SOLUBLE MEMBRANE ANTIGENS OF BRAIN TUMORS

I. Controlled Testing for Cell-Mediated Immune Responses in a Long Surviving Glioblastoma Multiforme Patient

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A patient with glioblastoma multiforme survived 18 years after diagnosis and underwent 20 operations for extracranial metastasis. An immunologic survey of the patient was made over a 1-year-period using *in vitro* tests of lymphocyte responsiveness and skin tests with control and tumor antigens isolated from autologous and allogenic brain cell membranes. Two tissue-associated soluble cell membrane antigens also present in normal white matter, and two tumor-associated antigens (TAA) produce cell-mediated immune responses in patients with brain tumors. One of these tumor-associated antigens predominates in meningioma cells. In addition some low molecular weight components appeared, which seemed to be unique for the glioblastoma cells from the long-surviving patient.

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LONG-TERM SURVIVAL OF PATIENTS WITH glioblastoma multiforme has been reported but is rare.[4] Long-term survival of a patient with metastatic glioblastoma is almost nonexistent. The unique subject of our present report is a glioblastoma patient who lived 18 years after diagnosis and who, during that period, underwent 20 operations for metastases. In an attempt to explain this longevity we conducted a longitudinal evaluation of both general and specific immune parameters, as well as comparative analyses of his tumor and sera for any unique properties. This 47-year-old white man was first seen by our Department of Neurosurgery on December 11, 1957, when he presented with the sole complaint of constant, generalized headaches of recent onset. Physical examination on the initial visit was unremarkable except for a mild left central facial paresis and a small hemorrhage in the right fundus. His headaches persisted. One week later he developed bilateral papilledema and a dense left hemiplegia. He was admitted to the hospital and diagnostic studies revealed the presence of a right frontal lobe mass lesion. On December 23, 1957, right frontotemporal craniotomy was performed with a relatively complete removal of a firm, solid, well encapsulated 53.2-g right frontal lobe tumor. Microscopically the tumor was typical of a glioblastoma multiforme (Fig. 1). It was a highly cellular tumor of hyperchromatic spongioblastic cells with areas of necrosis, pseudopalisading, and vascular endothelial proliferation. Few mitotic figures were evident.

The patient's postoperative course was unremarkable, with complete clearing of the left hemiplegia. He was discharged on the 13th postoperative day. Subsequent to discharge he received 24 treatments with a two million volt x-ray unit for a total dose of 6856 rads locally to the area of the tumor bed.

Three years after the initial operation a small subcutaneous metastatic tumor nodule was removed from the area of the original scalp flap. Microscopically the tumor was identical to the original glioblastoma. In the ensuing 15 years he had a total of 19 additional operative procedures for removal of metastatic nodules of the head and neck, each diagnosed as glioblastoma multiforme but with progressively more sarcomatous

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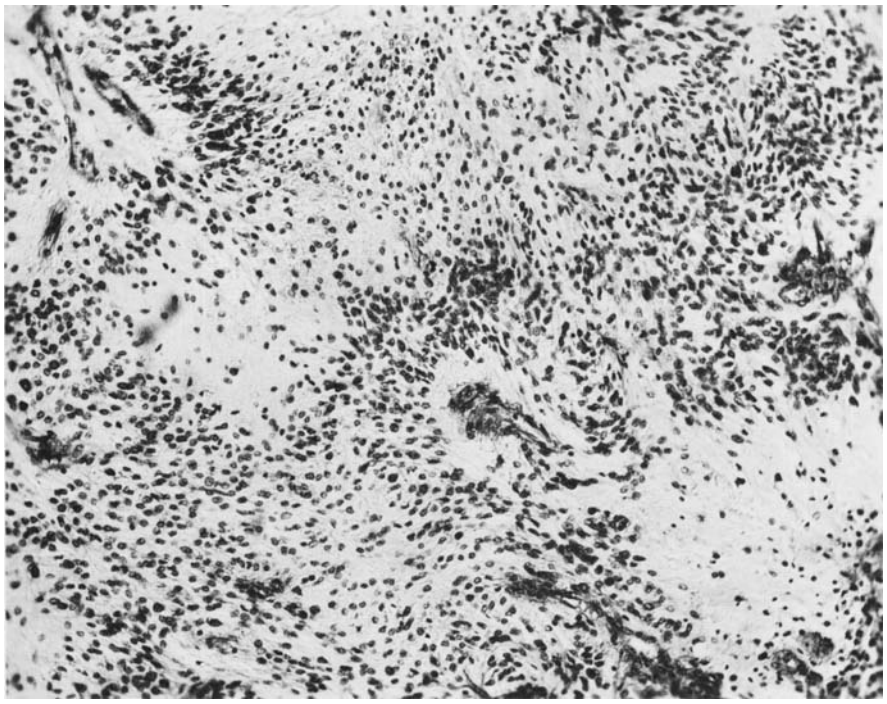

