Abstract. Today several findings indicate that a multifactorial strategy is the best strategy for treating cancer. Although radiotherapy, chemotherapy and surgery have been differently applied to treat human gliomas, no substantial improvement in life expectancy has been observed. Starting from 1992, the goal of our studies was to obtain new biological data on malignant astrocytomas to better understand the basic biology of the tumour and these are reviewed here. Immunotherapy may represent an available method in addition to the traditional therapeutic approaches. Starting from 1991, we set up a cellular model of lymphocytes obtained from peripheral blood of healthy patients treated with interleukin-2 (IL-2) in order to study the role of IL-2 in regulating lymphocytes activation. The lymphocytes responding to IL-2 treatment, named lymphokine-activated killer (LAK) cells, have a killer non MHC restricted activity, and are able to kill autologous and allogenic glioma cells. The interaction of LAK cells with various normal and transformed targets indicates that LAK cells recognize surface structures present both on normal and transformed cells. However, only the interaction with transformed cells induces lytic events and LAK cells can act as “surgical weapons” against tumour cells independently from their cell cycle. Much recent effort has focused on identifying the immune escape mechanisms used by glioma cells, in particular the modulation of the human leukocyte antigen (HLA) and antigen processing machinery component expression. Finally, another interesting field of research that will be presented is that of new tumour biomarkers of proliferation and apoptosis, cytokine/chemokine release and cytokine/chemokine receptors.

Gliomas are the most common type of primary brain tumour in adults and over the last 50 years the standard of care for these diseases has evolved slightly, but the clinical outcome remains unchanged. Further research to improve the treatment modalities is urgently needed because many clinical trials have not yielded convincing therapeutic benefit. Since 1992, the goal of our studies has been to obtain new biological data on malignant astrocytomas in order to better understand the basic biology of these tumours. It is well know that radiotherapy, chemotherapy and neurosurgery have been differently applied to treat human glioma with no substantial improvement in life expectancy. Today, various findings indicate that a multifactorial strategy is the best strategy for treating cancer and immunotherapy may represent a method in addition to the traditional therapeutic approach.

S.T. Rosemberg pioneered the development of immunotherapy for selected patients with advanced cancer and was the most cited clinician in the field of oncology for the 17 years from 1981 to 1998. He stated that “Biologic therapy differs conceptually from surgery, radiation therapy or chemotherapy because it acts not by directly attacking the tumour, but by stimulating the natural host defence mechanism to mediate cancer regression” (1). The rationale for immunotherapy in glioma was based on the detection of a significant number of lymphoid cells observed in these tumours. In fact, mononucleated inflammatory cell reaction has been observed in 30-60% of gliomas (2). This has suggested a possible immunological reaction on the part of the host but the specificity and the effect of the response have, however, been doubted (3, 4). For many years, our interdisciplinary group focused attention on the investigation of the interaction between immunocompetent cells and tumoral cells in the treatment of glioma, obtaining interesting biological data. However, the biological basis underlying the interaction between the immune system and tumoral growth is far from being explained.

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Starting in 1991-92, we set up an experimental cellular model of lymphocytes obtained from peripheral blood of healthy controls treated with interleukin-2 (IL-2) to study the role of IL-2 in regulating lymphocyte activation (5-7). The discovery that IL-2 confers oncolytic properties on lymphocytes determined the development of new strategies and innovative therapeutic protocols for human tumours. The lymphocytes which respond to IL-2 treatment, named lymphokine-activated killer (LAK) cells, have a killer, non major histocompatibility complex (MHC) restricted activity and are able to kill autologous glioma cells and allogenic glioma cells. All normal brain cells are LAK resistant. The interaction of LAK cells with various normal and transformed targets indicates that LAK cells recognize surface structures present on both normal and transformed cells. However, only the interaction with transformed cells induces lytic events and LAK cells can act as surgical weapons against tumour cells independently of the cell cycle (8-11). At present, the molecular mechanism of their killer activity is still unclear. Morphology and enzymatic patterns, studied by cytochemical techniques, showed that LAK cells differ from natural killer (NK) because they act through non-lysosomal type mechanisms. These cells represent a family of cells with many subsets, with a transient function inducible on CD-25-responding cells (12, 13). Another critical point regarding the IL-2 concentration (dose range: 10-1,000 pg/ml) used in the experiments *in vitro* should be noted: an increase in apoptotic cells with increasing dose was detected (14), with growth of selective lymphoid cells (15, 16). The most effective dose used in our experimental conditions was 100 pg/ml.

