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Secondary Antibody Responses to Thymus-independent Antigens in Mice with Brain Tumors

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Summary. **After transferring spleen cells from C57BL/6 (B6) mice into C57BL/10Cr (B10Cr) mice, secondary responses to trinitrophenylated lipopolysaccharide in the recipient B10Cr mice were investigated. Though we observed a primary response to TNP-LPS in the recipient B10Cr mice to which spleen cells of B6 mice were transferred, cells primed with TNP-LPS caused stronger IgG responses than virgin cells. The production and maintenance of memory B cells were hardly affected in glioma bearing mice. However, the isotype-switch of IgM to IgG on a secondary response appeared weaker in memory B cells from mice bearing brain tumors than in those from normal B6 mice. In spite of the memory-cell transfer of B6 mice, glioma-bearing B10Cr mice could not maintain the immunological memory. The isotype-switch was also incomplete in these mice. The antigen-presenting ability of peritoneal exudate cells from glioma-bearing B6 mice was also weaker than that of normal B6 mice. Thus, we observed a disturbance of antibody responses to the thymus-independent antigens in glioma-bearing mice, which is attributed to the impaired antigen presenting ability of the macrophages.**

INTRODUCTION

A second encounter with the same antigens leads to more rapid and greater responses. This immunological memory is thought to be brought about by the generation of memory B cells.[1] As thymus-independent responses do not usually show memory, the generation of memory B cells is considered to be linked to T cells. In certain mouse strains, however, a second response to the thymus-independent antigens, trinitrophenylated lipopolysaccharide (TNP-LPS), can be induced.[2] In this secondary response, only the LPS-reactive B cell can respond to the hapten TNP specifically.

If LPS-reactive B cells from C57BL/6 (B6) mice are transferred into a histocompatible LPS-nonresponding strain of C57BL/10Cr (B10Cr) mice, secondary responses obtained in the recipients can be attributed to the transferred B cells of B6 mice.[3] Because of the short life expectancy of transferred B cells in the recipients, memorial maintenance can also be ascribed to the newly formed cells caused by persisting antigens in the recipients.[4] Therefore, these experimental models seem very useful to study the mechanism of the immunological memory.

A role for the central nervous system in the regulation of immune responses, on the other hand, has been reported by others.[5,6] There is no doubt that immunological cells are exposed to brain messengers such as hormones and neurotransmitters,[7,8] and immunological organs are innervated by autonomic nerves.[9,10] Namely, there may be informative channels between the immune system and the central nervous system through which they communicate. For example, the expression of Ia antigens is detectable in several glioma cell lines[11] and some astrocytes can present the myelin basic protein to some T cell lines.[12] These reports indicate that immunological changes may be caused by gliomas.

Furthermore, some kinds of immunological abnormalities have been reported in patients with brain tumors,[13,14] and abnormalities of neurotransmitters can modulate the specific antibody response *in vivo*,[15] but the minute mechanism of this immunosuppression remains to be understood.

In this report, we studied the effects of brain tumors on the immunological function and, in particular, immunological memory using secondary antibody responses ascribed to a single cell population as mentioned above.

MATERIALS AND METHODS

Mice

Both B6 and B10Cr mice were obtained from Jackson Laboratory (Bar Harbor, ME, U.S.A.). All animals were between 6 and 12 weeks age. For each experiment, control and experimental mice were matched for age and sex. To make brain tumor models, 1-100×10^5 of C6 rat glioma cells were injected into these mice as previously described.[16] Briefly, after scalp incision, a burr hole was made in front of the coronal suture, 2.5 mm to the right of the midline. The glioma cells were suspended in a solution of RPMI 1640 (Roswell Park Memorial Institute, Buffalo, NY, U.S.A.). The tumor cell suspension (20 μl) was injected using a Hamilton syringe (Hamilton Co., Reno, NV, U.S.A.) with a 27-gauge needle, and injected to a depth of 3 mm from the outer table of the frontal lobe. After injection of the suspension, the wound was closed with clips. Ten days after the inoculation of C6 cells, mice were used as models bearing brain tumors. We chose this particular animal model of brain tumor because the C6 glioma cell caused sufficient immunological reaction to provide an adequate human model.

