

## An immunohistological study of the presence of inflammatory cells in malignant brain tumors

DIANA STANCA<sup>(1)</sup>, ȘTEFANIA CRĂIȚOIU<sup>(2)</sup>, VALERICA TUDORICĂ<sup>(1)</sup>,  
CARMEN ALBU<sup>(1)</sup>, OANA ALEXANDRU<sup>(1)</sup>, DENISA PÎRȘCOVEANU<sup>(1)</sup>,  
F. TRIFAN<sup>(1)</sup>, CORNELIA ZAHARIA<sup>(1)</sup>

<sup>1</sup> University of Medicine and Pharmacy Craiova, Department of Neurology, <sup>2</sup> University of Medicine and Pharmacy Craiova, Department of Histology

**ABSTRACT** The purpose of this study is to appreciate if there is an immune response in malignant brain tumors. Material and methods: Eleven astrocytomas of varying histological features were immunohistologically studied, using a panel of monoclonal antibody against CD20cy to highlight T-lymphocytes, CD3 for B-lymphocytes and CD68 as marker of the macrophages. Ki67 antibody was used as marker of proliferation. We evaluated the presence of these markers in peritumoral stroma and intratumoral tissue. Results: In our study, none of the astrocytic tumors studied showed B-lymphocytes. The presence of T lymphocytes was evidenced in all 11 selected cases. While low-grade astrocytomas showed positive CD3 immunostaining only in tumoral cells, anaplastic astrocytomas and glioblastomas revealed positive immunostaining also in perivascular space. As it was expected, mean values for proliferative marker Ki-67 revealed that there was a significant difference between the indices of low- (grade II) and high-grade tumors (grade III and IV) ( $P < 0.05$ ), but not between grade III and IV tumors ( $P > 0.05$ ). Conclusion: Our study demonstrated that the numbers of macrophages and T-lymphocytes in astrocytic tumors are related to highly malignant histological features. The presence of these cells in such a kind of neoplastic lesions raises questions concerning the possible immune response and the possible relation of this to prognosis.

**KEY WORDS** astrocytoma, glioblastoma, immunohistochemistry, T-lymphocytes, macrophages

### Introduction

The great structural complexity of the central nervous system is reflected by the diversity, ever-growing, of the new recognized neoplastic entities that affect nervous tissue. The classification and the grading of brain tumors is still based on morphological appearance, but the researches that were conducted in the recent years to understand the molecular mechanisms that lead to their development, have led to the opportunity to share the tumors with similar morphological features in different genetic subtypes. Solid tumors are composed not only of tumor cells, but they also contain a variety of stromal cells, including fibroblasts and inflammatory cells, that are represented by macrophages, neutrophils, mast cells and lymphocytes (1). The presence of the inflammatory cells in the tumoral tissue has been reported in several studies since the nineteenth century. These researches supported the hypothesis that inflammatory status to play a key-role in the development and in the progression of the tumors (2). The presence of the lymphocytes and macrophages in solid tumors has been well-documented over time (3), but the obvious role of these lymphoreticular cells, especially their antitumoral role is still unclear. The brain, that was characterized by the lack of the conventional lymphatic system and by the presence of blood-brain barrier, has long been regarded as an

“immune privileged” system (4, 5). But subsequent studies that have investigated the presence of the inflammatory cells in brain tumors, showed a depression of the systemic immune response in these neoplasms (6, 7) and, also, the presence of the mononuclear white blood cells in patients diagnosed with brain tumors (8, 9). Based on these observations, the purpose of this study is to appreciate if there is an immune response in the brain tumors.

### Material and methods

This study was performed on sections of primitive malignant brain tumors from 11 patients (6 men and 5 women, mean age  $60,9 \pm 8,08$  years). These patients were diagnosed, by neuroimaging methods (computed tomography and/or nuclear magnetic resonance) in Clinic of Neurology Craiova with primitive brain tumors and referred for surgery to the Neurosurgery Department of The Institute of Cerebrovascular Disease, Bucharest. The tumors specimens were fixed immediately after resection in 10% buffered formalin for 18-20 hours, being, after that, sent to the Research Center for Microscopic Morphology and Immunology of the University of Medicine and Pharmacy of Craiova, where they were further processed for inclusion in paraffin. Hematoxylin-eosin was used for routine staining and the histopathological diagnosis was performed in

accordance with The 2007 WHO Classification of Tumors of the Central Nervous System. For immunohistochemical study we used CD20cy antibody to highlight T-lymphocytes, CD3 antibody for B-lymphocytes and CD68 antibody as marker of the macrophages. Ki67 antibody was used as marker of proliferation. We evaluated the presence of these markers in peritumoralstroma and intratumoral tissue. The various dilutions, recommended by the manufacturer, are shown in Table 1.

