Surface Antigenic Characteristics of Human Glial Brain Tumor Cells

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SUMMARY

The surface antigenic characteristics of human glial brain tumor (HGBT) cells were studied by complement-dependent cytotoxic antibody assays and indirect membrane immunofluorescence. Eight permanent, well-characterized cell lines derived from human gliomas were used for analysis with antisera raised by hyperimmunization of nonhuman primates (Macaca fascicularis) with glioblastoma multiforme tissue or established HGBT cell lines. Exhaustive absorption of these antisera to remove predominantly anti-species activity rendered HLA nonreactive "preabsorbed" antisera, which reacted with a large panel of gliomatous and nongliomatous human tumor cells: 1 carcinoma, 2 sarcomas, 2 melanomas, 1 neuroblastoma, and 8 HGBT cell lines. Four lymphoblastoid lines and 2 carcinomas were unreactive. After further absorption with a human osteogenic sarcoma cell line, the antisera demonstrated significant levels of reactivity for 8 tested HGBT cell lines and no longer reacted with the nongliomatous cultured tumor cell lines. Therefore, extensive absorption of nonhuman primate anti-human glioma sera removed all activity for the nongliomatous cell lines tested, but it left significant reactivity against a glial tumor cell line-associated antigen(s) present on all 8 human glioma cell lines tested.

INTRODUCTION

Although several investigators have reported the humoral, cellular, or delayed cutaneous hypersensitivity reaction of brain tumor patients to various glioma tissue preparations, cultured glioma cells or glioma tissue, few attempts have been made to characterize the surface antigenic characteristics of malignant human glioma cells systematically. By the use of a heterologous rabbit anti-astrocytoma serum, Coakham (13) and Coakham and Lakshmi (14) described an apparently human astrocytoma-specific surface antigen; absorption of the complement-dependent cytotoxic antiserum with normal human adult or fetal brain homogenate or with human nonglial normal tissue did not reduce the cytotoxic titer. In contrast, Wahlstrom et al. (56) demonstrated antigens common to normal brain and glioblastoma multiforme cells in addition to apparent unique antigens common to cultured glioma cell lines detected in an indirect immunofluorescence assay with a rabbit antiserum raised against lyophilized human glioblastoma multiforme tissue (55).

We have investigated surface antigenic characteristics of human glioma cells with antisera raised in nonhuman primates to glioblastoma multiforme tissue or to cultured gliomatous cell lines. Immunization of nonhuman primates had previously been used to raise antisera that could distinguish human acute and chronic lymphocytic leukemia cells from those of acute myelogenous and chronic granulocytic cells (38-40); rabbit antiserum prepared by identical immunizations were simply broadly group reactive (7, 8, 15, 40). Similarly, production of operationally melanoma-specific antibody by immunization of monkeys with human melanoma cell lines (36) or melanoma tissue (52) has been reported. Because non-human primate antiserum can be carefully and extensively absorbed and tested for nonspecific or cross-reactive antibody activity, we have begun analysis of well-characterized, Mycoplasma-free, HLA-typed cultured human tumor cell lines of glial and nonglial origin with analytically absorbed nonhuman primate antisera. Results reported here demonstrate that the initial and continually predominant nonhuman primate antibody response to human glioblastoma multiforme tissue or cells is antispecies in nature, as detected by absorption analysis and direct testing for anti-HLA reactivity. Protocols involving multiple immunization have resulted in the production of antisera that, after extensive absorption for removal of the predominant antispecies activity, exhibit a reactivity profile, defined by sequential absorption analysis, that includes both reactivity for nonglial human tumor cells and residual reactivity associated with human glioma cells.