To further comprehend immune cell and tumour cell interactions in glioblastoma and to evaluate whether IL-2 may induce *in vitro* proliferation of tumour-infiltrating lymphocytes (TIL) capable of destroying tumour cells, cell imprints of patients affected by glioblastoma and early tumoral cell cultures obtained from the same patient were studied (17, 18). Morphological and cytochemical techniques were used. An interindividual variability was detected as regards the number of leukocytes infiltrating the tumour in the different samples. This could be explained by genetic differences between the patients or by disease progression. Therefore, we evaluated the increased percentage of activated lymphocytes which were positive in cytochemical reactions used as markers of cellular functions (19). A flow cytometric analysis was carried out to analyze the expression of lymphocyte function-associated antigen-1 (LFA-1) in glioblastoma patients. These proteins are involved in intercellular adhesion during the activation of immune functions. Intercellular cell adhesion molecules (ICAM-1) acts as a ligand for the LFA-1 and is considered as an accessory molecule in the activation of T-lymphocytes. An increased concentration of serum ICAM-1 has been repeatedly reported in patients with multiple sclerosis, viral encephalitis and other immunological diseases. In our glioblastoma patients (20), the serum level of ICAM-1 was not significantly increased in comparison with other tumours of the central nervous system and with the controls. The absence of any significant difference in LFA-1 expression in glioblastoma patients and controls suggests that the LFA-1/ICAM-1 system does not play an important role in down-regulation of immunoreactivity in glioblastoma.

Serum levels of IL-2 were significantly increased in all patients, whilst soluble IL-2 levels were high only in glioblastoma. These findings indicate that in malignant glioma there is stimulation of the immune system as evidenced by the presence of activated cells inside tumour tissue and soluble activating factors in serum (21). On the other hand, immunodepression in brain tumour patients is well documented: in these patients a reduced response to mitogens, functional deficiencies in circulating T-helper cells and blocking serum factors that repress the cytotoxic actions of T-cells have been described. Elevated soluble IL-2 serum levels in tumours of the CNS might be related to the immune depression observed in these patients (22, 23). The interaction of IL-2 with glial cells is intriguing because of the presence of T-activated cell (TAC) receptor on microglia and glial cells (24, 25). The effects of T-cell growth factor IL-2 on *in vitro* tissue fragments of human glioblastoma cells obtained immediately after surgery were investigated and the results showed that IL-2 induced both leukocyte proliferation and growth of the tumour cells. On the contrary the cultures maintained in the absence of IL-2 presented only poor leukocyte infiltration, with signs of degeneration and apoptotic phenomena in the tumour cells. Under our experimental conditions, IL-2 affected proliferation not only of infiltrating lymphoid cells, but also of tumour cells. Our data showed that immunotherapy with IL-2 may be either damaging or useful depending on the type and localization of the tumour, the mode of administration and dosage (26-28).

To continue our study, we determined the frequency of abnormalities in human leukocyte antigen (HLA) and antigen processing machinery (APM) component expression in malignant brain tumours by immunohistochemistry (29). This information contributed to our understanding of the immune escape mechanisms used by malignant brain tumours because HLA antigens mediate interactions of tumour cells with the host’s immune system. HLA class I antigens were lost in 50% of the glioblastoma multiform (GBL) lesions and in 20% of the grade 2 astrocytoma lesions stained. Selective HLA-A2 antigen loss was observed in 80% of the GBL lesions studied and in 50% of the grade 2 astrocytoma lesions stained. HLA class I antigen loss was significantly (*p*<0.025) correlated with tumour grade. Among the APM components investigated, tapasin expression was down-regulated in 20% of the GBL lesions analyzed; it was associated, although not significantly, with HLA class I antigen down-regulation and tumour grade. HLA class II antigen expression was detected in 30% of the
lesions analyzed (30). To the best of our knowledge, our study was the first to have analyzed HLA antigen and antigen processing machinery (APM) component expression in surgically removed malignant astrocytic tumours by immunohistochemical staining.

Selective loss of a HLA class I allospecificity is likely to have a negative effect on the efficacy of T-cell-based immunotherapy, which used the lost HLA class I allele as a restricting element. Abnormalities in HLA class I antigen expression seem to have clinical significance because the frequency of HLA class I antigen defects was significantly correlated with tumour grade, an important prognostic marker in astrocytoma (31, 32). Furthermore, the association between HLA class I antigen abnormalities and tumour grade argues in favour of a potential role of HLA class I antigen abnormalities in the clinical course of astrocytoma, although the frequency of total HLA class I antigen abnormalities was not associated with the disease-free interval or survival in our study. Alteration in APM component expression (namely of latent membrane protein 2, latent membrane protein 7, transporter associated with antigen processing 1 and 2, and tapasin) tends to correlate with defects in HLA class I antigen expression and, in some malignancies, is significantly associated with the clinical course of the disease (33, 34). Brain tumours are no exception to this rule because tapasin was down-regulated in a high proportion of GBL lesions and its down-regulation was frequently associated with defects in HLA class I antigen expression. These results suggest that abnormalities in APM component expression may underlie defective HLA class I antigen phenotypes in GBL lesions. The presence of HLA antigen defects in malignant brain tumours may provide an explanation for the relatively poor clinical response rates observed in the majority of the T-cell-based immunotherapy clinical trials conducted to date in patients with malignant brain tumours (35, 36).