Cell transfer experiments

Spleen cells were prepared from B6 mice by the method of Miller and colleagues.[17] After washing with Tris-buffered saline, 2×10^7 sells were injected intravenously into normal and/or tumor bearing B10Cr recipient mice.

Immunological response

TNP-LPS was prepared by the procedure described by Rittenberg and colleagues.[18] Briefly, 100 mg of LPS (Difco Laboratories, Detroit, MI, U.S.A.) was dissolved in 5 ml of cacodylate buffer, pH 6.9, and 60 mg of 2, 4, 6, -trinitro-benzenesulfonic acid (Pierce Chemical Co., Rockford, IL, U.S.A.) in 5 ml buffer was added at room temperature 2 h later. The optimal dose of 8 μg in 0.3 ml was used for i.p. priming and challenge injections into B10Cr recipient mice.

Measurement of antibodies

Anti-TNP antibodies were estimated by immunosorbent assay in the sera of immunized mice on Days 7 and 14 after booster injections. Enzyme-linked immunosorbent assay (ELISA) was a modification of the technique described by Guesdon and colleagues.[19] Briefly, dinitrophenyle-coated ELISA plates (Nunc, Roskilde, Denmark) were saturated with 2% bovine serum albumin for 6 h, washed, and incubated overnight at room temperature with 100 μl of different dilutions of the sera to be tested. After washing, rabbit anti-mouse isotype sera (Bionetics, Kensington, MD, U.S.A.) were added to the plate. After 2 h incubation at 37°C, the plate was washed and incubated for 90 min at 37°C with peroxidase-labeled sheep anti-rabbit sera (Sigma Chemical Co., St. Louis, MO, U.S.A.). Finally, the absorption was measured on a titertek multiscan photometer (Flow Laboratories, Irvine, U.K.) using coloration with the o-phenylenediamine tetraacetate substrate. The specificity of the antidinitrophenyle assay was determined by the binding inhibition by 2, 4-dinitrophenyle-glycine of the sera from TNP-LPS-immunized mice.

Antigen-presenting ability of peritoneal exudate cells in antibody response

Normal spleen cells from B6 mice suspended at 6×10^6/ml in RPMI 1640 with 10% fetal calf serum were cultured in a 60×15 mm plastic dish (No. 3002, Falcon) for 60 min at 37°C. Non-adherent cells were passed through a Sephadex G-10 column.[20] Effluent cells were used as a macrophage-depleted fraction. Unfractioned cells or macrophage-depleted spleen cells from B6 mice (5×10^5) were pulsed with TNP-LPS for 72 h. Then, anti-TNP antibodies in the supernatant were estimated by immunosorbent assay as mentioned above. A variable number of peritoneal exudate cells from B6 mice bearing glioma which had been injected i.p. with 5 ml of 3% thioglycolate broth were added to this culture system for the investigation of their antigen-presenting ability. Three days later, the same immunosorbent assay was carried out to detect anti-TNP antibodies in the culture soup.

RESULTS

Analysis of immune responses to thymus-independent antigens

We tested LPS responsiveness in a transfer model in which the spleen cells from an LPS-responder mouse strain (B6) were transferred i.p. into a histocompatible LPS-nonresponder mouse strain (B10Cr). As shown in Fig. 1, B6 mice showed primary and secondary responses to TNP-LPS molecule, whereas the B10Cr strain could not respond to this antigen.

