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# Cell-Mediated Immunotherapy: A New Approach to the Treatment of Malignant Glioma

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**Background:** *The dismal prognosis for patients harboring intracranial gliomas has prompted an intensive search for effective treatment alternatives such as immunotherapy. Our increased knowledge in basic immunology, glioma immunobiology, and molecular biology may lead to the development of effective, rational immunotherapy approaches.*

**Methods:** *The authors reviewed the literature on glioma immunology, the status of tumor vaccine therapy and on novel techniques to monitor the tumor-specific immune response.*

**Results:** *Experimental conditions currently exist whereby potent antitumor cell-mediated immune responses can be generated. However, clinically, no therapeutic regimen has proven effective. Obstacles to establishing an effective immunotherapy regimen are the lack of a well-defined glioma-specific antigen, the heterogeneity of tumor cells in gliomas, and the modulating effect of the glioma itself on the immune system. Unique strategies to overcome these barriers are being developed.*

**Conclusions:** *Novel strategies to generate an anti-glioma immune response through use of dendritic cell vaccination, directed cytokine delivery, gene-based immunotherapy, and reversal of tumor-induced immunosuppression are promising. These strategies carry the potential of overcoming the resistance of gliomas to immunotherapeutic manipulation and, undoubtedly, will become a part of our future therapeutic armamentarium.*

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## Introduction

Malignant gliomas are the one of the most devastating tumors in current clinical practice. Despite the improvement of local therapies, such as surgery and radiation, the mean survival time for patients carrying a diagnosis of glioblastoma multiforme remains virtually unchanged from a decade ago. On average, patients survive 12 to 18 months, with few patients surviving beyond 2 years.<sup>1,2</sup> In general, chemotherapy is only

marginally effective in the treatment of these lesions due to the difficulty of delivering drugs across the blood-brain barrier and the development of drug resistance by the tumor. Treatment failure in these patients is almost always local, believed to be due in part to the infiltrating nature of the tumor into the surrounding normal white matter. This makes complete surgical resection of these tumors virtually impossible, with a “cure” dependent on the ability to control these infiltrating tumor cells. The poor prognosis for patients with high-grade gliomas has led investigators to seek and develop new and innovative treatment modalities that carry the potential to eradicate these residual infiltrating tumor cells. Tumor vaccine therapy is one such approach. The recognition that lymphocyte infiltration into primary brain tumors is a favorable prognosticator for survival<sup>3,5</sup> has inspired a variety of approaches to utilize the immune system for the treatment of these tumors. Progress in our understanding of immunology and molecular biology in general, and specifically glioma immunobiology, has raised hope for the development of an effective vaccine therapy against this aggressive tumor.

## Can Immunotherapy Eradicate Malignant Brain Tumors?

### Generating an Immune Response

It is now evident that T-lymphocyte-mediated cellular responses play a critical role in the body’s ability to generate an antitumor immune response. The gen-

eration of an effective and specific T-cell-mediated immune response requires the activation of both the cytotoxic (CD8<sup>+</sup>) and the helper (CD4<sup>+</sup>) T-lymphocyte subsets (Fig 1). To activate a CD4<sup>+</sup>T-cell response, antigens must be presented to CD4<sup>+</sup>T cells in conjunction with a major histocompatibility complex (MHC) class II peptide. This is done in the presence of a number of co-stimulatory molecules (eg, CD28/B7 and CD40/CD40L). MHC class II peptides are expressed on the surface of professional antigen-presenting cells (APCs). Common APCs in the body include macrophages, B lymphocytes, bone-marrow-derived dendritic cells, Langerhans cells of the skin, and human endothelial cells. In the brain, candidate APCs include microglia, endothelial cells, capillary pericytes, and occasionally astrocytes themselves. Microglia are the most attractive candidates for APCs within the brain, accounting for 5% to 15% of the total cellular composition of the brain. These microglia are distributed throughout the central nervous system. APCs express not only the MHC class II molecule, but also co-stimulatory molecules, both of which are recognized by specific receptors on the T cell. T-cell recognition of the antigen-MHC class II complex is done through the T-cell receptor (TCR). Presentation of an antigen to the TCR in the absence of a co-stimulatory signal can lead to T-cell anergy, a mechanism potentially responsible for the induction of tolerance to self-antigens and possibly some tumor antigens.<sup>6</sup> Once CD4<sup>+</sup> cells have been activated, they proliferate and produce a variety of cytokines, (eg, interferon-gamma (IFN- $\gamma$ ), interleukin 2 [IL-2], IL-4, IL-7, IL-12), which serve to