FIG. 1. Photomicrograph of section of glioblastoma multiforme tumor, December 23, 1957, from long-surviving patient.

features, as seen in Fig. 2, a photomicrograph of a recent tumor nodule.

In June, 1973, he was started on chemotherapy: first with BCNU, then CCNU, then a combination of CCNU, methotrexate and cytoxan and finally again on BCNU at roughly 2-month intervals, depending on the patient's condition and blood counts.

The patient was readmitted in November, 1973, for removal of a subcutaneous nodule and

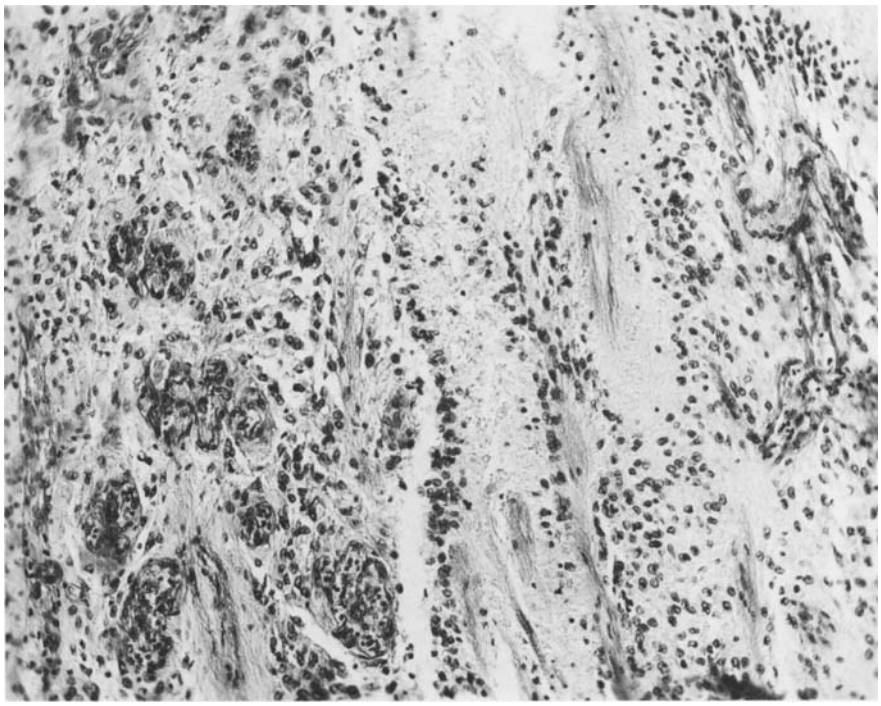

FIG. 2. Photomicrograph of a recent tumor nodule removed from the same patient (21st operation).