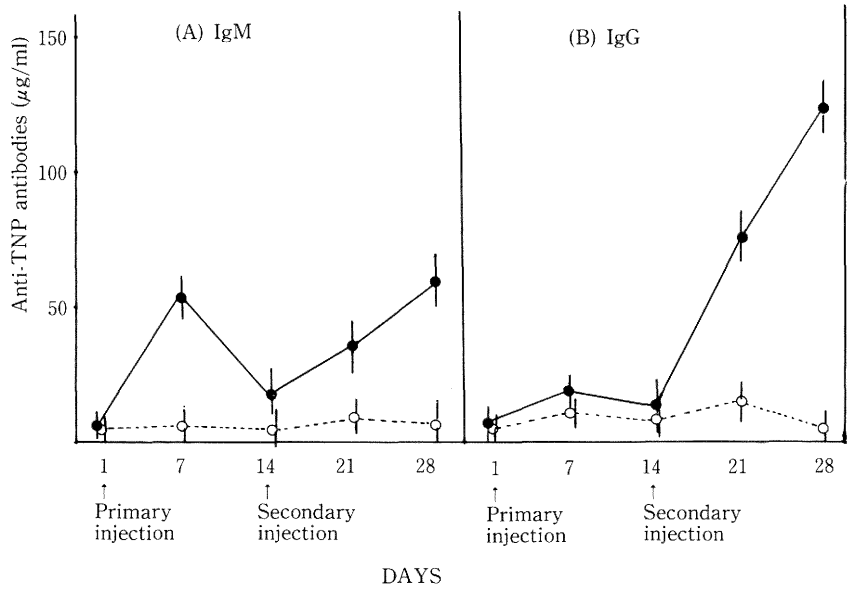

Fig. 1. Primary and secondary responses to TNP-LPS in B6 (●) and B10Cr (○) mice. Both mice were primed with 8 μg of TNP-LPS, and bled 7 and 14 days later. They were then boosted with TNP-LPS 2 weeks later, and bled 7 and 14 days after the challenge. The results are expressed as mean μg/ml ($\pm$SD) anti-TNP antibodies (6 mice per group) estimated by ELISA assay (A: IgM, B: IgG).

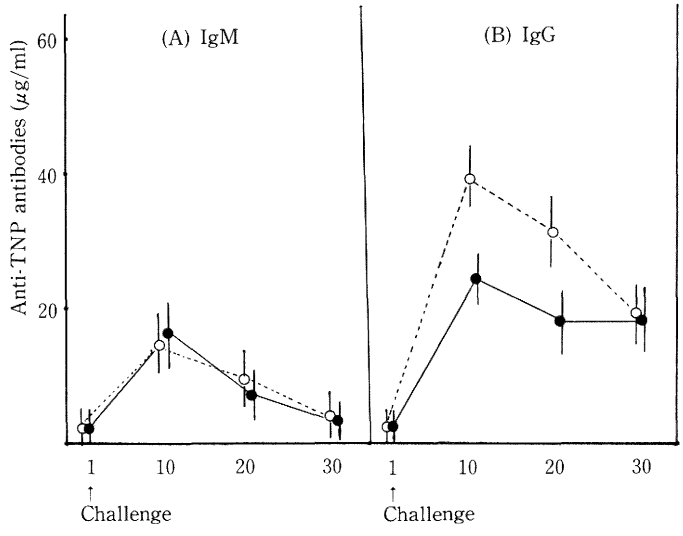

Fig. 2. Primary and secondary responses to TNP-LPS in B10Cr mice injected with spleen cells from B6 mice. B10Cr mice received 2×10^7 virgin (●), or TNP-LPS primed (○) spleen cells from B6 mice. After challenge injection of 8 μg of TNP-LPS, they were bled 10, 20, and 30 days later and sera were collected. The results are expressed as mean μg/ml ($\pm$SD) anti-TNP antibodies (8 mice per group) estimated by ELISA assay (A: IgM, B: IgG). Differences of IgG production in the two groups were statistically significant ($p<0.05$ (t-test)).

Though we tried another booster injection of tuberculin or LPS alone, no increase in the antibody levels toward these antigens was observed. From these results, this immunological reaction was considered to be specific to TNP-LPS alone.

After transferring spleen cells from B6 mice into B10Cr mice, secondary responses to TNP-LPS in the recipient B10Cr mice were examined. In this transfer model, memory responses to TNP-LPS were considered to be restricted to the LPS-reactive B cell alone. As shown in Fig. 2, we observed a primary response to TNP-LPS in the recipient B10Cr mice injected with spleen cells from B6 mice. However, the spleen cells from B6 mice primed with TNP-LPS caused stronger IgG responses in the recipient B10Cr mice than did virgin spleen cells from B10Cr mice. These data suggest that immunological memory induced by spleen cells from B6 mice primed with TNP-LPS can be maintained for less than 3 weeks.

Effect of brain tumor on immunological memory

On the other hand, the immune system is generally viewed as being regulated by a variety of internal mechanisms. Assuming a brain tumor affects immunological memory, we studied whether and/or how the brain could influence the thymus-independent secondary antibody responses, using this transfer model. Mice bearing C6 glioma cells appeared to be proper experimental models of brain tumors, regard-

Fig. 3. Photomicrograph of the intracerebral tumor at 21 days after injection of C6 glioma cells into the brain of B10Cr mice.