MATERIALS AND METHODS

Cell Lines Derived from Gliomas and Nonglial Tumors. The established permanent human tumor cell lines used throughout the work are described in Table 1. The possibility of HeLa cell contamination (32, 41) was excluded by glucose-6-phosphate dehydrogenase and phosphoglucomutase isoenzyme analyses (23, 51); phosphoglucomutase isotypes 1 and 3 for several of our lines have been published (3). Lack of intraspecies contamination of glial tumor cell
lines was determined by demonstration of their individually unique HLA antigen profiles (Table 1). The tumorigenicity of the established human tumor cell lines in congenitally thymus-deficient "nude" mice (genotype nu/nu NIH Swiss) was evaluated by the injection of up to 3 x 10^7 cultured cells s.c. After latency periods of 4 to 10 weeks, 2 of the gliomatous cell lines (D-54 MG and U-251 MG) and 3 of the nonglial tumor cell lines (PB, T-8, and KENT) showed progressive neoplastic growth in 11 of 16 injected nudes (6). The remaining cell lines are currently under study for tumorigenicity in nude mice.

The demonstrated partial cross-reactivity between antigens of normal brain and Mycoplasma (5) necessitated that all cell lines be checked on arrival and at every 10th passage level for Mycoplasma infection by inoculation of both broth and semisolid Mycoplasma media with scraped cells and cell culture supernatants from cultures grown longer than 3 days in antibiotic-free medium (2). None of the lines used in the studies reported here were Mycoplasma contaminated.

All cell lines, with the exception of suspension cultures Roswell Park Memorial Institute 1788, Jiyoye, Raji, and EB-3, were grown in monolayer culture in Richter's (45) improved minimal essential ZO4 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10 to 20% heat-inactivated FCS, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 584 mg glutamine per liter, and 50 μg gentamicin sulfate per ml. Suspension cultures were initially grown in Roswell Park Memorial Institute Medium 1640 supplemented with 10% FCS and additional glutamine and gentamicin; all have been converted successfully to growth in 10% FCS-ZO. To minimize any possible variation in cell properties that might occur with long-term culture, we frequently froze large quantities of cells at a given passage level in 10% dimethyl sulfoxide-ZO (v/v) for later use.

Production of Antisera. Nonhuman primates of the species Macaca fascicularis were used. A surgically removed human glioblastoma (right parietal lobe) was used to prepare cell suspensions of viable tumor cells (WR-GBM) by gentle teasing and resuspension through a syringe. Replicate samples of 0.5 mg were viably frozen in cell culture medium with 20% AB+ serum and 10% dimethyl sulfoxide and stored in liquid nitrogen for subsequent immunization. Monkey M5-4 was given an initial dose of 250 mg WR-GBM s.c. in 8 sites over the chest and outer aspects of the extremities. Thirty days later an identical dose in 1 ml serum-free medium plus 1 ml H37 Ra CFA (Difco Laboratories, Detroit, Mich.) was given i.d. in the same sites. The animal was bled 7 and 14 days after each subsequent monthly immunization of 250 mg WR-GBM (immunizations 1 to 7) and of 125 mg WR-GBM (immunizations 8 to 11); the final immunization again was administered with an

