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Immunology

Serological analysis of cell surface antigens of malignant human brain tumors

(astrocytoma/human cancer/tissue culture/cancer immunology)

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ABSTRACT **Sera from 30 patients with astrocytoma were tested for antibody reacting with cell surface antigens of cultured autologous astrocytoma cells. Ten percent of the patients had antibody detectable by mixed hemadsorption assays, ≈50% by immune adherence and protein A assays, and 100% by anti-C3-mixed hemadsorption assays. Absorption analysis of reactive sera with autologous, allogeneic, and xenogeneic cells permitted the definition of three classes of astrocytoma cell surface antigens. Class I antigens showed an absolute restriction to autologous astrocytoma cells. Class II antigens were shared by all astrocytomas tested and could be detected also on neuroblastoma, sarcoma, and some (but not all) melanoma cell lines; these antigens were not found on cell lines derived from carcinomas or normal tissues. Class III antigens were widely distributed on cultured normal and malignant cells of human and animal origin. In this series, sera from 2 patients recognized class I antigens, 4 patients' serum recognized class II antigens, and 13 patients' sera recognized class III antigens. Absorption tests have shown that the AJ (class II) antigen of astrocytoma is serologically related to the previously described AH (class II) antigen of melanoma; in tests of nine melanoma cell lines, there was a correspondence between the AJ and AH phenotypes. This method of autologous typing provides a way to classify the cell surface antigens of astrocytomas and to assess the clinical significance of humoral immunity to these antigens.**

An underlying assumption of much work in cancer immunology is that cancer cells can be distinguished from normal cells by the presence of distinctive antigens on the surface of the cancer cells. The evidence supporting this assumption comes primarily from transplantation studies in inbred mice and rats with tumors induced by chemical carcinogens, such as methylcholanthrene, or by viruses, such as polyoma. The repeated demonstration that these tumors are immunogenic in syngeneic hosts represents the cornerstone of the field of cancer immunology.

Despite the enormous literature that has centered on the question of tumor-specific antigens of human cancers, the existence of such antigens must still be considered in the realm of speculation, not fact. The general impression by many in the field of tumor immunology as well as those in related fields, that tumor-specific antigens have been demonstrated in many types of human cancers, is simply not justified. The critical issue with regard to tumor-specific antigens is, of course, the question of specificity, and demonstrating the specificity of a serological or cell-mediated reaction is orders of magnitude easier in the mouse than in man. Lacking inbred mouse strains, hyperimmune antisera, and transplanted tumor cell lines with defined antigenicity, the human cancer serologist seeking evidence for tumor-specific antigens is faced with an exceedingly difficult task. Heteroimmune sera prepared against human cancer cells that at first have the appearance of tumor specificity have in every instance, when analyzed sufficiently, turned out to be directed against normal cellular products, either present in higher concentrations in tumor cells or found in a restricted normal cell population. Surveys of human sera for reactivity with cell surface antigens of allogeneic tumor cells, the basis of a vast number of reports in the literature, are rarely, if ever, interpretable in terms of tumor-specific reactions, because the unknown participation of alloantibodies in the reactions observed is extremely difficult to exclude.

To develop as unambiguous a serological typing system as possible, we turned several years ago to analyzing autologous serum reactivity to cell surface antigens of human tumors (1–4). In the case of solid tumors, the need for target cells that can be repeatedly tested has restricted our studies to tumor types that can be adapted to tissue culture growth with some degree of regularity—e.g., melanoma, renal cancer, and astrocytoma. Absorption tests with normal and malignant cells from autologous, allogeneic, and xenogeneic sources have provided the way to analyze the specificity of autologous reactions and to determine the distribution of the detected antigen on various cell types. By applying this approach to the study of malignant melanoma, the most compelling evidence to date for the existence of tumor-specific antigens in human cancer has been obtained (1, 2). We now report the results of a comparable serological analysis of human astrocytomas.