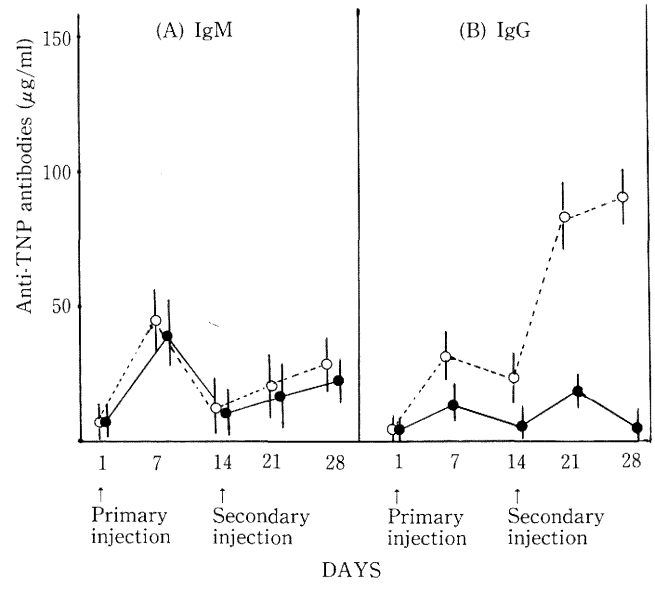

Fig. 4. Primary and secondary responses to TNP-LPS in B6 mice bearing brain tumors. Normal saline or 1×10^6 C6 glioma cells were injected into the frontal lobe of B6 mice. Seven days later, these mice were primed with 8 μg of TNP-LPS and bled 7 and 14 days later after the challenge. The results are expressed as mean μg/ml ($\pm$SD) anti-TNP antibodies (7 mice per group) estimated by ELISA assay (A: IgM, B: IgG). ○, Normal saline was injected; ●, 1×10^6 C6 cells were injected. Though IgM production was hardly affected, there were statistically significant differences in IgG production between normal and glioma bearing mice ($p<0.02$ (t-test)).

less of sex or strain. The average survival of mice inoculated with a million glioma cells was 40 days. Fig. 3 shows their photomicrograph. All mice were confirmed as having glioma in their frontal lobes. In all experiments, models of brain tumors were injected with 1×10^6 of C6 10 days previously in their frontal lobes. At this point, all mice showed no altered eating or drinking behavior. Their spleens weighed the same as those of the control group. Although there were some problems in a non-syngeneic model and/or in glioma-specific immunosuppression, our transfer model was considered to yield a result similar to a syngeneic model. The results of the experimental transfer models comparing normal and tumor models are summarized in Fig. 4 and 5. The results in Fig. 4 suggest that the production and the maintenance of memory B cells are hardly affected by the presence of brain tumors. However, the IgM to IgG isotypeswitch on a secondary response appeared weaker in memory B cells from mice bearing brain tumors than in those from normal B6 mice. All subtypes of IgG including IgG2b were disturbed in this impaired secondary response (Table 1). In the presence of brain tumors, moreover, B10Cr mice could not maintain the immunological memory in spite of the cell transfer of effective memory B cells from mice (Fig. 5).

Antigen-presenting ability

Antigen-presenting ability of the peritoneal exudate cells from B6 mice bearing glioma was examined

Table 1. Subclasses of IgG detected in the sera of glioma-bearing B6 mice after TNP-LPS stimulation

Response[a)]	IgG1	IgG2a	IgG2b	IgG3[b)]
Primary response				
Control	54± 9	7±3	38±8	<1
Glioma-bearing	52± 8	6±3	41±7	<1
Secondary response				
Control	58± 9	7±4	33±7	<1
Glioma-bearing	74±12	6±2	19±5	<1

[a)] IgG subclasses were determined by ELISA using the sera of the control or glioma-bearing B6 mice.
[b)] The values (mean±SE) are expressed as percentages of total IgG.