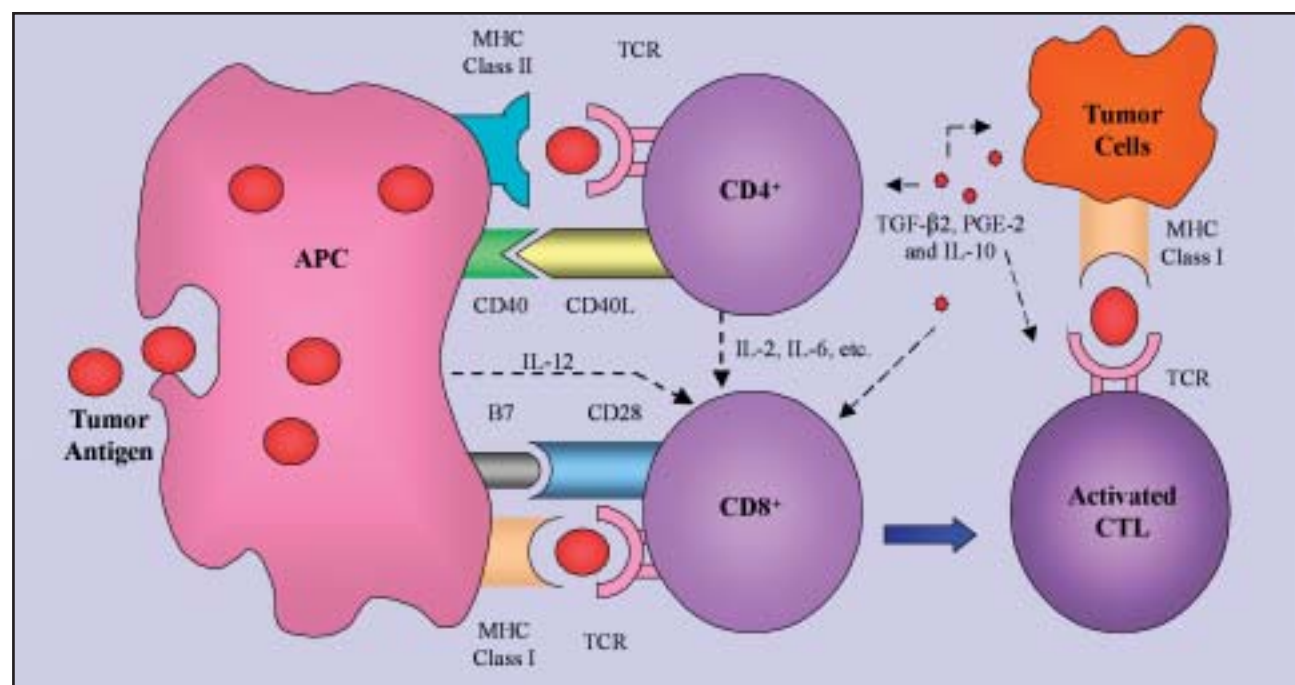


Fig 1. — The T-cell-mediated immune response (APC = antigen-presenting cell, CTL = cytotoxic T lymphocyte, TCR = T-cell receptor, TGF = transforming growth factor, MHC = major histocompatibility complex).

amplify the immune response. These cytokines are critical in fully activating the CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) response.

CD8<sup>+</sup> CTLs are activated by the presence of tumor antigen coupled to an MHC class I peptide. This tumor antigen-MHC class I peptide complex binds to the antigen-specific TCR on the CD8<sup>+</sup> T lymphocyte and stimulates T-cell activation. Unlike MHC class II peptides expressed only on APCs, nearly all cells in the body express MHC class I peptides. However, full activation of the CD8<sup>+</sup> T lymphocyte into a CTL capable of tumor cell lysis requires the presence of cytokine (eg, IL-2, IL-6). This cytokine secretion is provided through CD4<sup>+</sup> “helper” T-cell activation. Activated CTLs are then capable of “targeted” tumor cell killing through one of two primary mechanisms (Fig 2). The first is through the secretion of perforin, which causes dissolution of the tumor cell lipid membrane, thereby leading to tumor cell swelling and ultimately tumor cell death.<sup>7</sup> The second is by inducing programmed cell death (apoptosis) through the Fas/Fas ligand pathway. During the tumor cell lysis, CTLs themselves are not injured by either of these two processes. The CTLs are able to subsequently disengage themselves from the target cell surface following delivery of their lethal hit. Therefore, the CTL is capable of further migration through tissue and subsequent target cell kills.