Table 1

Permanent human tumor cell lines used in this study*  

<table>
<thead>
<tr>
<th>Cell line designation*</th>
<th>Diagnosis of tumor of origin</th>
<th>Passage level</th>
<th>Laboratory of origin or source</th>
<th>HLA specificities expressed†</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-18 MG</td>
<td>Glioblastoma multiforme</td>
<td>86</td>
<td>Bigner (6)</td>
<td>A1, 3, B8</td>
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<tr>
<td>D-37 MG</td>
<td>Glioblastoma multiforme</td>
<td>91</td>
<td>Bigner (6)</td>
<td>A1, 2</td>
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<tr>
<td>A-172 MG</td>
<td>Glioblastoma multiforme</td>
<td>129</td>
<td>Toddaro (19)</td>
<td>A1, w24, B7, 8</td>
</tr>
<tr>
<td>D-54 MG</td>
<td>Mixed anaplastic glioma</td>
<td>60</td>
<td>Bigner (6)</td>
<td>A1, 3, w30, 31</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>Anaplastic glioma</td>
<td>555</td>
<td>Pontén (44)</td>
<td>A1, 2, 3, w24</td>
</tr>
<tr>
<td>U-373 MG</td>
<td>Anaplastic glioma</td>
<td>316</td>
<td>Pontén (44)</td>
<td>A1, B12</td>
</tr>
<tr>
<td>U-105 MG</td>
<td>Anaplastic glioma</td>
<td>307</td>
<td>Pontén (44)</td>
<td>A2, w24</td>
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<tr>
<td>U-343 MG</td>
<td>Anaplastic glioma</td>
<td>220</td>
<td>Pontén (44)</td>
<td>A2, 3, B5</td>
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<tr>
<td>PB</td>
<td>Bladder carcinoma</td>
<td>462</td>
<td>Levy (33)</td>
<td>NT</td>
</tr>
<tr>
<td>T-24</td>
<td>Bladder carcinoma</td>
<td>50</td>
<td>Bubenik (10)</td>
<td>NT</td>
</tr>
<tr>
<td>DU-145</td>
<td>Prostatic carcinoma</td>
<td>63</td>
<td>Mickey‡</td>
<td>NT</td>
</tr>
<tr>
<td>RD</td>
<td>Rhabdomyosarcoma</td>
<td>56</td>
<td>ATCC CCL 136</td>
<td>NT</td>
</tr>
<tr>
<td>2-T</td>
<td>Osteogenic sarcoma</td>
<td>410</td>
<td>Wahlström (55)</td>
<td>A2</td>
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<tr>
<td>T-8</td>
<td>Melanoma</td>
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<td>Levy (33)</td>
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</tr>
<tr>
<td>KENT</td>
<td>Melanoma</td>
<td>67</td>
<td>Levy (33)</td>
<td>NT</td>
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<tr>
<td>RPMI 1788</td>
<td>IgM-secreting lymphoblastoid line</td>
<td>Unknown</td>
<td>ATCC CCL 156</td>
<td>A2, B7</td>
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<td>Jiyoye</td>
<td>Burkitt lymphoma</td>
<td>57</td>
<td>ATCC CCL 87</td>
<td>NT</td>
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<td>Raji</td>
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<td>EB-3</td>
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<td>ATCC CCL 85</td>
<td>NT</td>
</tr>
<tr>
<td>IMR-32</td>
<td>Neuroblastoma</td>
<td>47</td>
<td>ATCC CCL 127</td>
<td>NT</td>
</tr>
</tbody>
</table>

* In addition to the permanent cell lines listed, we have 10 meningioma cell lines of finite in vitro life span in liquid nitrogen storage, and we have available for testing frozen stock of passages 1 to 4. Similarly, 2 normal adult brain cell lines of finite in vitro life span are also available.

† HLA typing and interpretation by the standard AO-VA technique (24, 57), generously performed by Dr. Metzgar, Dr. Mohanakumar, and Dr. Ward. NT, not tested.

‡ D. D. Mickey, personal communication.

* Cell lines obtained from the American Type Culture Collection, Rockville, Md.

The abbreviations used are: ZO, zinc option medium described in text; FCS, fetal calf serum; WR-GBM, whole glioblastoma multiforme tissue used for immunization; CFA, complete Freund's adjuvant; i.d., intradermal; MCT, microcytotoxicity test; IMIF, indirect membrane immunofluorescence; FI, fluorescent index; PBL, peripheral blood leukocytes.
equal volume of CFA, for a total of 2.25 g WR-GBM per 11 immunizations. Monkey M5-30 was given an initial multiplets site inoculation of 8 x 10⁶ U-251 MG cells s.c., which were freshly grown and were harvested by scraping, followed 30 days later by a 2nd i.d. injection of 2 x 10⁶ cells in CFA. Monthly immunizations and bleedings were performed as with M5-4, for a total of 17 x 10⁶ U-251 MG cells per 12 immunizations. Incomplete medium (without serum) or Dulbecco’s phosphate-buffered saline [8% NaCl, 0.02% KCl, 0.02% KH₂PO₄, and 0.22% Na₂HPO₄.2H₂O (calcium and magnesium free; Grand Island Biological Co.)] was used for all manipulations involving immunizing materials, to avoid induction of antibodies to FCS (25).

Absorption of Antisera. All absorptions were performed under aseptic conditions. Antisera were inactivated by heating at 56°C for 30 min and absorbed on an equal volume of washed, packed human AB+ erythrocytes until they could no longer agglutinate these cells (40). Further absorption of antispecies activity was performed by six 1-hr incubations at room temperature of RBC-absorbed antisera with crude human cadaveric spleen homogenate at a ratio of 0.5 ml antiserum to 250 mg (wet weight) homogenate per absorption; this procedure was also used for absorption with WR-GBM frozen tumor material (two 1-hr absorptions).