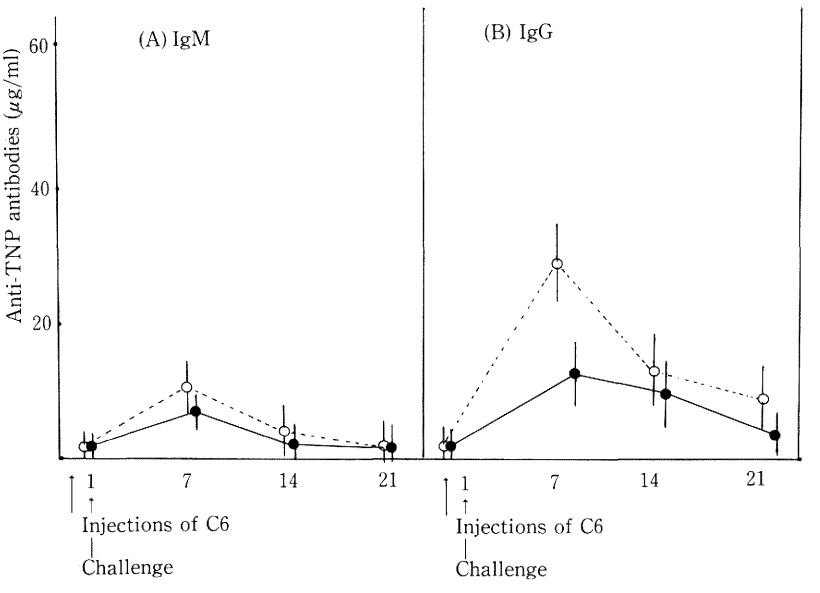

Fig. 5. Primary and secondary responses to TNP-LPS in B10Cr mice bearing brain tumors who had also received primary spleen cells from B6 mice. Normal saline (○) or 1×10^6 C6 glioma cells (●) were injected into the brains of B10Cr mice injected with 2×10^7 spleen cells of B6 mice primed with TNP-LPS. Following the challenge with TNP-LPS next day, sera were collected 7, 14 and 21 days later. The results (A: IgM, B: IgG) are expressed as mean μg/ml (±SD) anti-TNP antibodies (5 mice per group) estimated by ELISA assay. In glioma-bearing mice, differences in the two groups that were statistically significant ($p<0.01$ (t-test)) were noted both in the production of IgM and IgG.

using macrophage-depleted spleen from B6 mice for the antibody responses to TNP-LPS.

As shown in Fig. 6, though there are other potential biological variables, the impaired ability of antigen presentation of those spleen cells appeared to be one important factor for the immunological disturbance. The disturbance of the isotype-switch also suggests that the T cell help may be affected by the brain, because the differentiation of B cells into IgG secreting B cells is considered to require T cell help. Although other factors such as the number of cytokines or general stress might bring about this immunosuppression, T cell help seemed to be disturbed mainly in glioma-bearing mice.

DISCUSSION

The immune system has the ability to learn. While the first encounter of the B cell with antigens leads to the slowly rising synthesis of an antibody, some B cells persisting as memory cells cause a rapid and greater secondary response. This secondary response is therefore more effective, and the B cells are described as showing a memory of the antigen in question.[21] On the other hand, attention has been paid to the role of the brain in the regulation of immune responses. Immunological cells are exposed to brain messengers such as hormones and neurotransmitters. Immunological organs are innervated by autonomic nerves. Receptors for these messengers have also been demonstrated in some immunological cells.

In order to investigate the effects of a brain tumor on the immune system, we have studied basic mechanisms of immunological memory using a transfer model in which secondary responses could be ascribed to a single celll population. Moreover, the effects of brain tumors on immunological memory were examined to understand the immunosuppressive mechanism observed in mice bearing brain tumors.

For the study of secondary antibody responses to thymus-independent antigens, we used a transfer model in which spleen cells of LPS-responder mice (B6) were transferred into histocompatible LPS-non-responder mice (B10Cr). In these B10Cr mice, the primary and secondary antibody responses to TNP-LPS were not detected (Figs. 1 and 2). Though immunological barriers between B6 and B10Cr mice must be taken into consideration, we believe that B10Cr mice did not recognize the transferred B6 cells as foreigners because we observed that the decreasing rate of B6 cells in the recipient B10Cr mice was not affected by any previous immunization of the recipients with B6 spleen cells. This secondary memory response transferred by B6 spleen cells, however, was maintained for only 2-3 weeks (Fig. 2). Therefore, this immunological memory seemed to require a continuous B cell supply and/or residual antigen presentation for its maintenance.

