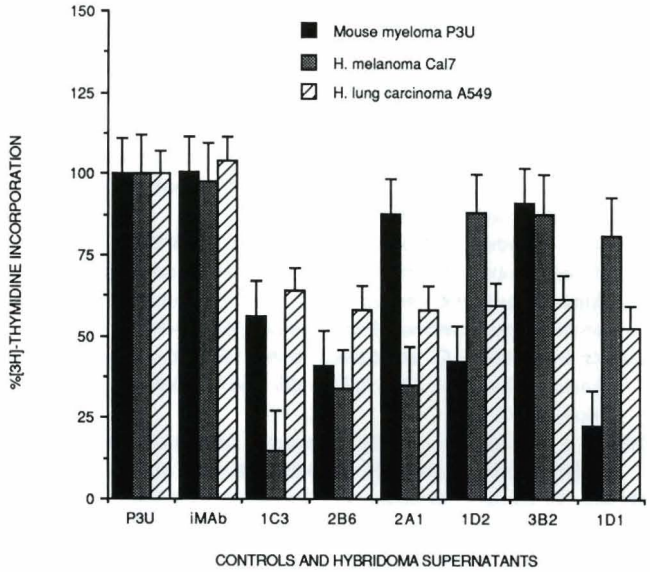

Fig. 4. Incorporation of (^{3}H)-thymidine to three different tumoral cell lines, after a one hour exposure to mAbs. Values are the average of triplicates in two separate experiments (mean ± SD) and are referred as percentage of control using conditioned supernatants by myeloma line P3U1. Abbreviations: H, human; iMAb, irrelevant monoclonal antibody.

intermediate filament (Liwnicz et al., 1986). However, the dramatic reduction in (^{3}H)-thymidine incorporation by T2 cells after incubation with all the selected mAbs in our panel suggests that some specific antigens are shared by all T2 subclones. In addition, these antigens are common to other glioma cells such as T1 (Fig. 3) and also to other human glioma tumors as determined by immunohistochemistry (data not shown).

Four of the selected mAbs appeared to recognize epitopes shared by several human glioma and also other human cell types. For instance, cytotoxic effects of mAb2B6 and its immunoreactivity indicate that there are glioma antigens present in haematopoietic or melanoma cells as observed by other investigators (Carrel et al., 1982; Wikstrand and Bigner, 1982; Nanda et al., 1989). However, antibodies 1D2 and 3B2 were not cytotoxic to melanoma cells, and showed similar discriminative binding as monoclonals selected by RIA assays (Narayan et al., 1986). MAb3B2, which does not crossreact with adult human brain, haematopoietic cells, melanoma and myelona cells nor primary astrocytes, might serve as a marker for glioma tumors. Although this antibody does not recognize epitopes in foetal rat brain, further immunohistochemical studies in human brain tissue are needed to prove that it does not react with neural onco-foetal antigens.

Some of the antibodies reported here might have some therapeutical value and might also help as valuable probes to study the biology of brain tumors.

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References

Bigner D.D., Bigner S.H., Ponten J., Westermark B., Mahaley M.S., Ruoslahti E., Herschman H., Eng L.F. and Wikstrand C.J. (1981). Heterogeneity of genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. J. Neuropathol. Exp. Neurol. 40, 201-229

Cairncross J.G., Mattes M.J., Beresford H.R., Albino A.P., Houghton A.N., Lloyd K.O. and Old L.J. (1982). Cell surface antigens of human astrocytoma defined by mouse monoclonal antibodies: Identification of astrocytoma subsets. Proc. Natl. Acad. Sci. USA. 79, 5461-5645.

Carrel S., de Tribolet N. and Gross N. (1982). Expression of HLA-DR and common acute lymphoblastic leukemia antigens on glioma cells. Eur. J. Immunol. 12, 354-357.

Coakham H. (1974). Surface antigen(s) common to human astrocytoma cells. Nature 250, 328-330.

Dillman R.O. (1987). Principles of cancer biotherapy. Raven Press. New York.

Fischer D.K., Chen T.L. and Narayan R.K. (1988). Immunological and biochemical strategies for the identification of brain tumor-

associated antigens. J. Neurosurg. 68, 165-180.

Fogler W.E., Sun L.K. and Klinger M.R. (1989). Biological characterization of a chimeric mouse human IgM antibody directed against the 17-1A antigen. Cancer Immunol. Immunother. 30, 43-50.

Galfré C., Howe S.C., Milstein C., Butcher G.W. and Howard J.C. (1977). Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 266, 550-552.

Kennett R.H. and Gilbert F. (1979). Hybrid myelomas producing antibodies against a human neuroblastoma antigen present on fetal brain. Science 213, 1120-1121.

Liu C.J., Grandes P., Matute C., Cuenot M. and Streit P. (1989). Glutamate-like immunoreactivity revealed in rat olfactory bulb, hippocampus and cerebellum by a monoclonal antibody and sensitive staining method. Histochemistry 90, 427-445.

Liwnicz B.H., Archer G. and Soukup S.W. (1986). Continuous human glioma-derived cell lines UC-11MG and UC-302MG. J. Neurol. Oncol. 3, 373-385.

Matute C. and Streit P. (1986). Monoclonal antibodies demonstrating GABA-like immunoreactivity. Histochemistry 86, 147-157.

Matute C., Liu C.J., Grandes P., Cuenot M. and Streit P. (1987). Glutamate-like immunoreactivity revealed in rat brain by monoclonal antibody and sensitive staining method. Soc. Neurosci. 13, 1562 (Abstract).

McCarthy K.D. and de Vellis J. (1980). Preparation of separate astroglial and oligodendroglial cultures from rat cerebral tissue. J. Cell Biol. 85, 890-902.

McComb R.D. and Bigner D.D. (1985). Immunolocalization of monoclonal antibody-defined extracellular matrix antigens in human brain tumors. J. Neurol. Oncol. 3, 181-186.

Nanda A., Liwnicz B. and Atkinson B.F. (1989). Monoclonal antibodies with cytotoxic reactivities against human gliomas. J. Neurosurg. 71, 892-897.

Narayan R.K., Heydorn W.E. and Creed G.J. (1986). Protein patterns in various malignant human brain tumors by two dimensional gel electrophoresis. Cancer Res. 46, 4685-4694.

Sánchez-Gómez M.V., Conde-Guerri B., Sinués Porta E., Doñate-Oliver F. and Matute C. (1989). Monoclonal antibodies to a human glioblastoma cell line inhibiting proliferation in vitro. Eur. J. Neurosci. (Suppl 2), 19. (Abstract).

Schnegg R.C., Diserens A., Carrel S., Acolla R.S. and de Tribolet N. (1981). Human glioma-associated antigens detected by monoclonal antibodies. Cancer Res. 41, 1209-1213.

Trouillas P. (1971). Carcino-fetal antigen in glial tumors. Lancet, 2, 552.

Wahlstroem T., Linder E. and Saksela E. (1974). Tumor-specific membrane antigens in established cell lines from gliomas. Cancer 34, 274-289.

Wikstrand C.J. and Bigner D.D. (1979). Surface antigens of human glioma cells shared with normal adult and fetal brain. Cancer Res. 39, 3235-3243.

Wikstrand C.J. and Bigner D.D. (1982). Expression of human fetal brain antigens by human tumors of neuroectodermal origin as defined by monoclonal antibodies. Cancer Res. 42, 267-275.

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