MATERIALS AND METHODS

Tissue culture procedures

Astrocytomas. Sterile tumor specimens were processed for tissue culture as described (1). The frequency of subculturing different astrocytoma lines ranged from once every week to once every 6 weeks. Maintenance of a high cell density was essential for successful passaging. Of the 45 astrocytoma cultures initiated in our laboratory, subculturing and repeated serological testing was possible in 25 cases; longer-term cultures (>15 passages) could be obtained with 9 astrocytomas. As described by Ponten and McIntyre (5), two main types of astrocytoma cultures were observed. The first type (16/25 astrocytomas) grew in an irregular, highly variable pattern. The cytoplasm of the cells was frequently drawn out into long protrusions, resulting in a complex network of interlacing cells. The second type (9/25 astrocytomas) consisted of spindle-shaped cells that grew in parallel fashion and had a tendency to cluster and heap up. This latter type showed the most vigorous growth *in vitro*. Astrocytoma cultures and sera from patients AA, AB, AC, AD

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Abbreviations: MHA, mixed hemadsorption; IA, immune adherence; C3-MHA, anti-C3 mixed hemadsorption; PA, protein A; VBM, Veronal buffer medium; P_i/NaCl, phosphate-buffered saline.

and AE were provided by Michael A. Bean (Virginia Mason Research Center, Seattle, WA). Established astrocytoma cell lines A382, U138MG, U178MG, U251MG, U343MG, U373MG, and T98 were provided by Jørgen Fogh of Sloan-Kettering Institute (see ref. 6).

Fibroblasts. Fibroblasts were cultured from biopsied scalp or arm skin.

Other Cell Lines. For derivation of other cell lines, see refs. 1–3, 6. P5A, a cell line derived from a human osteogenic sarcoma, was provided by E. Beth and G. Giraldo (Sloan-Kettering Institute). The rat glioblastoma cell line (EA 285) was established by H. Cravioto (New York University Medical Center). Cell lines derived fron normal monkey brain (MA 100 and MA 116) were obtained from Microbiological Associates (Bethesda, MD).

Serological procedures

The mixed hemadsorption (MHA) assay, immune adherence (IA) assay, and qualitative and quantitative absorption procedures were performed as described (1–3).

Anti-C3-MHA (C3-MHA) Assay. The C3-MHA assay described by Irie *et al.* (7) was modified in the following way: EAC 14 cells were incubated with guinea pig C2 and C3 (Cordis Laboratories). After washing with Veronal buffer medium (VBM), the EAC 1423 cell suspension was incubated with a subagglutinating concentration of goat anti guinea pig C3 (Cappel Laboratories). Tumor cells were seeded in wells of 3040 microtest plates (Falcon Plastics) as described for MHA and IA assays (1, 2). After incubation of target cells with serial dilutions of autologous serum at either 4° or 24° for 1 hr, the wells were washed three times with VBM/5% fetal bovine serum and once with VBM alone. To each well, 0.05 ml of a 1:50 dilution of preselected guinea pig serum was added as a source of complement and the mixture was incubated at 37° for 30 min. The wells were again washed as before. Then, 0.05 ml of 0.05% (vol/vol) suspension of indicator cells was added to each well and incubated at 37° for 30 min. After this final incubation, the plates were washed once with VBM and evaluated by light microscopy. Individual positive cells were scored as described for the IA assay (2). In each experiment, control wells incubated with VBM/5% fetal bovine serum alone were included; as a rule these had <1% positive cells. Antibody titer refers to the highest serum dilution that showed 10% positive cells.

Protein A (PA) Assay. For the preparation of PA indicator cells, staphylococcal protein A (Pharmacia Fine Chemicals) was conjugated to the surface of human O^+ erythrocytes with 0.01% $CrCl_3$. Indicator cells were washed twice in VBM and resuspended in VBM. After incubation of target cells with serial dilutions of autologous serum at 4° or 24°, the wells were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (P_i/NaCl) containing 5% gamma globulin-free fetal bovine serum; 0.05 ml of a 0.2% (vol/vol) suspension of indicator cells was added to each well. After a 45-min incubation at 24°, the wells were washed once in the same P_i/NaCl/fetal bovine serum and evaluated under a light microscope. PA assays were evaluated according to the same criteria as C3-MHA assays.