**Table 1: Antibodies used in this study**

Antibody	Epitope/Marker	Dilution	Antigenic recovery	Source
CD20cy	B-lymphocytes	1:100	Citrate, pH6	Dako
CD3	T-lymphocytes	1:100	Citrate, pH6	Dako
CD68	Macrophages/monocytes	1:100	Citrate, pH6	Dako
Ki-67	Proliferation marker	1:50	EDTA, pH9	Dako

**Table 2: Clinical and demographic data**

WHO grade	No.	Gender		Mean age (years)	Mean level of education	Involved hemisphere and location of tumor		Mean size (cm)
		Male	Female			Right	Left	
<b>Grade II:</b> Diffuse astrocytomas	2	2	0	48	12	2 - P	0	3,5
<b>Grade III:</b> Anaplastic astrocytomas	4	4	0	61	11,75	1 - F 1 - P	2 - F-P	3,75
<b>Grade IV:</b> Glioblastomas	5	0	5	61,5	7,8	2 - F 2 - F-P 1 - T-P	0	5,35
<b>Total</b>	<b>11</b>	<b>6</b>	<b>5</b>	<b>56,83</b>	<b>9,95</b>	<b>9</b>	<b>2</b>	<b>4,2</b>

## Results

The clinical and demographic data about patients and data on locations of the tumor processes are summarized in Table 2.

As we have been shown in the table above, we remarked that the mean age of the patients with diffuse astrocytomas (48 years) was significantly statistic lower than the mean age of the patients with anaplastic astrocytomas (61 years) or glioblastomas (61,5 years), respectively.

In terms of macroscopic appearance, it was difficult to differentiate between diffuse astrocytomas and anaplastic astrocytomas; the anaplastic tumors, however, had a more net limit in comparison with the diffuse ones.

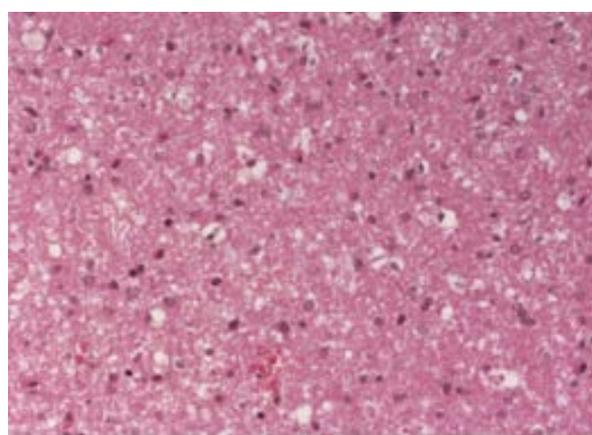
Histopathological investigation of these tumors revealed that the most numerous studied lesions were malignant (4 anaplastic astrocytomas and 5 glioblastomas), while diffuse astrocytomas encountered only 2 cases. On sections, the diffuse astrocytomas showed well differentiated

The colored slides were investigated using a Nikon Eclipse 90i microscope, equipped with aapocromate plan objective, a 5 megapixel CCD sensor and a morphometric imagistic analysis (Nikon NIS-Elements). Areas of interests were photographed and stored in \*TIFF format images, in a database.

The expression of the CD20cy, CD3 and CD68 markers was scored by counting the number of positive cells and evaluating the signal intensity. The intensity of staining was graded as negative (-) when there was no staining, weak (+) when less than 5% of cells labeled, moderate (++) when between 5 and 50% of cells labeled and strong (+++) when more than 50% of cells labeled.

The Ki-67 labelling index was calculated as a percentage of labeled nuclei per 1000 cells. One thousand tumor cells were counted in several areas of the tissue where positively stained nuclei were evenly distributed. But in those cases with uneven distribution of positive nuclei, the tumor cells were counted in the areas with highest density of positive nuclei by visual analysis [10].

neoplastic astrocytes in a microcystic tumor matrix, with moderate cellularity and nuclear atypia, but without mitotic activity (Fig.1), while histopathological examination of anaplastic astrocytomas notified diffuse increased cellularity, nuclear atypia and mitotic activity.