Extensive absorption and backtesting were required to render the sera devoid of anti-HLA reactivity. Procedures used for HLA absorption and testing are detailed in "Results."

Antisera were absorbed with cultured cell lines at a ratio of 1 x 10⁶ washed, packed cells per ml serum for 1 hr at room temperature and then overnight at 4°C (12). All antisera were ultracentrifuged at 26,384 x g for 1 hr after absorption to remove anticomplementary activity, treated with sodium azide (final concentration, 0.02%), and kept at 4°C for immediate testing or stored at −70°C for later testing. The effectiveness of absorption on nonglial cell lines and the detection of remaining glial cell reactivity were monitored by a modified version of the trypsin blue dye exclusion MCT, AO-VA method (24, 57). The only necessary alterations for monitoring glial cell cytotoxicity in the leukocyte assay were (a) the use of 20% FCS-ZO medium for suspension of 0.02% EDTA-harvested glial cells, (b) an increase in cell concentration to 2 x 10⁶ cells/ml, and (c) gentle aspiration of wash volumes.

IMIF Assay. The indirect live-cell membrane immunofluorescence assay has been described (12, 21). The secondary antiserum used was a fluorescein-conjugated rabbit anti-monkey IgG (Cappel Laboratories, Downingtown, Pa.). The only modification introduced for cultured human glial cell lines was the reduction of target cell number to 1.5 x 10⁶ cells/microtiter plate well (Falcon Plastics, Oxnard, Calif.) for reaction with 30 μl of various dilutions of primary antisera. A minimum of 200 cells/test well were scored for membrane immunofluorescence under dark-field UV with a Zeiss universal microscope with a VZ condenser, BG38 excitation, KP500 interference, and 50 barrier filters. FI’s per antiserum dilution were calculated as

\[ \text{FI} = \frac{(A - B)}{A} \times 100 \]

where A is the percentage of nonfluorescing cells with preimmune serum and B is the percentage of nonfluorescing cells with antiserum (29).

[^14C]Nicotinamide Release Assay. The [¹⁴C]nicotinamide assay (31) has been fully described (9); only minor variations of the method were introduced for use with cultured human glial tumor cells. Briefly, 1 to 10 x 10⁶ 0.125% trypsin-0.02% EDTA-harvested human glial tumor cells were seeded in each well of a Terasaki test plate (Falcon Plastics No. 3034) in 10 μl complete ZO and 20 μCi [¹⁴C]nicotinamide (60 μCi/mmol; Amersham/Searle Corp., Arlington Heights, III.) and allowed to reach confluence by an 18- to 36-hr incubation at 37°C. After removal of unincorporated label, 5-μl quantities of appropriate serum dilutions were added, followed by a 15-min incubation at 37°C, addition of rabbit complement, another 15-min incubation, and supernatant sampling to assess [¹⁴C]nicotinamide release; cpm were determined in a Beckman LS-333 liquid scintillation counter. Maximum release was determined by the addition of 10 μl of 0.5% Triton X (Roehm & Haas Co., Philadelphia, Pa.) to control wells; background release was determined by complement and medium controls. The percentage of specific [¹⁴C]nicotinamide release was determined by the formula

\[ \frac{\text{test} - \text{background} \times 100}{\text{max} - \text{background}} \]

Results were then expressed as the percentage of specific release based on the medium and complement control; as established in the murine system, specific release ≥20% was considered significant (9).

