THE YALE JOURNAL OF BIOLOGY AND MEDICINE 50 (1977), 397–403

Malignant Glial Neoplasms: Definition of a Humoral Host Response to Tumor-Associated Antigen(s)

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Received August 16, 1976

There is increasing evidence that human tumors possess tumor-associated neo-antigens. The host mounts an immunological response to these antigens, as evidenced by the detection of circulating humoral antibodies in a variety of human neoplasia.

An indirect immunofluorescent antibody technique was employed to detect antibodies to tumor-associated antigens in the sera of patients with malignant gliomas. Viable single cell suspensions were used to demonstrate antibodies to surface contents of tumor cells and cell preparations were snap-frozen at −160° C to demonstrate antibodies to cytoplasmic components of tumor cells. After incubation with serum, the preparations were treated with polyvalent sheep antihuman globulin conjugated to isomer-1-fluorescein isothiocyanate, washed, and examined with a Leitz incident fluorescent microscope.

Of the 17 sera from histologically proven malignant glial neoplasm patients, 2 (11%) were positive for an autologous surface antibody reaction. Five (23%) of 21 were positive for an autologus cytoplasmic antibody, however, 10 (47%) of 21 of the sera gave a positive reaction for cross-reacting cytoplasmic antibodies when tested with a battery of tumor cells obtained from different patients with malignant glial tumors.

No reaction was observed with normal brain tissue. Absorption studies indicated the presence of a tumor-associated antigen.

This study demonstrated that certain patients with malignant gliomas possess circulating antibodies to cytoplasmic components of their own tumor cells. The fact that a number of sera cross-reacted with tumor cells obtained from different patients suggests that antigenic cross-reactivity exists between malignant glioma cells from different patients. It is suggested that with further refinement, immunofluorescent detection of antibodies could evolve as a useful diagnostic adjunct in malignant glioma.

INTRODUCTION

A wide variety of human neoplasms have been shown to carry tumor-associated antigens and to elicit, in the host, cell-mediated [1] and humoral [2] immunity as well as serum factors [3] that are capable of abrogating in vitro manifestations of cell-mediated immunity.

Humoral antibodies directed against human tumor cells or constituents have been extensively sought and have been demonstrated in sera of patients with a variety of neoplasms [4–7]. Though the possible in vivo role of such antibodies is unclear, there is suggestive evidence that humoral antibodies play a role in the control of disease and prognosis of human tumors [8,9].

We are reporting our preliminary study which indicates that sera of patients with malignant glial neoplasms have antibodies demonstrable by indirect immunofluorescence. These antibodies are directed against intracytoplasmic antigens of autologous and allogeneic tumor cells.

This work was supported in part by the Robert E. and May R. Wright Foundation, Award #41.

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MATERIALS AND METHODS

Tissue specimens were obtained from a consecutive series of 21 patients with malignant glial tumors. The mean age of the population was 50 years with a range of 33–71 years. All patients had relatively brief clinical histories (less than 6 months) and had been on dexamethasone therapy for a period of 2–5 days with a mean of 3 days. Tissue specimens were obtained at the time of surgery when subtotal excision of the tumors was accomplished. Serum specimens were obtained in a simultaneous fashion. To insure uniformity of opinion regarding the pathological material, all specimens were reviewed by the director of the Cajal Neuropathological Laboratories at the Los Angeles County-University of Southern California Medical Center. The degree of malignancy observed was considered to be at the level of either a malignant astrocytoma or glioblastoma multiforme in all cases.

In addition to the serum samples from 21 patients with malignant glial tumors, serum specimens were available from 6 patients with meningioma, 10 patients with breast carcinoma, and 14 patients with melanoma. A group of 15 individuals with non-malignant disease was included as controls.

After dissection and removal of tissue for routine pathological evaluation, the specimen was transferred in Hank's balanced salt solution. The tissue was mechanically minced to form a single cell suspension. The final concentration of cells was adjusted to 1 x 10^6 cells per ml.

A portion of the cell suspension was used to prepare cell smears for cytoplasmic immunofluorescence studies. A drop of cell suspension was placed on a microscopic glass slide, air dried, and snap frozen in a mixture of iso-pentane and liquid nitrogen at $-160°$ C [2]. The slides were stored at $-70°$ C. Prior to use, the slides were removed from cold storage, air dried, and washed in Phosphate buffered saline (PBS) for 10 minutes with constant agitation.