**Figure 1: Diffuse astrocytoma, HE, 20x.**

Histopathological analysis of the glioblastomas revealed poorly differentiated, highly anaplastic and pleomorphic tumor cells that had increased nuclear atypia and highly mitotic activity. On sections, we noticed intense vascular proliferations and areas of necrosis (Fig.2).

**Results of immunohistochemical analysis**

Immunohistochemical staining with the antibodies used in this study was assessed semi-quantitatively from percentage of tumor cells staining, and the results are shown in Table 3.

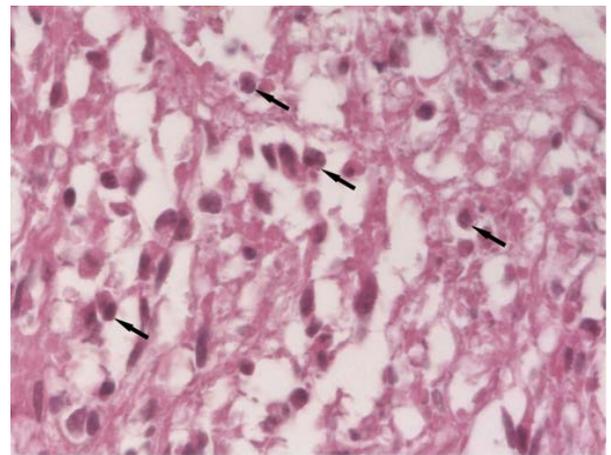


Figure 2: Glioblastoma, HE, 60x.

Table 3: Immunohistochemical analysis

Tumor type	No. of cases	CD20cy expression				CD3 expression			CD68 expression				Ki-67 index
		+++	++	+	-	Intratumoral (intensity)	Perivascular (intensity)	-	+++	++	+	-	
Diffuse astrocytoma	2	0	0	0	2	2 (++)	0	0	0	0	1	1	3.5
Anaplastic astrocytoma	4	0	0	0	4	2 (+)	2 (++)	0	1	2	0	1	11.5
Glioblastoma	5	0	0	0	5	0	5 (+++)	0	5	0	0	0	16.2

In our study-group, none of studied sections had presented reactivity to CD20cy antibody.

The two cases of diffuse astrocytomas showed positivity to CD3 antibody in tumoral cells with moderat intensity, while the fragments of both anaplastic astrocytomas and glioblastomas revealed the presence of T-lymphocytes that were around vessels (Fig.3) with higher intensity than in low-grade glioma.

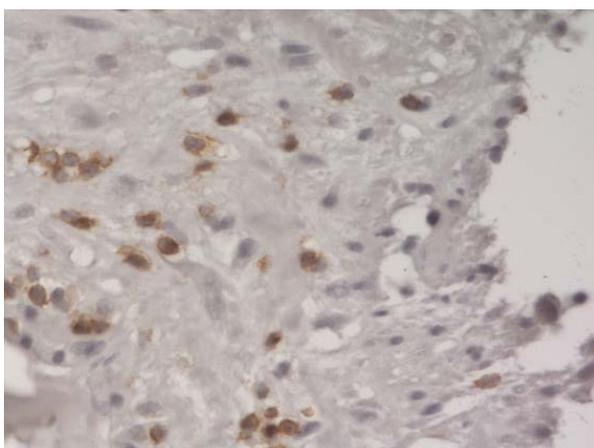


Figure 3: Glioblastoma, CD3 (+) in perivascular space, 40x.

In anaplastic astrocytomas we found the invasion of tumor parenchyma in two cases, while

the sections of the glioblastomas studied did not reveal the presence of intratumoral invasion.

The study of the CD68 immunorexpression revealed that 81,3% (9 cases) of astrocytic tumors expressed this antibody with variable intensity. The two cases in which CD68 was absent were represented by one case with diffuse astrocytoma and one case with anaplastic astrocytoma. Low intensity was showed by a case of diffuse astrocytoma; moderate intensity was showed by 2 cases of anaplastic astrocytomas, while an anaplastic astrocytoma and all 5 glioblastomas had expressed CD68 with maximum intensity (Fig.4).