RESULTS

Removal of Residual Anti-HLA Activity from Antisera Preliminarily Absorbed with RBC and Spleen Homogente. After preliminary absorption of the nonhuman primate antisera with human RBC (5 to 6 times) and spleen homogenate (6 times) for removal of antispecies reactivity, both the antisera significantly reacted by MCT with a subset of PBL samples from a panel of 10 donors representing a population cross-section of HLA antigens. The 4 reacting PBL populations shared a common specificity, HLA-A2, which indicated that the observed serum activity was probably directed against HLA antigens. Because the HLA type of the patients from whom brain tumor material was excised was known and our permanent cell lines had been HLA typed, selective HLA absorption based on prediction of the reactivity in the primate antisera was possible. However, as demonstrated by Fritze et al. (17) and observed with our cell lines (Table 1), accurate HLA typing of cultured cell lines is complicated by (a) the weak reactivity of HLA antisera derived from multiparous women or the possible recognition by anti-"human la" or anti-Fc receptor-reactive components in HLA-typing sera of such depressed specificities in cultured glial cells (7, 18, 46). Therefore, both antisera were absorbed on PBL of HLA-typed volunteers chosen to represent a broad spectrum of known...
HLA specificities. Buffy coat leukocytes were prepared as described by Mohanakumar et al. (40); twice in succession, sera were absorbed at room temperature at a ratio of 10^6 cells/ml for 1 hr (total exposure, 2 x 10^6 cells). The completeness of HLA absorption of test antisera was determined by MCT; final determination of the absence of HLA-specific reactivity by the more sensitive and antiglobulin method (28) was kindly performed in the laboratory of Dr. Frances Ward. Absorbed antisera were tested against a representative HLA panel and were not used for testing until they were nonreactive on such a panel; antiserum absorbed and tested thusly were designated "preabsorbed sera."

Preliminary Analysis of Primate Antiseras. The anti-WR-GBM serum and the anti-cell culture line U-251 MG serum (anti-MG 251) were initially screened for reactivity against U-251 MG target cells after sequential absorptions with human RBC, human spleen homogenate, and human peripheral WBC. Results of [3H]nicotinamide analysis are presented in Charts 1 and 2. Although sera obtained after immunizations 1 to 6 were largely unreactive with glioma target cells after removal of antispecies and anti-HLA activity, those obtained after immunizations 7 to 12 retained anti-glioma target cell activity when they were HLA unreactive. The predominant activity even in late-bleed antisera, however, was antispecies in nature (Chart 1); unabsorbed anti-WR-GBM serum (11th bleeding) contained significant reactivity for the glioma cell line U-251 MG with a 50% cytotoxic titer of approximately 1/160. A fall in serum titer (50% cytotoxic titer, approximately 1/12) was observed after extensive absorption with human AB+ RBC and human spleen homogenate and human WBC; significant reactivity remained after further absorption with 2-T sarcoma cells (50% cytotoxic titer, approximately 1/4). Absorption of anti-WR-GBM serum with the immunizing material (WR-GBM; twice) removed all antibody activity. Similarly, analysis of late-bleed (9th bleeding) anti-MG 251 serum revealed that the initial high titer for U-251 MG cells found with unabsorbed antiserum (50% cytotoxic titer, 1/2000) was largely antispecies in nature; the titer of anti-MG 251 serum after preabsorption and 2-T sarcoma cell absorption was not appreciably different (50% cytotoxic titer, approximately 1/12) from the analogous titer for anti-WR-GBM serum.

Reactivity of Primate Antiseras Anti-WR-GBM and Anti-MG 251 for Cultured Human Glioma Cells as Determined by MCT. The MCT was found to be the most serologically economical and rapid means of determining the reactivity profile of absorbed sera for a relatively large panel of target cells. Therefore, the MCT was used extensively to monitor the progress of serum absorption. Glioma and nonglioma monolayer-cultured human tumor cells, however, were not as suitable a target cell population in MCT assays as were PBLS, the cell population for which the assay was designed. Although background cell death of PBLS in medium and complement control wells was routinely <5% in our studies, analogous background levels with monolayer-cultured target cells were occasionally >10 to 15%, although the viability of the starting test cell suspension was ascertained to be >95%. Because of this relatively high background, only tests with background cell death levels <15% were used, and the data presented corrected for background cell death. Because data obtained with the MCT are subjective, the data are presented as the approximate percentage kill in the presence of test antiserum and complement. On the basis of MCT analysis, serum obtained 14 days after the 11th immunization with WR-GBM tissue and serum obtained 7 days after the 9th immunization with cultured gliomatous cell line U-251 MG were selected for all analyses reported here.