the first of four immunologic evaluations. The tests chosen for immunosurveillance were lymphocyte responsiveness to mitogen phytohemagglutinin (PHA), determination of thymus-derived rosette forming cells (T cells), both of which measure general immune responsiveness *in vitro,* and skin tests to measure *in vivo* delayed hypersensitivity to recall and specific tumor antigens. One week postoperatively and 6 weeks after any chemotherapy, his general immunocompetence was evaluated with the first PHA and T-cell determinations. Tumor antigens for the skin tests were obtained from allogeneic glioblastoma cells. In March, 1974, he was again admitted for removal of a nodule. His chest roentgenogram, which had been clear of any active disease, now showed evidence of multiple bilateral pulmonary nodules compatible with more diffuse metastasis. Two days postoperatively and 1 week after chemotherapy he was again evaluated using the same *in vitro* and *in vivo* tests for general and specific immune reactivity. Included were tests with antigens from normal gray and white matter and other brain tumors.

Eight months after the second testing a third set of skin tests was performed using recall and specific autologous tumor membrane antigens isolated from the patient's own tumor cells. These same antigens were used in a fourth testing 2 weeks after the third set. T-cell determinations were performed with each test series as a longitudinal study of the patient's general immune reactivity.

A comparison was made of polyacrylamide gel electrophoretic (PAGE) patterns of the patient's tumor cell membrane components compared with the separation patterns of normal brain tissue and tissue from other intracranial tumors, including glioblastoma cells from short-surviving patients. A comparison was also made between the PAGE separation patterns of serum from our patient and serum from a patient matched for sex, age, type and grade of tumor but whose postoperative course was more typical of glioblastoma patients, living only 5 months after diagnosis.

Throughout the testing period the patient's condition slowly but progressively deteriorated. The pulmonary nodules on chest roentgenogram became larger and more numerous. He developed subcutaneous metastasis with greater frequency and he became more debilitated. EMI scans performed in November, 1974, and again in March, 1975, failed to show any intracranial tumor recurrence and only mild ventricular dilatation. The patient died in July, 1975, 9 months after testing. Autopsy showed extensive metastasis of his tumor to his lungs but his brain showed no evidence of tumor, either grossly or microscopically.

MATERIALS AND METHODS

The method of Chretien *et al.* was used for the PHA determinations.[2,12] Lymphocytes isolated from the patients blood sample were grown to a monolayer of 2×10^6 cells in 80% culture medium (RPMI 1640) and 20% autologous serum with streptomycin and glutamine added. Mitogen phytohemaglutinin-P was added and the cultures incubated at 37°C in 5% CO_2 atmosphere for 54 hours. Three hundred millicuries of tritiated thymidine were added. The cultures were reincubated for 72 hours and terminated. The cells were washed and lysed, and the nucleoprotein was precipitated. The radioactivity of each precipitate was then determined. Normal mean values for PHA and T-cell determinations were performed concurrently, as the tests on our patient were run with a large series for another purpose.

The method described by Wybran *et al.*[15] was used for the T-cell determinations. Lymphocytes isolated from heparinized blood samples were washed in PBS (pH 7.3) and concentrated to 5×10^6 cells/ml. Sheep red blood cells were added in a ratio of eight red cells per lymphocyte and 500 lymphocytes were examined for the percent of rosette formation, a rosette consisting of three or more red cells per lymphocyte; the total white blood cell count was used for calculating T cells/mm^3.

Specimens of allogenic and autologous glioblastoma cells, autologous glioblastoma tissue cultures, normal brain tissue and other intracranial tumor specimens were prepared by methods described elsewhere.[9] The concentrated solubilized membrane protein was subjected to separation by polyacrylamide gel electrophoresis (PAGE) to isolate specific antigens for skin testing. Intradermal injections of 0.1 ml of concentrated sterile antigen were made on the patient's back and the amount of induration recorded at 24 and 48 hours. Serum analysis of serum samples from the long-surviving patient and samples from a matched glioblastoma patient who survived only 5 months postoperatively were compared using the PAGE method of separation. Reactions to the entire spectrum of antigens in low protein concentrations[8] prepared from the patient's tumor and normal brain tissue, other intracranial tumors,

TABLE 1. Lymphocyte Reactivity to Mitogen Phytohemaglutinins (PHA)

Dose per min.	First testing	Second testing	Normal mean
Scintilation counts	37,200	56,700	81,000

and tumor tissue from short-surviving glioblastoma patients were recorded in appropriate fashion and positive reactions assessed according to our criteria.[8,9] Recall antigens were used as described previously.[9]

RESULTS

The dose per minute scintilation counts in the PHA determination are recorded in Table 1. Although the counts for both first and second testing periods were below normal there was a partial increase in activity in the second testing, when the patient's condition had deteriorated because of more generalized metastases. This increased responsiveness is also seen in the T-cell determinations (see Table 2).