RESULTS

Reactions of Autologous Sera with Surface Antigens of Cultured Astrocytoma Cells. Sera from 30 patients were tested for reactivity to cell surface antigens of autologous astrocytomas. In all but a few instances, each serum was tested in the four serological assays: MHA, IA, C3-MHA, and PA. Table 1 summarizes the results of these tests. The MHA test detected antibody in 3 of 30 patients, whereas the C3-MHA test detected antibody in all patients. With both IA and PA tests, approximately half the patients were found to have autologous serum reactivity. In most cases, serum titers were highest with C3-MHA tests. The three exceptions to this finding were sera from patients AE, AW, and BC in which PA titers were higher than C3-MHA titers.

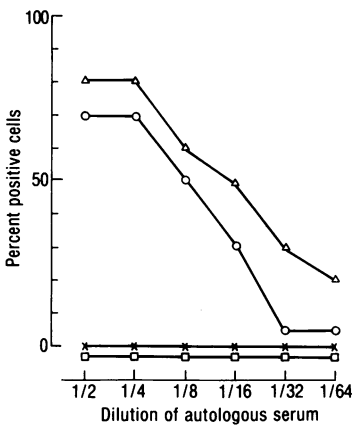

FIG. 1. Reactivity of serum from patients BC for surface antigens of autologous astrocytoma cells in four serological test systems: △, PA, ○, C3-MHA; ×, IA; □, MHA.

Fig. 1 illustrates the serum reactivity of patient BC for autologous astrocytoma cells, as determined in the four serological test systems. Antibody was detected by PA and C3-MHA tests but not by MHA and IA tests.

Reactions of Autologous Sera with Surface Antigens of Normal Fibroblasts. Cultures of fibroblasts were available from 20 of the 30 patients (Table 1). Autologous sera did not react with these cells in MHA, PA, or IA assays. However, reactions were observed in C3-MHA tests with sera from three patients (AA, AB, and AC). Sera from the two other patients tested (AD and BC) were negative. Fig. 2 illustrates the reactions of AC and BC sera with autologous fibroblasts and astrocytoma cells. Serum from patient AC reacted equally well with normal fibroblasts and astrocytoma cells, whereas serum from patient BC reacted with astrocytoma cells but not fibroblasts.

Absorption Analysis of Autologous Serum Reactivity for Astrocytoma Cells. In 15 cases, autologous serum reactivity was high enough in at least one of the four assays to permit analysis of the reaction by absorption tests.

Fig. 3 illustrates individual absorption tests with sera from patients AC, BC, AJ, and AK. Table 2 summarizes the results of absorption studies performed with these sera. Antigens detected by AC and BC sera on autologous astrocytoma cells were restricted to autologous astrocytoma cells. Other cell types, including autologous fibroblasts, did not absorb autologous reactivity.

In contrast to AC and BC antigens, the antigen detected by AJ serum on autologous astrocytoma cells was detected on all 14 astrocytoma cell lines tested. In addition, neuroblastoma, sarcoma, and some (but not all) melanoma cell lines absorbed autologous AJ reactivity. This antigen has not been found on other cell lines derived from malignant or normal cells, including fibroblasts from patients whose astrocytoma or melanoma cells express AJ antigen.

The antigen detected by AK serum was found on all cultured neoplastic or normal cells tested, whether of autologous, allogeneic, or xenogeneic origin. Human erythrocytes, platelets, lymphocytes, or granulocytes or sheep erythrocytes did not absorb AK reactivity.