Another interesting field of research is that of tumoral biological markers. In particular, we studied a novel indicator of proliferation by an immunohistochemical method: minichromosome maintenance protein 7 (MCM). In comparison with Ki-67 and proliferative cell nuclear antigen (PCNA), a stronger increase of the MCM labelling index in relation to tumour aggressiveness was observed. Our results suggest that the cell cycle-associated MCM proteins are not only useful markers of proliferation, but also valid aids for diagnosis in cerebral glioblastoma (37, 38). In addition, p53 is a cell cycle regulator that has been well recognized as the key molecule that triggers induction and in the control of cell proliferation and apoptosis. Apoptosis and proliferation are two processes intimately coupled that occur simultaneously in tumour tissue. We correlated p53 expression with apoptotic index (AI) and the cell proliferation index (PI) in pilocytic astrocytoma and GBL: while a correlation of p53 expression with AI and PI was found in pilocytic astrocytoma, in glioblastoma it was not found because of the mutated p53 phenotype (39). Platelet-derived growth factor receptors (PDGFR) also regulate several processes in normal cells including cellular proliferation, differentiation and migration, and are widely expressed in a variety of malignancies. In astrocytoma, PDGF ligand and receptors are often over-expressed and PDGFR activity deregulation has been linked to pathogenesis. In our research, we found that astrocytoma cells express PDGFRα and respond to PDGF mitogenic action in a grade-dependent manner. Exogenous PDGF induces human astrocytoma cell line proliferation (40). Concerning the effects of ionizing radiation, a specific study on cell survival, cytokine release and cytokine receptors in tumor glioblastoma cells exposed to different types of ionising radiation (low and high LET) is in progress (41).

Many scientific centres in the world have studied the immunological aspects of tumour host interactions and carried out therapeutic trials. The data obtained from these studies are now an important source for developing further strategies (42). A systematic understanding of the molecular basis of the trafficking and biodistribution of immune cells is important for the development of more efficacious cancer immunotherapies. The accumulation of effective immune cells in tumour tissues is crucial in order to control the growth of tumour cells. Recent characterization of various chemokines and chemokine receptors in the immune system has increased our knowledge of the regulatory mechanisms of immune cell recruitment in controlling the systemic pharmacokinetics of immune cells and, in particular, focus on their receptors and their use in cancer immunotherapy (43). In some types of cancer, such as glioblastoma, a chemo- and radioresistant tumour, immune recruitment could enable new approaches to be developed. It is necessary to concentrate on the relationship between the tumour and its host and on cytokines that regulate their relationships in the microenvironment.

An open unsolved problem is the ability to identify genetic alterations in cancer antigens that drive glial cell transformation and malignant progression resulting in tumour-specific changes in protein expression. Thus, the identification of individual proteins or protein clusters differentially expressed in neoplastic tissues can provide valuable tools for improving of the diagnosis of astrocytoma patients and in identifying novel strategies for therapeutic intervention. Moreover, the monitoring of changes in the protein composition of malignant cells can be useful for a better understanding of gliomagenesis. Recently, many efforts have been devoted to discovering new biomarkers through the proteomic analysis of glioma cell lines and patients affected by astrocytoma. More than one hundred differently expressed proteins have been identify and some of these might represent novel targets to specifically arrest the neoplastic transformation (44). In order to identify novel astrocytic tumor molecular markers, the importance of the doppel gene (PRND) as a useful molecular marker for glioma.
diagnosis has been underlined and its expression is associated with the human astrocytic tumour progression (45-48). Growth factor receptor, kinases and vascular endothelial growth factor (VEGF) inhibitors may lead us to new insights in glioma biology. Two essential aspects of glioma therapy remain still to be achieved: local control of the primary tumour and blockade of tumour cell invasion of normal brain (49). A new prospective is the study of regulatory T-cells (Treg) that are often found in human tumours. The functional characteristics of these cells have not yet been totally evaluated due to their cell number and the inability to adequately distinguish the activated and regulatory T-cell population. Tumour infiltrating FOXP3+ CD4 T-cells, unlike FOXP3– T-cells, were unable to produce IL-2 and interferon-gamma upon ex vivo stimulation, indicating that FOXP3 expression is a valid biological marker for human Tregs even in the tumour microenvironment (50, 51).

In conclusion, the best strategy for glioma is not the use of IL-2 since its receptor is inducible in glia. New studies may be focused e.g. on natural killer cells and their receptors I and II, or on the study of IL-2 resistance mechanisms.

References