The results of the first two skin tests are recorded in Table 3. The antigens were obtained from the PAGE separation of tumor cell membrane extracts from a patient with glioblastoma with a survival of only 3 months postoperatively. The gel column was divided into four regions and each was tested separately. The results show the patient to be completely anergic when first tested but 4 months later he showed an increased reactivity to both recall antigens and to PAGE regions 2 and 3. Not shown are positive responses to control PAGE region 2 of normal white matter and region 3 of meningioma cells. All other regions and tests with separated antigens of gray matter were negative.

Eight months after the second skin tests with control and allogenic tumor cell antigens the third and fourth skin tests using isolated autologous tumor cell antigens were performed 2 weeks apart. In the third skin test the patient showed a good response to recall and both high and low concentrations of tumor antigens (see Table 4). Two weeks later he showed an increase response using only one half the concentration of antigenic tumor material tested previously.

The PAGE separation profiles presented in Figure 3 show protein bands from our long-surviving glioblastoma patient (No. 6) differing from bands of the short-surviving glioblastoma patient (No. 3) in bands 1, 2, 7, 15, 16, 18, 19 and 20. Of these, the carbohydrate portion of band number 1 is also present in normal white matter. Band 2 was present in normal white matter and astrocytoma (gel 5). Band 7 was present in white matter, band 15 was present in

TABLE 2. Determination of Thymus-derived Human Rosette-Forming Cells (T-cells)

	First testing	Second testing	Third testing	Fourth testing	Mean value (control)
T-cells/ mm^3	607	854	1058	1338	1510/mm^3

TABLE 3. Skin Tests Using Recall Antigens and Allogenic Tumor Cell Antigens from a Short-Surviving Patient (Fig. 3) PAGE Column Number 5

Antigens used in skin test	First testing Induration at 48 hours	Second testing* Induration at 48 hours
	(mm)	(mm)
SKSD	0	5
Mumps	0	7
Glioblastoma		
Region 1	0	4
Region 2	0	6
Region 3	0	7
Region 4	0	4

* Second testing: Positive responses at 5- to 7-mm induration also occurred with PAGE region 2 of allogeneic white matter and region 3 of meningioma cells. All other regions, as well as all regions of gray matter, were negative.

TABLE 4. Skin Tests Using Recall Antigens and Autologous Tumor Cell Antigens from the Long-Surviving Patient with Glioblastoma. Pooled Regions 3 and 4 (Figure 3, gel 6)

Antigens used in 3rd testing	Induration at 48 hours (mm)
SKSD	12
Mumps	11
Pooled glio. low molecular weight antigens (5 μg protein/0.1 ml)	7
Pooled glio. low molecular weight antigens (50 μg protein/0.1 ml)	10

Antigens used in 4th testing	Induration at 48 hours
SKSD	20
Mumps	8
Pooled glio. low molecular weight antigens (5 μg protein/0.1 ml)	5
Pooled glio. low molecular weight antigens (25 μg protein/0.1 ml)	16

the meningioma (gel 4), and band 19 was present in the astrocytoma (gel 5). Therefore, the only unique bands in the long-surviving glioblastoma patient's membrane material were in the protein of band 1 and the low molecular weight proteins 16, 18 and 20.