INDIRECT IMMUNOFLUORESCENCE

An indirect immunofluorescence method was used on snap frozen tumor cells or single cell suspensions to demonstrate antibodies to cytoplasmic or surface constituents, respectively. The reactions between patients' serum and autologous, as well as allogeneic tumor cells, were examined. In the immunofluorescent test, tumor cell suspensions or smears were incubated with serum diluted 1:8 in PBS for 20 minutes, rinsed, and incubated with sheep anti-human Ig conjugated to isomer-1-fluorescein isothiocyanate (obtained from Burroughs Wellcome Laboratories). For control purposes, tumor cells were also incubated each time with known positive and negative sera and with fluorescein labeled anti-human globulin alone without prior incubation with human serum.

Sheep anti-human globulin Ig conjugated to isomer-1-fluorescein isothiocyanate was obtained from Burroughs Wellcome Laboratories. These conjugates were further purified by chromatography as previously described [10].

Microscopic examination was done with a Leitz incident microscope with a high intensity UV lamp using an HBO mercury lamp with a K490 primary exciting filter and K515 suppression filter. The positive reaction gave brilliant apple-green fluorescence.

Cell counts were performed to determine the percentage of cells which fluoresced after the application of the test serum. A preparation was considered positive if 30% or more cells showed positive fluorescence.

SERUM ABSORPTION

Routinely, a small portion of glial tumor from each case was removed and stored at −70° C for absorption studies. Small pieces of tissue obtained from temporal and frontal lobectomies performed for lesions of traumatic etiology were pooled and used as a source of normal brain tissue. Pooled tumor tissue or normal brain tissue were suspended in PBS to remove blood and minced with a pair of scissors in a petri dish. The coarse homogenate was centrifuged at 5,000 x G for 30 minutes at 4° C, the pellet was resuspended in PBS, and homogenized in a Waring blender. The emulsion was frozen and thawed several times and centrifuged at 7,000 x G for 30 minutes at 4° C. The pellet was lypholized and stored at 4° C.

For use, the required quantity of lypholized material was resuspended in 10 volumes of PBS for 30 minutes, centrifuged at 7,000 x G for 30 minutes, and the supernatant discarded. It may be pointed out here that during this procedure, we may have lost the soluble component of cellular material.

In absorption tests, 0.5 ml of serum diluted to 2 ml with PBS was mixed with 1 gm of reconstituted homogenate. The mixture was stirred gently for 3 hours at room temperature, overnight at 4° C, and centrifuged at 7,000 x G for 30 minutes to recover the maximum amount of serum [7].

RESULTS

Results, summarized in Table 1, indicate that 2 (11%) of 17 glioma sera gave a positive immunofluorescence reaction to surface components, and 5 (23%) out of 21 sera reacted with intracytoplasmic components of autologous tumor cells.

When large pieces of tumor were available, serum antibody reactions to allogeneic tumor cells were studied.

Sera from 11 glioma patients were tested with tumor cells from 4 patients. No reaction for serum antibodies to surface antigen of allogeneic tumor cells were noticed. Similarly, for studies of serum antibodies to cytoplasmic antigens of tumor cells, sera from 6 patients were reacted with their tumor cells. The results are shown in Table 2.

Sera from patients M.D., C.L., T.M., and J.B. did not show autologous reaction (reaction with the patient's own cells). However, sera from two patients, T.M., and V.B., showed allogeneic reaction (reaction with cells obtained from other patients) for antibodies to intracytoplasmic antigens (Table 2).

Antigen preparations from patients A.C., E.G., and V.B. showed higher frequency of cross-reaction for antibodies to cytoplasmic antigens (i.e., cells from patient A.C. reacted with all 6 sera, whereas cells from E.G. and V.B. reacted with at least 4 out of 6 sera tested) than those obtained from patients M.D., C.L., and T.M. (Table 2). In this study, antigen preparations from former groups of patients (i.e., A.C., E.G., and V.B.) were used routinely to study serum antibodies to cytoplasmic antigens of malignant glioma. Frequency of the reactive sera was comparable in all 3 substrates. Ten (47%) of 21 sera from glioma patients showed positive fluorescent reaction, however, 1 (7%) of 15 sera from a normal population, 1 (10%) out of 10 sera from patients with carcinoma of the breast, 2 (14%) of 14 sera from patients with melanoma, and 1 out of 6 sera from meningioma patients were reactive with cytoplasmic components of tumor cells (Table 3).