## Glioma Immunology

The central nervous system has long been considered an immunologically privileged site. However, many recent studies have demonstrated that this privileged state is not absolute and can be easily overcome.<sup>8</sup> The presence of active immunity within the central nervous system has significant implications for neuro-oncology and supports the possibility of manipulating the immune system to create an effective therapy against gliomas. The discovery of tumor-associated antigens expressed by gliomas and the ability to artificially enhance class I and II MHC-restricted antigen presentation have further strengthened this possibility.

It is now evident that T lymphocytes play an important role in the antitumor immune response.<sup>9</sup> The expression of antigens on the surface of tumor cells that can be recognized by the cellular elements of the immune system is the indispensable condition needed to generate a specific antitumor immune response. Although a universal glioma-specific antigen has not been found, several tumor-associated antigens shared by histogenetically related tumors have been identified, such as tenascin, gp240, an altered epidermal growth factor receptor isoform (EGFRvIII), tyrosinase, tyrosinase-related proteins 1 and 2, gp100, MAGE-1, and MAGE-3.<sup>10-14</sup> Several of these antigens have been shown

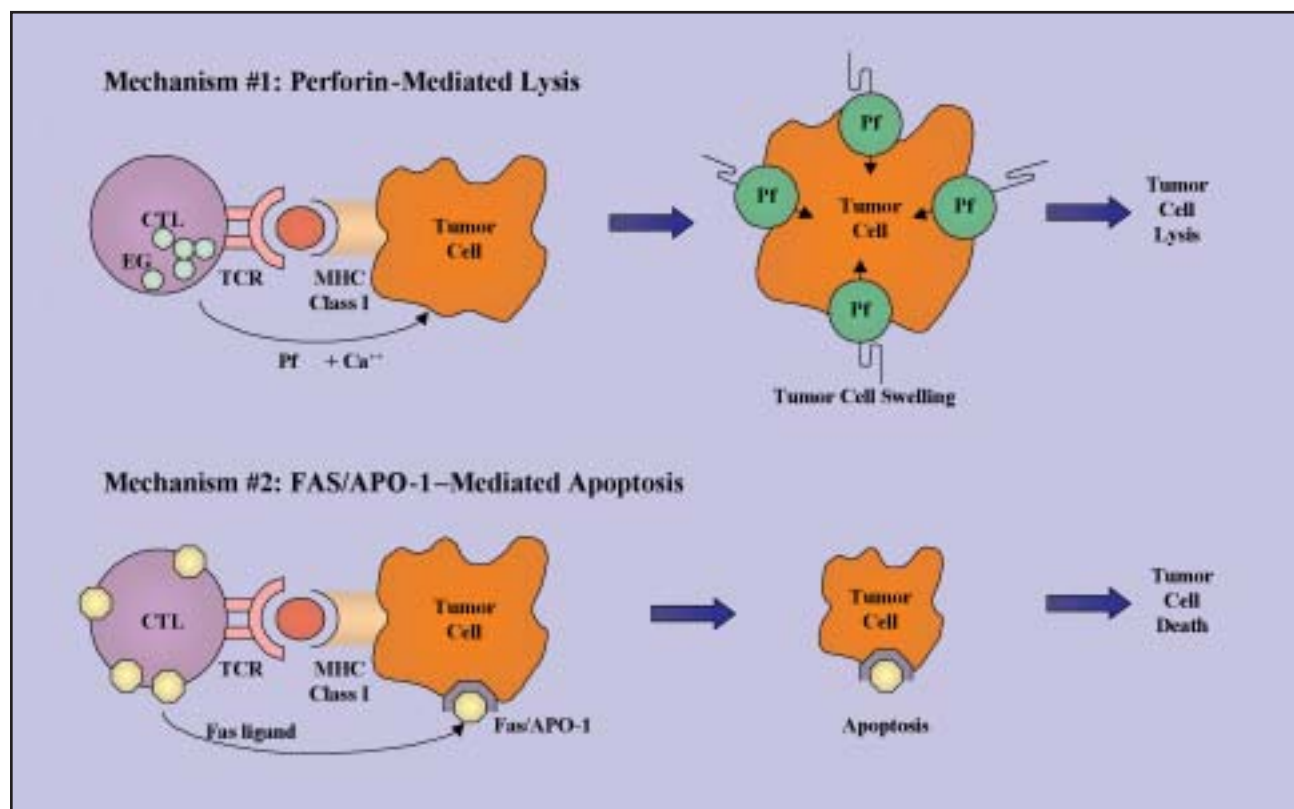


Fig 2. — Mechanisms of T-cell-mediated cytotoxicity (EG = endoplasmic perforin granules, TCR = T-cell receptor, CTL = cytotoxic T lymphocyte, PF = perforin, MHC = major histocompatibility complex).

to be capable of generating a tumor-specific immune response in the laboratory<sup>15</sup> and have led the way to using immunotherapy in the treatment of patients with malignant glioma.<sup>16,17</sup> Clinically, however, it remains to be proven whether these antigens can induce an effective antitumor immune response or even serve as useful immune targets in the clinical setting.

Generally, gliomas not only express a variety of tumor-associated antigens, but also have the ability to present these antigens to T cells. Gliomas have been shown to express low levels of class I MHC, which can be increased both *in vitro* and *in vivo* after appropriate stimuli, such as IFN- $\gamma$  exposure.<sup>18</sup> Therefore, gliomas are capable of presenting tumor-specific antigens to cytotoxic T cells via the class I MHC pathway.<sup>18</sup> Most human gliomas cell also have been shown to express Fas/APO-1 (CD95) and Fas ligand, which can cause glioma cells to undergo Fas/Fas-ligand-mediated apoptosis, the major mechanism for T-cell-mediated cytotoxicity.<sup>18,19</sup>