It was of interest to explore further those antigens that induced cell-mediated immune responses by study in a selected group of patients. Response by the long-surviving glioblastoma patient to allogeneic glioblastoma antigens (Table 3) was confined to PAGE region 2 and 3, while response to autologous antigens (Table 4) was confined to pooled PAGE regions 3 and 4. Antigens unique to the long-survival glioblastoma tumor cells were present in region 4. Therefore, further separation of short-survival glioblastoma material was undertaken to split PAGE regions 2 and 3 into 2 separate sections (a and b) for further identification of reactive material. PAGE region 2a contained band 2, which is present in normal white matter but present in greater quantities in astrocytoma: a tissue-associated antigen. Only one patient (J.K., Table 5) gave a positive reaction to this antigen. PAGE region 2b contained band 7, which is also present in normal white matter, and two patients (R.L. and V.M.) responded to this tissue-associated antigen. PAGE region 3a produced no positive reactions. PAGE region 3b contained band 15,

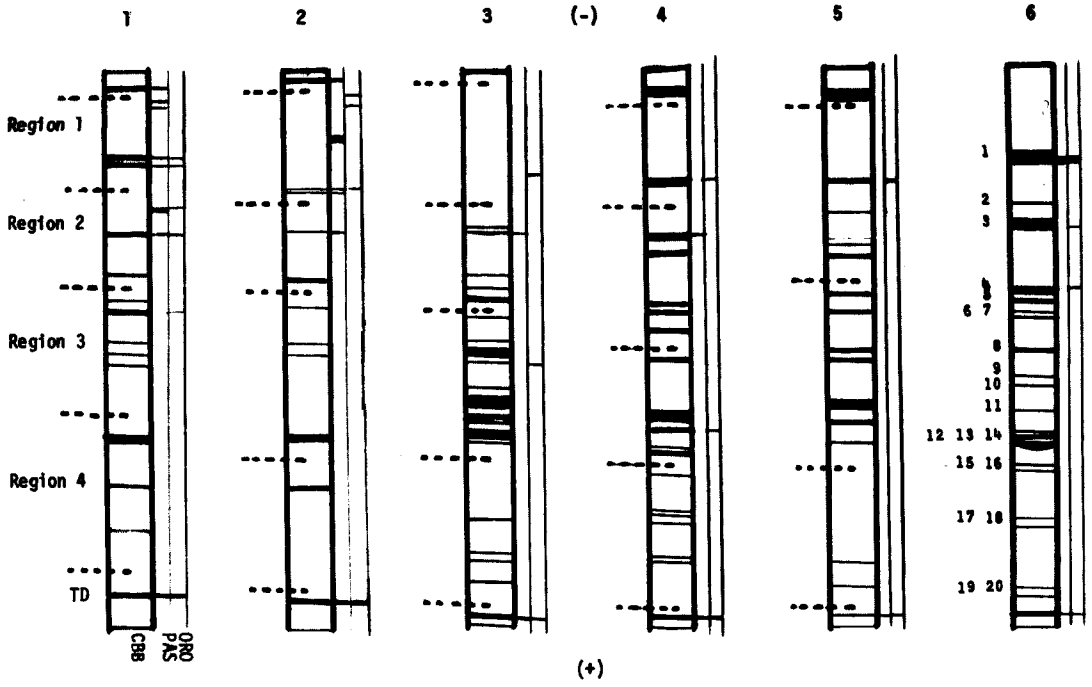

FIG. 3. Separation by polyacrylamide gel (3.5%, 4.75%, 7%, and 10% stacked gels) using bromphenol blue tracking dye (TD), and stained for protein with Coomassie brilliant blue, for carbohydrate using periodic acid-Schiff stain (PAS), and for lipid using oil red O stain (ORO). Three separations each of soluble membrane material for staining purposes were performed using 1) 108 μg protein per gel of gray matter of normal brain, 2) 103 μg protein per gel of white matter of normal brain, 3) 96 μg protein per gel glioblastoma (Kron), 4) 100 μg protein per gel of meningioma, 5) 117 μg protein per gel of astrocytoma, and 6) 100 μg per gel of a tissue culture preparation of glioblastoma from the patient with long survival. The materials were separated into regions, and a series of unstained gels from each of the first five groups were sliced (see dotted lines) and materials from each region eluted, concentrated by ultrafiltration and used for various studies. Protein bands of the glioblastoma patient with long survival (number 6) which differed from protein bands of the glioblastoma patient with short survival (number 3) are: 1, 2, 7, 15, 16, 18, 19 and 20. Of these bands, band number 1, the carbohydrate portion only, is also present in the normal white matter. Band number 2 is also seen present in the normal white matter and in the astrocytoma (number 5) separations. Band number 7 is also seen present in the white