Comparing the results of these fundamental experiments with those obtained from the glioma-bearing mice models, we also studied the effect of brain tumors on secondary antibody responses to thymus-independent antigens. Memory B cells of glioma-bearing B6 mice showed almost the same response as those of normal B6 mice. However, the IgM to IgG isotype-switch in a secondary response was not complete in the glioma-bearing B6 mice (Fig. 4). This switch-disturbance seemed to be due to the impaired production of all IgG subtypes (Table 1). In spite of the cell transfer of the primed B cells from B6 mice, on the other hand, B10Cr mice could not preserve the memory function in the presence of brain tumors. B10Cr mice with brain tumors also showed an incom-

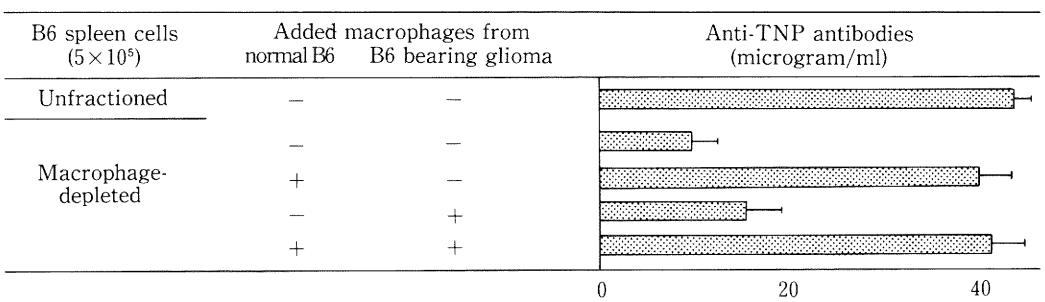

Fig. 6. Antigen-presenting ability of peritoneal macrophages from B6 mice bearing gliomas (n=8) in TNP-LPS responses. The mean level of anti-TNP antibodies of unfractionated cells is 45.6±9.4 μg/ml. The level of adherent cell-depleted fractions is 12.8±5.5 μg/ml. Following this, 1×10^5 of macrophages from B6 mice were added to B6 spleen cells. Peritoneal macrophages from glioma bearing B6 mice had no effect on this antibody responses.

plete isotype-switch in a secondary response (Fig. 5). These data suggest that the brain tumor may affect the differentiation and maintenance of B cells rather than the production of memory B cells. However, continuous presentation of residual antigens might also be disturbed in these states, as thymus-independent antibody responses were generated only by primed spleen cells from B6 mice in our transfer model.

The disturbance of immunological memory in brain tumor bearing mice was considered to be induced mainly by the impaired antigen presentation ability, since our previous experiments showed that continuous antigen persistence might lead to the maintenance of immunological memory. The results of Fig. 6 support this hypothesis, because the antigen-presenting ability of peritoneal exudate cells from glioma-bearing B6 mice was very weak compared with that of normal B6 mice. Furthermore, the disturbance of the isotype-switch upon a secondary antibody response suggests that T cell-help becomes very weak in these tumor bearing mice. Thus, it is clear that immunological memory observed in the secondary antibody response to thymus-independent antigens is closely connected with the brain activity. One of the major functions in the brain is to process information on changes in the external and internal environments detected by receptor organs.[22-24]

Our studies suggest that the brain may be informed by the intrusion of antigenic macromolecules, and that the brain may play an important part in the maintenance of immunological memory through the mechanism of persistent antigen presentation.[25] Several other mechanisms for the regulation of specific immune responses in the nervous system have also been presented; these include: that receptors for a number of neurotransmitter substances are presented on leukocytes, that stimulation of such receptors results in altered immunological activity,[26,27] and that a humoral response to antigens correlates with altered "firing" rates in hypothalamic neurons.[28] The immunological ability of the brain to recognize foreign elements is very weak.[29] These minute mechanisms are not clear, but our data suggest that the immunological memory is closely connected with the brain. Of course, there are many other factors affecting immunity in glioma-bearing mice. Though our model is non-syngeneic and immunosuppression may be affecting our results, this particular experimental model served well to study secondary immunological responses using the cell-transfer system.

The host response to the glioma may alter the impaired immunity described above. However, these studies are useful in understanding abnormal immunological states observed in patients with malignant brain tumors.[30]

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