Quantitative Absorption Analysis of AJ Antigen Expression by Autologous and Allogeneic Cell Lines. Quantitative

Table 1. Summary of serological tests of sera from patients with astrocytomas: Demonstration of autologous antibody to surface antigens of cultured astrocytoma cells

Patient	Age, yr; sex	Astrocytoma grade	Tissue culture passages tested	Maximal antibody titer* MHA	IA	C3-MHA	PA	Antigen class†
AA‡	39;F	IV	7–9	1/2	1/2	1/16		III (C3-MHA)
AB‡	61;M	III-IV	6–10	1/2	–	1/16	–	III (C3-MHA)
AC‡§	28;M	II	2–12	–	1/8	1/16	–	I (C3-MHA)
AD‡	62;F	III	4–7	–	1/2	1/8		III (C3-MHA)
AE	64;M	III	4–6	–	–	1/8	1/16	III (C3-MHA, PA)
AF§	53;M	II-III	1–12	–	–	1/4	–	
AG	35;M	III	1–4	–	–	1/16	1/4	III (C3-MHA)
AH‡	52;F	III	1–2			1/64	–	III (C3-MHA)
AI	57;M	III	1–6	–	–	1/16	1/2	
AJ§	59;F	IV	1–18	1/8	1/32	1/128	1/4	II (C3-MHA)
AK‡§	57;M	IV	1–10	–	1/16	1/64	1/2	III (C3-MHA)
AL‡	44;M	IV	1–2	–	–	1/8	–	
AM	62;F	II	1–5	–	–	1/32	1/8	III (C3-MHA)
AN	49;M	IV	2–4	–	–	1/16	–	III (C3-MHA)
AO	49;F	III	1	–	1/2	1/32	–	III (C3-MHA)
AP‡	55;F	IV	1–6	–	1/8	1/64	1/2	
AQ‡	62;M	IV	1	–	–	1/8	1/2	
AR‡	71;M	IV	1	–	–	1/16	–	
AS‡§	57;M	III-IV	1–10	–	1/2	1/16	1/2	III (C3-MHA)
AT‡	53;M	III-IV	1	–	–	1/2	–	
AU‡	35;M	IV	1	–	–	1/16	–	III (C3-MHA)
AV‡	64;M	III-IV	1	–	–	1/2	1/2	
AW‡	63;M	III-IV	1–3	–	–	1/16	1/64	
AX	51;M	IV	2	–	–	1/4	–	
AY‡	54;F	IV	2	–	–	1/4	–	
AZ‡	50;M	IV	1–3	–	1/8	1/64	1/4	
BA‡	58;M	III-IV	2	–	–	1/32	–	
BB	45;M	IV	1–3	–	1/8	1/64	1/2	
BC‡	46;F	III	1–10	–	–	1/16	1/64	I (PA); III (C3-MHA)
BD‡	18;F	III	2	–	1/4	1/32	–	

* Titers generally did not differ by more than one serial dilution between incubations at 4° and 24°. – indicates titer <1/2. In 23/30 cases, two or more serum specimens were obtained (interval between specimens 1 week to 12 months). Serum titers did not show significant changes and the specificity of the antibody as determined by absorption analysis remained the same.

† See *Results* and *Discussion*. Class I, antigens restricted to autologous astrocytoma cells (e.g., BC); class II, antigens present on allogeneic as well as autologous tumor cells but not detected on any normal cell type (e.g., AJ); class III, antigens present on a wide variety of cultured cell types (normal and malignant; autologous. allogeneic, and xenogeneic).

‡ Autologous skin fibroblasts were also cultured.

§ Cultured astrocytoma cells of patients AC, AF, AJ, AK, and AS were implanted (10^7 cells) subcutaneously into *nu/nu* mice. Progressive tumors were observed in the case of AF, AJ, and AK astrocytomas.

absorption tests were performed with several cell lines expressing AJ antigen. As measured by absorption capacity per cell, U20S sarcoma expressed the same amount of AJ antigen as did AJ astrocytoma. In comparison with AJ astrocytoma, SK-MEL-13 melanoma and U251MG astrocytoma expressed approximately 5 times more AJ antigen and SK-NMC neuroblastoma expressed only one-fifth.