matter. Band number 15 is also present in the meningioma separation; band number 19 is also present in the astrocytoma separation. This would mean that the only unique bands present in the long survival glioblastoma patient material are the protein portion of band number 1 and low molecular weight bands number 16, number 18 and number 20. For this particular study primary passage of biopsied cells was used for simultaneous analysis; however, the profile agreed with the previous PAGE profiles of primary tissue preparations from five separate biopsies.

TABLE 5. Skin Tests for Delayed Hypersensitive Reactions to Separated Soluble Cell Membrane Antigens from a Short-surviving Glioblastoma Patient

Short-surviving glioblastoma PAGE regions in skin tests*	TAA antigens present Band number	(Where present)	E.K. Glioblastoma multiforme	J.K. Astrocytoma grade II	R.L. Glioblastoma multiforme	V.M. Meningioma	F.S. Lymphocytic lymphoma with frontal metastases	S.B. Glioblastoma multiforme
			(mm induration at 48 to 72 hours)					
1			0	0	0	0	3	0
2a	2	(white matter astrocytoma)	4	5	4	0	0	0
2b	7	(white matter)	3	3	5	7	0	1
3a			0	0	4	0	0	4
3b	15	(meningioma)	5	4	4	7	9	6.5
4	19	(astrocytoma)	8	5	5	3	0	5
Dermatophytin						8	5	6
Candida			0	8	0			
SKSD			0	0	1	15	6	14

* Positive test: >5 mm induration after 48 hours, using 20 to 25 μg protein per test.

which was present in high quantity in meningiomas as well as in the long surviving glioblastoma. Positive reactions were seen in four of six patients tested. Band 19 was the only unique band present in PAGE region 4 and was seen associated with astrocytoma; all of the patients with glioblastoma reacted to this antigen (Table 5). The tests showed at least two tissue-associated antigens (numbers 2 and 7) and two tumor-associated antigens (numbers 15 and 19) which produce CMI responses in patients with brain tumors. One TAA (number 15) is associated mainly with meningioma and not glioblastoma with one exception, the long-survival glioblastoma. It evokes response in patients with short-survival glioblastoma as well. These antigens would account for most of the cell-mediated immune responses seen in short-surviving patients. Antigens 16, 18 and 20 were unique for the long-surviving glioblastoma patient. This patient had responded to skin tests of the tissue antigens in PAGE region 2 of normal white matter and short-survival glioblastoma tissue, to region 3 of meningioma cells and to the unique antigens and TAA 19 contained in region 4.

The comparison of the PAGE separation of serum (Fig. 4) from our long-surviving patient