The reactivity profiles of sera anti-WR-GBM and anti-MG 251 for a panel consisting of 9 nongliomatous and 6 gliomatous cultured human tumor cell lines are presented in Charts 3 and 4. Serial 2-fold dilution titrations (1/2 to 1/22)
Surface Antigens of Human Glioma Cells

Chart 3. Reactivity of nonhuman primate anti-WR-GBM serum at a dilution of 1/2 for cultured human tumor cells of gliomatous and nongliomatous origin as measured by trypan blue dye exclusion MCT. A, reactivity after exhaustive absorption on human RBC (5 times), spleen homogenate (6 times) and WBC (2 times) until HLA unreactive (pre-absorbed antiserum (Pre-abs)); B, reactivity of preabsorbed antiserum after absorption on 2-T osteogenic sarcoma cells (2 times).

Chart 4. Reactivity of nonhuman primate anti-MG 251 serum at a dilution of 1/2 for cultured human tumor cells of gliomatous and nongliomatous origin as measured by trypan blue dye exclusion MCT. A, reactivity after exhaustive absorption on human RBC (5 times), spleen homogenate (6 times) and WBC (2 times) until HLA unreactive (pre-absorbed antiserum (Pre-abs)); B, reactivity of preabsorbed antiserum after absorption on 2-T osteogenic sarcoma cells (2 times).

were performed; for comparison the percentage of stained cells at a serum dilution of 1/2 is shown. Because both the preabsorbed antisera showed high levels of reactivity for glioma cell lines and a wide panel of nonglial human tumor cell lines, most notably the osteogenic sarcoma cell line 2-T, aliquots of preabsorbed anti-WR-GBM and anti-MG 251 were absorbed with 2-T. Absorption with 2-T cells removed all antibody activity for the nonglial tumor cell lines tested, leaving significant levels of antibody reactivity for the 6 gliomatous cell lines tested. Absorption with the immunizing material (WR-GBM) removed all detectable antibody activity for all tumor target cell lines.

Reactivity of Primate Antisera Anti-WR-GBM and Anti-MG 251 for Cultured Human Gliomatous Cells as Determined by [12^C]Nicotinamide Assay. The patterns of reactivity defined for the anti-WR-GBM and anti-MG 251 sera by MCT were extended and substantiated by the more sensitive and reproducible [14^C]nicotinamide assay (Charts 5 and 6). Preabsorbed anti-WR-GBM serum showed significant reactivity (>20% specific [14C] release) for the entire panel of glial and nonglial target tumor cells. In contrast to MCT test results, anti-WR-GBM serum also showed significant reactivity for D-54 MG cells by [14C]nicotinamide assay. Absorption with 2-T osteogenic sarcoma cells removed all significant reactivity for nongliomatous cell lines, including the neuroblastoma cell line IMR-32. Absorption of anti-WR-GBM serum with the immunizing material (WR-GBM) removed all antibody activity for gliomatous lines.

Results of similar [14C]nicotinamide analysis of the activity of anti-MG 251 serum for the panel of glioma and nonglial cell lines is presented in Chart 6; significant reactivity with target cell lines of glioma and nonglial origin was present in preabsorbed anti-MG 251 serum; absorption with the osteogenic sarcoma tumor cell line 2-T removed activity for the nonglial tumor cell lines, leaving significant levels of antibody reactivity for the 6 gliomatous cell lines tested. Absorption with the immunizing U-251 MG cell line removed all detectable antibody activity for all tumor target cell lines.

Chart 5. Reactivity of nonhuman primate serum anti-WR-GBM for cultured human tumor cells of gliomatous and nongliomatous origin as measured by [14C]nicotinamide release assay. O and △, reactivity after exhaustive absorption on human RBC (5 times), spleen homogenate (6 times), and WBC (2 times) until HLA unreactive (preabsorbed antiserum (Pre-abs)); △ and ▽, reactivity of preabsorbed antiserum after absorption on 2-T osteogenic sarcoma cells (2 times); ◊, reactivity of anti-WR-GBM serum after absorption with the immunizing material, WR-GBM.
Reactivity of Primate Sera Anti-WR-GBM and Anti-MG 251 for Cultured Human Glioma Cells as Determined by IMIF. The binding of sera anti-WR-GBM and anti-MG 251 after preabsorption and osteogenic sarcoma 2-T cell absorption (2 times) was measured by IMIF (Table 2; Fig. 1). The 2-T osteogenic sarcoma-absorbed antisera were not bound by a carcinoma (PB), the tested nongliomatous cell lines, or the absorbing sarcoma cell line 2-T; all 5 glioma cell lines tested at the same dilution showed FI's >90%, with high-intensity staining of typical membrane antigens. The intensity of staining was uniform within a cell population; in no case was capping observed (Fig. 1).