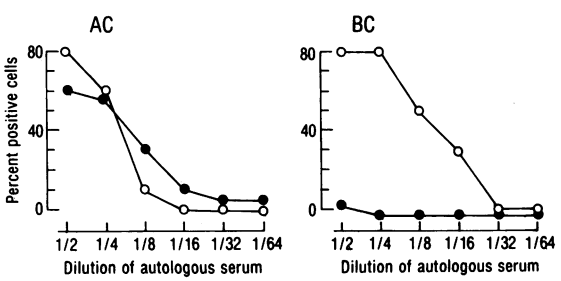

FIG. 2. C3-MHA reactivity of sera from patients AC and BC for surface antigens of autologous fibroblasts (●) and astrocytoma cells (○).

Relationship of AJ Astrocytoma Antigen to AH Melanoma Antigen. The AH antigen of melanoma represents an example of a shared cell surface antigen of melanoma defined in an autologous typing system (2). In our original study, AH was found on 5 of 12 melanoma cell lines but not on any other cell type. Comparison of the AJ typing of the nine melanoma lines shown in Table 2 with their known AH phenotype revealed that six lines were AJ^+AH^+ (SK-MEL-13, -23, -27, -29, -31, -42) and three lines were AJ^-AH^- (SK-MEL-28, -37, Mel-101D). No example of an AJ^+AH^- or AJ^-AH^+ cell line was found. Thus, the AJ astrocytoma antigen and AH melanoma antigen appear to be serologically related.

Stability of Antigen Expression by Cultured Astrocytoma Cells. Astrocytoma cells that could be repeatedly subcultured were tested over successive passage generations for reactivity with autologous sera. For example, astrocytoma AJ was tested between passages 1 and 18 (10 months), astrocytoma AK between passages 1 and 10 (8 months), astrocytoma AC between passages 2 and 12 (12 months), and astrocytoma BC between passages 1 and 10 (6 months). In general, only minimal fluctuations in the strength of the autologous reaction and in anti-

Table 2. Summary of absorption analyses: Reaction of autologous sera with cell surface antigens of cultured AC, BC, AJ, and AK astrocytoma cells

Absorbed with	AC (C3-MHA)*	BC (PA)	AJ (C3-MHA)	AK (C3-MHA)
Astrocytoma:				
AC	+	−	+	+
AE	−	−	+	+
AF		−	+	
AJ	−	−	+	+
AK	−	−	+	+
AS	−	−	+	+
BC	−	+	+	+
A 382	−	−	+	+
U 138 MG	−	−	+	+
U 178 MG	−	−	+	+
U 251 MG	−	−	+	+
U 343 MG		−	+	
U 373 MG		−	+	
T 98	−	−	+	+
Neuroblastoma:				
SK-NMC	−	−	+	+
SK-NSH	−	−	+	+
Melanoma:				
SK-MEL-13	−	−	+	
SK-MEL-23			+	
SK-MEL-27			+	+
SK-MEL-28			−	+
SK-MEL-29		−	+	+
SK-MEL-31			+	
SK-MEL-37			−	
SK-MEL-42			+	
Mel 101D			−	
Mixed mesodermal tumor:				
MB		−	+	+
Sarcoma:				
P5A	−	−	+	
SAOS 2			+	+
U2OS			+	+
Carcinoma:				
Bladder, T24		−	−	+
Breast, A1Ab			−	+
Cervix, ME180	−	−	−	+
Colon, HW			−	
Colon, HT 29		−	−	
Kidney, SK-RC-NS	−	−	−	+
Larynx, HEp2			−	
Lung, SK-LC-LL		−	−	
Ovary, SK-OV-3		−	−	+
Burkitt lymphoma Raji		−	−	+
Meningioma BM or SA	−	−	−	
Normal glial cells TF	−	−	−	
Fetal brain:				
Cultured	−	−	−	+
Not cultured		−	−	−
Fibroblasts:				
AC	−		−	
AK				+
AS			−	
BC		−	−	+
MB			−	
SA			−	
Leukocytes, platelets, erythrocytes	−	−	−	−
Rat glioblastoma EA 285	−	−	−	+
Monkey brain MA 100 or MA 116		−	−	
Sheep erythrocytes	−	−	−	−
Fetal bovine serum	−	−	−	−

+, Absorption of autologous reactivity; −, no absorption of autologous reactivity.
* Autologous sera (serological assay).