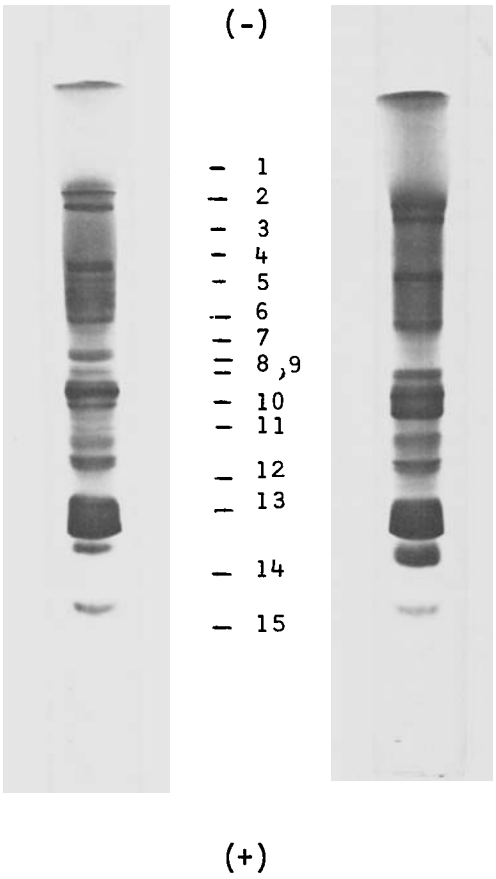

FIG. 4 Analysis of components of sera from A (left) our long-surviving glioblastoma patient and (B, right) short-surviving patient (Wat) are similar. Seven hundred micrograms of each sera were separated; CBB stain. Components, numbered 1 to 15 are: 1) beta lipoprotein; 2) haptoglobin; 3) alpha 2-macroglobulin; 4) beta 2-glycoprotein; 5) beta 1c and beta la globulins; 6) alpha 1-lipoprotein; 7) ceruloplasmin; 8) transferrin; 9) hemopexin; 10) alpha 2-HS-glycoprotein; 11) Gc-globulin; 12) albumin; 13) alpha-1-acid-glycoprotein; 14) thyroxine-binding prealbumin; 15) prealbumins, unknown components. Components 13 and 14 differ markedly in milligram percent levels: patient number 1 prealbumin is 152% and α_1-acid glycoprotein is 254%; patient number 2 prealbumin is 47% and α_1-acid glycoprotein is 422%.

and from a matched patient who survived only 5 months postoperatively failed to show any significant qualitative difference. However, there was a striking difference in the quantitative measurements of two prealbumin components. The short-surviving glioblastoma patient had a lower prealbumin level (47 mg/100 ml) and a higher α1-acid glycoprotein level (422 mg/100 ml) than the long-surviving patient (152 mg % prealbumin and 254 mg % α_1-acid glycoprotein). This contrast is even apparent in the photograph (Fig. 4). As will be reported elsewhere, we find that it is the ratio of these two components which gives a fairly sensitive index of the degree of malignancy, α_1-acid glycoprotein/prealbumin. For the short-surviving patient this index is 8.94 and for the long-surviving patient this index is 1.67.

DISCUSSION

Immunocompetence surveillance of cancer patients is useful in following the course of the disease and its relation to conventional therapy.[2,7,11] Several studies have indicated this possibility for patients with brain tumors.[10,13,14] Because of the long survival of our patient with repeated extracranial metastasis of this aggresive tumor, we felt that immune testing was indicated. Early in the testing the PHA responsiveness, T-cell determinations, and skin tests indicated that the patient was relatively anergic. The most reasonable explanation for the initial anergic state of the patient is that it represented a state frequently noted in glioblastoma patients often associated with a poorer prognosis.[2,5] Therefore it is possible that the patient was in a state favoring advancement of his tumor before there was any clinical indication of more extensive metastases. With further testing on the second test 4 months later and in the third and fourth tests 1 year later he showed a progressive increase in immune responsiveness in all tests performed both *in vivo* and *in vitro*. The patient was still able to respond antigenically to his tumor, though the test values such as the T-cell determination never reached the normal mean values. One explanation is that the rapid growth of his tumor, possibly because of his initial anergic state, finally surpassed his immune response and he succumbed to his disease. The fact that with advancing disease the parameters improved possibly reflects a uniqueness of response for this particular patient, since this pattern is reversed in patients with short-term glioblastoma.

Meningioma-related TAA 15 was present in cells of the long-survival glioblastoma but not seen in short survival glioblastoma materials and may suggest a different immunogenetic pattern. In a separate report we show the presence of a dicentric chromosome marker that appears to be associated with meningioma cells and is also present in the cells of the long-surviving glioblastoma patient. The unique antigens found in the prealbumin anodic portion of the PAGE gels, present only in this patient's tumor cells, as well as those identified for other brain tumors, must be further characterized.

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