Chart 6. Reactivity of nonhuman primate serum anti-MG 251 for cultured human tumor cells of gliomatous and nongliomatous origin as measured by [%H]nicotinamide release assay. O and △, reactivity after exhaustive absorption on human RBC (6 times), spleen homogenate (6 times), and WBC (2 times) until HLA unreactive (pre-absorbed antiserum) for the designated target cell; ● and △, reactivity of preabsorbed antiserum after absorption on 2-T osteogenic sarcoma (2 times); ○, reactivity of anti-MG 251 serum after absorption with the immunizing cell line, U-251 MG (2 times).

Table 2
Analysis of the activity of monkey Anti-WR-GBM serum and monkey Anti-MG 251 serum for cell lines of nonglial and glioma origin as determined by IMIF

Test antisera were compared in IMIF assay at a dilution of 1/2. The absorption profile of both the test antisera is as follows. The standard preabsorption regimen consisted of absorptions of human erythrocytes (5 to 6 times), human spleen homogenate (6 times), and PBL (2 times). Absorption on 2-T osteogenic sarcoma cells at a density of 10^5 cells/ml of serum for 1 hr at room temperature, followed by overnight absorption at 4°C, was done 2 times.

Reactivity for target cells (FI)*

<table>
<thead>
<tr>
<th>Antisera exhaustively preabsorbed and absorbed with 2-T osteogenic sarcoma cells</th>
<th>Non-glial origin</th>
<th>Glioma origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB 2-T T-8 RPMI*</td>
<td>D-37 MG D-54 MG U-251 MG U-105 MG</td>
</tr>
<tr>
<td>Anti-WR-GBM Bleeding 11</td>
<td>4 1 0 0 0</td>
<td>99 100 99 100 100</td>
</tr>
<tr>
<td>Anti-MG 251 Bleeding 9</td>
<td>3 2 0 0 0</td>
<td>100 100 100 100 99</td>
</tr>
</tbody>
</table>

* FI = [(A - B)/A] x 100, where A is the percentage of nonfluorescing cells with preimmune serum and B is the percentage of nonfluorescing cells with antiserum (29).

* RPMI, Roswell Park Memorial Institute.
DISCUSSION

The few published and unpublished reports of human glioma-associated tumor antigens (13, 14, 30, 43, 50, 55) have not definitively considered whether spontaneous glial brain tumors possess qualitatively or quantitatively distinctive surface antigens. We have begun a carefully controlled, systematic immunological investigation of the surface antigenic characteristics of cultured human gliomatous cell lines. Such an investigation requires (a) a large cell line panel well-characterized by species- and organ-specific markers and demonstrably free of HeLa cell and Mycoplasma contamination; (b) antisera of sufficiently high titer to permit exhaustive absorption analysis, (c) analysis of antibody activity performed through a series of well-controlled, sequential, and exhaustive absorptions, and (d) more than 1 measure of antibody activity.

The necessity for extensive antibody absorption is well demonstrated in Charts 1 and 2. The predominant antibody activity present was antispecies (antihistocompatibility antigen) in nature, as shown by the diminution in titer after absorption with human RBC, spleen homogenate, and WBC. This was especially true of early (3rd through 6th bleeding) serum samples in which little or no antibody activity remained for target gliomatous and nongliomatous tumor cell lines after exhaustive absorption to remove HLA-reactive antibody. Subsequent (7th through 13th bleeding) preabsorbed serum samples retained significant levels of antibody activity for target cells; this was similar to the length of immunization protocol required to produce operationally specific chimpanzee anti-melanoma antibodies (52).