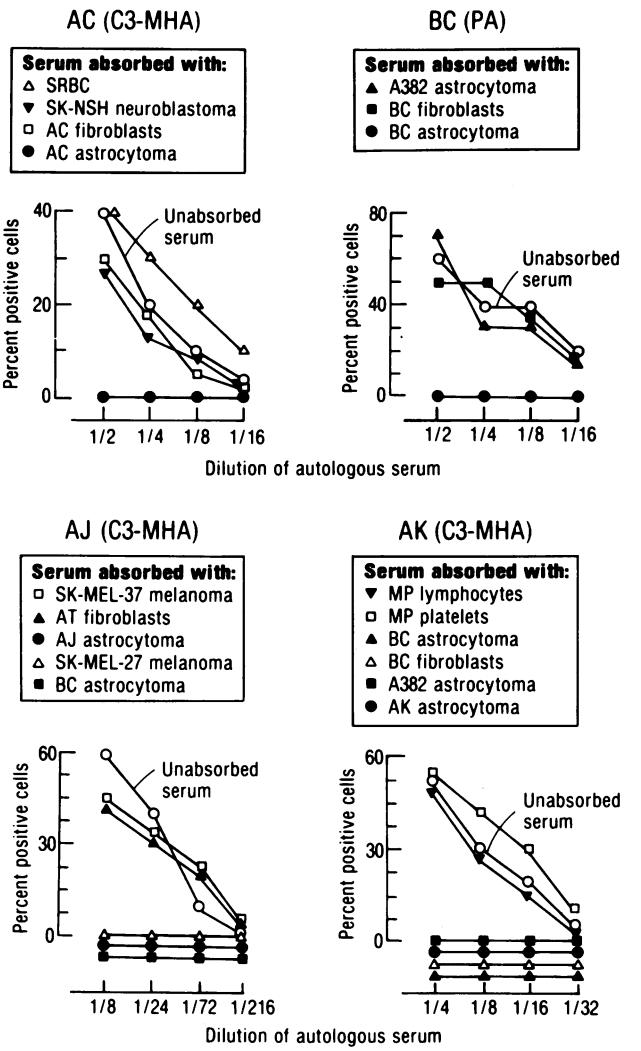

FIG. 3. Examples of individual qualitative absorption tests with four sera. C3-MHA reactivity of AC serum was absorbed by autologous AC astrocytoma cells but not by AC fibroblasts or other cells. PA reactivity of BC serum was absorbed by autologous BC astrocytoma cells but not by BC fibroblast or A382 astrocytoma. C3-MHA reactivity of AJ serum was absorbed by autologous astrocytoma cells, allogeneic astrocytoma cells, and melanoma SK-MEL-27 but not by melanoma SK-MEL-37. C3-MHA reactivity of AK serum was absorbed by allogeneic astrocytoma cells and fibroblasts but not by lymphocytes, platelets, or granulocytes.

body titer were observed. The specificity of the reactions, as determined by absorption analysis, also remained constant.

DISCUSSION

Three classes of cell surface antigens of cultured astrocytoma cells have been defined in this study by absorption analysis of autologous sera. AC and BC antigens, which will be referred to as class I antigens, represent individually distinct or unique antigens that show an absolute restriction to autologous astrocytoma cells. The AJ antigen, a class II antigen, represents a class of shared tumor antigens, being expressed by allogeneic as well as autologous astrocytoma cells and by neuroblastoma, sarcoma, and melanoma cell lines but not by other types of malignant cells or by normal cells. In contrast, the AK antigen, a class III antigen, has the most widespread distribution; various cultured cells, both normal and neoplastic, of autologous, allogeneic, or xenogeneic origin express AK antigen. These three classes of astrocytoma cell surface antigens have their parallel in the antigens defined in a similar manner on melanoma cells, with

the AU, BD, and BI melanoma antigens representing class I antigens (1–3), the AH melanoma antigen representing a class II antigen (2), and class III antigens being commonly detected by sera from melanoma patients (3). Class III antigens are also the ones most commonly detected by sera from astrocytoma patients (Table 1). Because antibodies to class III antigens are frequently not detectable in direct tests with autologous fibroblasts, even though the presence of class III antigens on fibroblasts is revealed by absorption tests, these antigens can be mistaken for tumor-specific antigens. For this reason, rigorous absorption analysis is essential to distinguish antigens belonging to class III from those belonging to class I or class II.