Titer of serum antibodies to intracytoplasmic antigens among glioma patients varied between 1:8 to 1:32. Immunofluorescence was principally limited to the cytoplasm of the cells (Fig. 1).

TABLE 1
Indirect Immunofluorescent Reaction of Sera Obtained from Malignant Glioma Patients with Patient's Own Tissue

Nature of Reaction	# of Sera Tested	Sera Reacted	% Positive
Membrane Reaction	17	2	11
Cytoplasmic Reaction	21	5	23

TABLE 2
Indirect Immunofluorescent Reactions of Sera Obtained from Glioma Patients with Cytoplasmic Components of Autologous and Allogeneic Tumor Cells

Sera	Cells					
	A.C.	E.G.	M.D.	C.L.	T.M.	V.B.
A.C.	+	+	–	–	+	+
E.G.	+	+	+	+	+	+
M.D.	+	–	–	–	–	+
C.L.	+	–	–	–	–	–
T.M.	+	+	+	+	–	+
V.B.	+	+	+	+	+	–

NOTE: (The results within the central diagonal lines are reactions with autologous cells.)

TABLE 3
Indirect Immunofluorescence Reaction of Sera Obtained from Malignant Glioma Patients and Various Control Groups Reacting with Malignant Glioma Cells

	Fluorescence Localization			
	Cell Surface		Cytoplasm	
Diagnosis	Sera Reacted/ Total	% Positive	Sera Reacted/ Total	% Positive
Malignant Glioma	0/11	0	10/21	47
Meningioma	–	–	1/6	16
Normal	–	–	1/15	6
Ca. Breast	–	–	1/10	10
Melanoma	–	–	2/14	14

In order to establish the specificity of the reaction, glioma sera were reacted with normal brain tissue. In none of the sera was a reaction realized.

In another experiment, strongly positive sera (titer 1/32) from 3 glioma patients were absorbed separately with normal human muscle, brain, and pooled glioma tissue and reacted again with tumor cells (Table 4). While absorption with normal tissues had little or no effect, the absorption of sera with tumor cells significantly removed the reactivity.

It is impossible to say that the number of glial cells used in the absorption experiment was equal between the glioma tissue and normal brain. However, no immunofluorescence was appreciated when positive sera were reacted against normal brain tissue. Additionally, the amounts of antibody which we are attempting to study are of such a small order of magnitude that absorption with normal brain, which is composed predominantly of glial cells, would presumably be equivalent to absorption with equal amounts of glioma tissue.

Evidence for specificity of the reactions for glioma antigen was further sought by reacting sera of glioma patients with cell preparations obtained from various types of

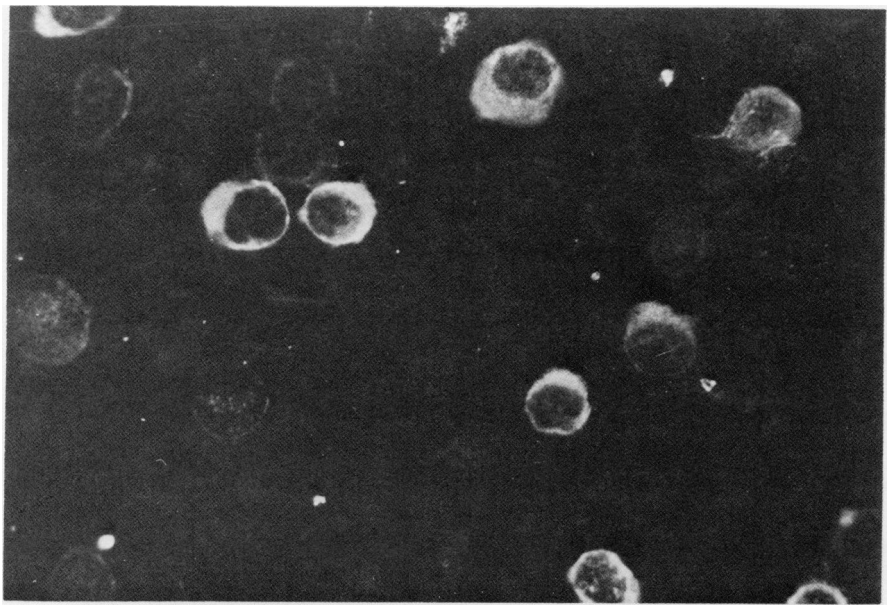

FIG. 1. Intracytoplasmic immunofluorescent reaction demonstrated in snap frozen glioma cell preparation.