It is not possible at this stage to compare the titers or reactivity profiles of the 2 antisera. The increase in antibody activity observed after immunization with U-251 MG (Charts 1 and 2) was attributable to an increase in antibody activity removable by thorough absorption with a panel of normal human peripheral WBC. This is consistent with the report by Metzgar and Miller (37), demonstrating the ease of anti-HLA antibody induction in nonhuman primates and the frequent positive reactivity of cultured human cell lines for more specificities than are required to yield a "full house" with standard HLA-typing antisera (17). The demonstration of high levels of antispecies and histocompatibility activity in hyperimmune anti-WR-GBM and anti-MG 251 sera necessitated that any determination of specific antibody reactivity proceed with HLA-unreactive antiserum (preabsorbed antiserum). Of all cultured tumor cell lines tested, only the lymphoblastoid lines were unreactive with preabsorbed serum. The evidence for such broadly cross-reactive "phase-specific" or "oncofetal" antigens is extensive, including the well-described carcinoembryonic antigen (20), a-fetoprotein (1), ß-oncofetal antigen (16), and an oncofetal antigen common to cultured melanoma, breast carcinoma, sarcoma, normal skin, normal muscle, and normal 22-week fetal brain cell lines (26). Similarly, recent reports of serologically detected antigens common to tumor and normal tissue (4) or shared by sarcomas and carcinomas (47) can be accounted for by the expression of normally repressed gene products.

The reactivity of both the preabsorbed antiserum for a large panel of monolayer cultured glioma and nonglial cell lines suggested that the detected activity was simply directed against membrane characteristics common to cultured cells (27) or phase-specific antigens of rapidly dividing cells (53). The serum anti-WR-GBM, raised against whole glioblastoma multiforme tissue, could not be inferred to contain such activity, unless it is postulated that antigens attributable to the in vivo transformed state (neoplasia) and to the in vitro transformed state (long-term cultured lines) are highly cross-reactive. That we might exclude the possibility of anti-"cultured cell" activity, both anti-WR-GBM and anti-MG 251 sera were absorbed with the long-term cultured osteogenic sarcoma cell line 2-T. The removal of antibody activity for all non-glioma cell lines by 2-T absorption established that the remaining significant levels of activity for gliomatous cell lines (Charts 5 and 6; Table 2) were not solely attributable to the long-term cultured status of the cell lines. Further evidence that the reactivity detected here is not directed against a common cultured cell characteristic is provided by the demonstration of the failure of both the HLA-unreactive antiserum to react with long-term suspension-cultured lymphoblastoid lines (Charts 3 and 4; Table 2). Such apparent glioma target cell line-associated reactivity is potentially similar to the leukemic type-specific reactions reported by Mohanakumar et al. (40) or the apparent common human sarcoma antigen recognized specifically with sera from patients with nonsarcomatous neoplasias (34).

The demonstration that cultured human "neurosarcoma
tumor antigen" (34) expression is cell cycle dependent (11) (high concentration is detectable by immunofluorescence in the mid-G1 phase as opposed to negligible levels in G2 and M phases) stresses the importance of consistent selection of target cell populations. Culture conditions of both the glioma and nonglial target cells used were identical; cells were used at confluence for both [*4C]nicotinamide and IMIF assays in our study.

In conclusion, the data presented here establish that hyperimmunization of nonhuman primates with either whole human glioblastoma multiforme tissue or cultured human glioma cells results in the production of antisera that, after exhaustive absorption of species-specific and HLA activity, contain significant levels of activity for glioma and nonglial cultured human tumor cells. Subsequent absorption with cultured human sarcoma cells removes antibody activity for nongliomatous target cells, although significant antigenomas target cell line activity is present. Whether the glioma-associated antigen(s) detected by these antisera is expressed by normal adult glial cells, fetal glial cells, or other fetal tissues is presently under study.

ACKNOWLEDGMENTS

The authors express their appreciation to Debra Eveland, Melody Faircloth, Fred Warren, and Charles Pegram for technical assistance, to Dr. Frances Ward for expert advice and HLA testing, and to Joan Parks and Fleta Ware for secretarial assistance.

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Surface Antigenic Characteristics of Human Glial Brain Tumor Cells

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