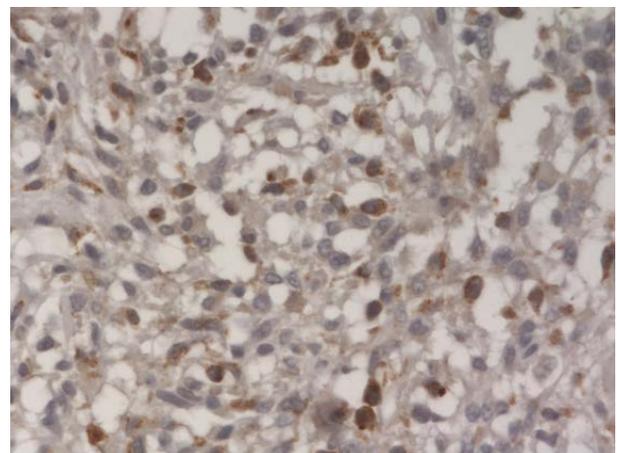


Figure 4: Glioblastoma, CD68 (+),40x.

These aspects had led to the conclusion that the expression of CD68 was significantly correlated with the degree of malignancy.

Immunohistochemical data and estimates for mean values for proliferative marker Ki-67, revealed that mean values for grade II, III and IV tumors were for the whole material 3.5, 11.5 and 16.2, respectively. There was a significant difference between the indices of low- (grade II) and high-grade tumors (grade III and IV) ( $P < 0.05$ ), but not between grade III and IV tumors ( $P > 0.05$ ).

## Discussions

Many reports have shown that patients that were diagnosed with glioblastomas expressed deficiencies in cell-mediated immunity (11, 12). However immunohistological studies demonstrated different levels of infiltrates with mononuclear cell (13), consisting predominantly of T-lymphocytes (14) within malignant gliomas, and several studies suggested a correlation between the level of lymphoid cell infiltration and the patient survival time (15). Although these studies emphasizes the idea that immune reactions appear at the level of nervous system too (16, 17), the particular location of the tumor in a place that was long while thought to be "immune privileged" may have consequences for the presumed antitumoral immune response, which should not be considered similar to that observed in other tissues.

The presence of B cells could not be evidenced in astrocytic tumors in any researches; however, most astrocytomas are infiltrated by T lymphocytes (18). The study conducted by Yang and colleagues showed the presence of T-cell infiltrates in glioblastomas, and also showed that perivascular infiltration with these cells is significantly higher in glioblastomas than in low-grade astrocytomas (19). The study showed that in perivascular space of glioblastomas there was an inflammatory infiltrate richer in T cells than into intratumoral compartment, while in the low-grade astrocytomas there were similar amounts of T cells into the both compartments.

There are also articles that support the existence of macrophage and microglial infiltration in astrocytomas with different grades of malignancy (20). This study support the idea that there is a statistically significant correlation between the intensity of macrophage infiltration and degree of malignancy of astrocytic tumors (19, 21). These observations, taken together,

suggest that highly malignant tumors, particularly glioblastomas, induce a unique immune response.

In our study, none of the astrocytic tumors studied showed B-lymphocytes. However, the presence of T lymphocytes was evidenced in all 11 selected cases. While low-grade astrocytomas showed positive CD3 immunostaining only in tumoral cells, anaplastic astrocytomas and glioblastomas revealed positive immunostaining in perivascular space, too. These aspects are consistent with those observed by Yang I and colleagues (19).

Studying the CD68 immunostaining on sections, we remarked that CD68 expression was significantly increased in high-grade astrocytomas compared with low-grade astrocytomas.

Analysis of the proliferative potential of astrocytic tumors showed an obvious correlation between histological grade of the neoplasms and the intensity of the immunoexpression of Ki-67. This marker is therefore useful in predicting the biological behavior and brain tumor growth, proliferative activity being positively correlated with tumor grade and with patient prognosis.

## Conclusion

Our study demonstrated that the numbers of macrophages and T-lymphocytes in astrocytic tumors are related to highly malignant histological features. The presence of these cells in such a kind of neoplastic lesions raises questions concerning the possible immune response and the possible relation of this to prognosis. Taken together, all of these features may help to assess the likely aggressive behavior of the astrocytic tumors.

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*Correspondence Address: Diana Stanca MD, PhD Student, Department of Neurology, University of Medicine and Pharmacy of Craiova, StrPetruRares nr. 4, 200456, Craiova, Dolj, Romania, E-mail: diana\_stanca@yahoo.com*