The class I antigens of astrocytomas and melanomas resemble the individually distinct or unique antigens demonstrable by transplantation techniques on various experimental tumors (see ref. 8). It was once thought that these unique antigens were characteristic of chemically induced tumors and that virus-induced tumors had shared antigens that were common to all tumors induced by a particular virus. We now know that this distinction is not absolute; chemically induced tumors can have shared antigens and virus-induced tumors can express individually distinct antigens. The extensive polymorphism of individually distinct or class I antigens is a fascinating puzzle for tumor biologists. The nature of these antigens has been the subject of much speculation, with their origin being ascribed to host or viral genes that have been mutated, derepressed, or otherwise rearranged, or to epigenetic errors in membrane synthesis. The availability of serological reagents that detect these antigens in human melanoma and astrocytoma and in experimental tumors should make it possible to define their chemical nature and, by means of somatic cell hybrids, to ask whether class I antigens are specified by distinct chromosomal loci.

Two class II antigens have been defined by autologous typing of cultured human cancer cells: the AJ antigen of astrocytoma and the previously described AH antigen of melanoma (2). In comparing the AJ and AH phenotypes of a series of melanomas, it became evident that these two antigenic systems were related; melanomas were either AJ^+AH^+ or AJ^-AH^- but not AJ^+AH^- or AJ^-AH^+. One possibility that could account for the sharing of an antigen by astrocytomas and melanomas comes from the fact that these tumors are derived from cells of neural crest origin. According to this view, the AJ and AH antigens represent serologically related differentiation antigens of neural crest-derived tissues and this would be consistent with the finding that neuroblastomas also are AJ^+. This possibility seems unlikely for two reasons: fetal brain (12–16 weeks' gestation) lacks AJ antigen; and sarcomas, which are of mesodermal origin, type AJ^+. In this regard, it may be relevant that AJ astrocytoma showed marked sarcomatous changes; it is conceivable that this might have provided the immunogenic stimulus for the production of antibody to an antigen shared by sarcomas and neural crest-derived malignancies. Another possibility that cannot be excluded at present is that AJ antigen is related to a virus that is restricted to AJ^+ tumors. Once again, the availability of serological probes that define class II antigens should permit us to gain insight into the genetic origin of these antigens.

From this study of astrocytomas and our continuing study of melanomas, it is clear that the simultaneous use of several serological test systems minimizes the risk of missing autologous serum reactivity. This can be ascribed to differences in the inherent sensitivity of the various techniques and to the fact that they detect different classes of immunoglobulins. For example, the reactivity of BC serum, which is detected by C3-MHA and PA assays, would have been missed if only MHA and IA tests had been performed. In addition, the AU melanoma antigen (1), which was originally defined by MHA, cannot be detected by IA, and the AH melanoma antigen (2), originally defined by IA, cannot be detected by MHA. Another advantage of using more than one assay is the possibility of detecting antibodies to different specificities in the same serum. This is what we observed in absorption analysis of sera from patient BC; PA assays detected a class I antigen and C3-MHA assays detected a class III antigen (Table 1).

Because cultured cells form the basis for these serological studies of surface antigens of human cancer cells, a major impediment to future progress in this area is the general inability to grow many types of human cells, both normal and malignant, in tissue culture. In our experience, approximately 50% of astrocytomas and 20–30% of melanomas will propagate sufficiently to permit repeated serological testing of autologous sera. With other tumor types, such as breast, lung, and colon, serological study by autologous typing is possible in less than 5% of cases. Defining conditions for the successful culture of a greater proportion of human cancers and, wherever possible, the comparable normal cell population will be necessary before a comprehensive and meaningful classification of the surface antigens of human cancers can be achieved.

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