TABLE 4
Indirect Immunofluorescence: The Effect of Serum Absorption with Various Tissue Preparations on the Titer of the Cytoplasmic Antibodies to Malignant Glioma Cells

Tissue Used for Absorption	Sera from Patient		
	A	B	C
Original Titer	1/32	1/32	1/32
Muscle	1/32	1/32	1/16
Normal Brain	1/16	1/32	1/16
Pool Malignant Glioma Tissue	1/4	1/4	1/2

human tumor tissue, i.e., melanoma, carcinoma of the breast, and lung and ocular melanoma. The frequency of reactions of glioma patients' sera in tests with other types of tumor cells was low (Table 5) as compared to that of glioma tumor cells.

DISCUSSION

In our study, the majority of sera from glioma patients (15 sera of 17 patients) lacked circulating antibodies to surface antigens of glioma cells (Table 1). Similar findings have been reported by Delpech et al. [11], and Eggers [12] in 1972 who, using indirect immunofluorescent antibody technique, reported that only 1 of 17 sera of glioma patients gave evidence of antibodies to membrane antigens.

In these studies, the lack of detection of circulating antibodies to surface antigens of tumor cells could be due to the already in vivo binding of globulin to surface antigens of tumor cells as demonstrated by Tabuchi and Kirsch [13]. Utilizing a rabbit anti-human IgG source for FAB fragments, Tabuchi and Kirsch conjugated antibody with horseradish peroxidase and demonstrated the presence of the peroxidase reaction product predominately on the membranes of 4 of 9 glioblastoma

TABLE 5
Indirect Immunofluorescence: Reaction of Sera Obtained from Glioma Patients with Cytoplasmic Components of Tumor Cells Obtained from Patients with Melanoma, Ocular Melanoma, Carcinoma of Breast, or Carcinoma of Lung

	Sera Tested	# Positive	% Positive
Melanoma	10	0	0
Ocular Melanoma	10	1	10
Ca. Breast	12	0	0
Ca. Lung	15	2	13

preparations. This observation suggested the presumptive antigen-antibody complex on the surface of glioblastoma cells in vivo and implied that IgG can cross a vascular capillary barrier in certain human glioblastomas.

We routinely reacted the tumor cells with fluorescein isothiocyanate (FITC) conjugated to antihuman globulin. These tests failed to demonstrate antigen-antibody binding to membranes of tumor cells.

Our results demonstrated that a large number of sera (10 of 21) from patients with malignant gliomas reacted with cytoplasmic antigens of allogeneic tumors, whereas reactivity among sera from various control groups was significantly reduced, ranging from 6% in a normal population to 16% in meningioma patients (Table 3). This difference in the fluorescence reaction among patients with gliomas and control donors strongly suggests the presence of circulating antibodies to antigens associated with the cytoplasmic components of the tumor cells in these patients.

Prior to use, the substrate (i.e., cell smear preparations) as well as tissue homogenate for absorption studies was washed in PBS. This process may eliminate a part or all of the soluble fraction of the antigenic component. Therefore, it is possible that this test may be detecting antibodies to a part of the antigenic component of glioma cells, i.e., possibly an insoluble fraction.

The observations that in our studies positive sera did not react with the normal brain tissue and that the absorption of these sera with normal brain tissue did not abolish the activity while absorption with tumor tissue removed the activity (Table 4), suggest that the serum antibodies reported here are tumor associated. Allogeneic cross-reactivity of antibodies to cytoplasmic antigens in various glioma patients suggests the presence of common, tumor-associated antigens in these patients.

Reactivity of some of the sera with intracytoplasmic antigens of allogeneic tumor cells and not with autologous tumor cells is difficult to explain. It is conceivable that glioma cells may have more than one antigenic component (i.e., individual, specific, or common) among all gliomas, and that the prevalence of antibodies to various components may vary in relation with the various stages of the disease.

Tissue typing, which was not performed on the patients, might have been useful in assessing the presence or absence of antibodies in these sera.

A number of studies have been directed toward the detection and definition of a humoral response to glial tumors. In 1965, Mitts and Walker [14] undertook investigations which demonstrated that culture growth of malignant glial neoplasms was inhibited by the addition of autologous sera. Kornblith et al. [15] demonstrated that postoperative sera caused hyperpolarization of the membranes of glial cells in culture. In another publication, Kornblith et al. [16] applied an in vitro microcytotoxicity assay to the study of the immune response in human astrocytomas. The sera of 17 of 26 patients harboring glial neoplasms produced significant cytotoxicity when tested against allogeneic astrocytoma cells. These studies demonstrated no particular relationship to the histological grade of the patient's tumor. Three of the 46 sera from

normal controls, and 1 of 13 sera from patients with non-neoplastic central nervous system disease were also cytotoxic in this series. None of 4 sera from meningioma patients was positive.

The reactions of 6% of sera in the normal control group (though in low titer) with glioma cells requires an explanation. Similar findings have been previously reported in other human neoplasms [2,6]. It is possible that these sera reacted nonspecifically with tumor cells. It is also equally possible that the detection of antibodies to cytoplasmic antigens in such cases may be related to future malignancy. In such instances, therefore, followup of fluorescent antibody study, including absorption of these sera with normal and tumor tissue and careful clinical examination, would be of special interest.

Studies on a large population of patients with a number of central nervous system neoplasms are in progress in our laboratory. If the present findings prove to be consistent in a large group of glioma patients, the implications of such studies could be of immense importance in screening of patients and consequently for the early detection and management of these neoplasms.

REFERENCES

1. Hellstrom KE, Hellstrom I: Cellular immunity against tumor antigens. Adv Cancer Res 12:167, 1969
2. Lewis MG, Avis PJG, Phillips TM, et al: Tumor associated antigens in human malignant melanoma. Yale J Biol Med 46:551, 1973
3. Heppner GH: Blocking antibodies and enhancement. Ser Haematol 5:41, 1972
4. Klein G, Klein E, Clifford P: Search for host defense in Burkitt's lymphoma. Membrane immunofluorescence test on biopsies and tissue culture cells. Cancer Res 27:2510, 1967
5. Hellstrom KE, Hellstrom I: Immunity to neuroblastoma and melanoma. Ann Rev Med 23:19, 1972
6. Morton DL, Malingrave RA: Human osteosarcoma: Immunologic evidence suggesting an associated infectious agent. Science 162:1279, 1968
7. Sheikh KMA, Quismorio RP, Fliou GJ, et al: Breast carcinoma: Serum antibodies to tumor-associated antigens (in preparation)
8. Lewis MA, Ikonopisof RL, Nairn RC: Tumor-specific antibodies in human malignant melanoma and their relationship to the extent of the disease. Br J Med 3:547, 1969
9. Sheikh KM, Brownstein S, Lewis MA: Tumor-specific antibodies in ocular melanoma and their relationship to tumor cell type. Amer Assoc Cancer Res, May 10, 1975
10. Lewis MA, Sheikh KM, Avis P, et al: The identifications of foetal antigens in human bone marrow cells. Differentiation 2:307, 1974
11. Delpech B, Delpech A, Clement J, et al: Etude immunochimique et immunologique des tumeurs due cerveau humain. Int J Cancer 9:374, 1972
12. Eggers AE: Autoradiographic and fluorescence antibody studies of the human host immune response to gliomas. Neurol 22:246, 1972
13. Tabuchi K, Kirsch WM: Detection of IgG on glioblastoma cell surfaces. Proc Amer Assoc Neurol Surgeons, April 1974, pp 51a–h
14. Mitts MG, Walker AE: Autoimmune response to malignant glial tumors. Neurol 15:474, 1965
15. Kornblith PL, Prieto A Jr, Pollin DA: Alterations in glial and mesenchymal tumor cell membrane resistance with heteroimmune and autologous sera. Ann NY Acad Sci 159:585, 1969
16. Kornblith PL, Dohan JF, Wood WC, et al: Human astrocytoma: Serum mediated immunologic response. Cancer 34:152, 1974

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