Despite the existence, intracranially, of the conditions necessary for the generation of cell-mediated immune responses, effective antitumor immune responses against intracranial gliomas have not been effectively generated in the clinical setting. To date, efforts to reliably manipulate the immune system to promote tumor regression in the brain have been universally disappointing. Factors contributing to the ability of glioma to escape the host immune system are its poor overall immunogenicity, its failure to express specific glioma antigens, and the modulatory effects of the tumor on the immune system.<sup>20</sup> Local lymphocyte infiltration into glioma has been well documented. However, the correlation between the extent of T-lymphocyte infiltration and its effect on prognosis is controversial. Whereas some studies have strongly supported a relationship between the extent of lymphocyte infiltration and improved survival,<sup>3,5</sup> others have shown no significant benefit.<sup>21,22</sup> Histologically, little sign of tumor rejection has been noted in the presence of lymphocyte infiltration and as a result, these tumor-infiltrating lymphocytes are thought to be functionally compromised. Indeed, a range of immunological defects, particularly effecting cell-mediated immunity, have been identified in patients with malignant glioma. These patients have been shown to exhibit cutaneous anergy with an abnormal delayed hypersensitivity,<sup>23,24</sup> a reduced number of circulating T lymphocytes,<sup>24</sup> a depressed lymphocyte proliferative response to mitogen,<sup>23,25</sup> a decreased antibody response,<sup>24</sup> and a deficient antibody-mediated and T-cell-mediated cytotoxicity *in vitro*.<sup>23,24</sup> The fact that this immunosuppression can be partially reversed by surgical removal of the tumor<sup>26</sup> strongly suggests that the presence of tumor itself is a major factor responsible for this immunosuppressed state.

Studies supporting glioma-induced immunosuppression have shown a down-regulation of T-lymphocyte activity from T lymphocytes harvested from both autologous brain tumor patients and normal patients in the presence of glioma supernatants. This was demonstrated using the supernatant from both human glioblastoma cell lines and fresh, surgically removed glioblastomas.<sup>27,28</sup> It is now evident that the impaired lymphocyte responses in glioma patients are a result of immunosuppressive factors produced by glioma cells *in situ*.<sup>29</sup> Of the numerous immunosuppression factors identified to date, the most well-characterized is transforming growth factor-beta 2 (TGF- $\beta$ 2), originally called glioblastoma cell-derived T-cell suppressor factor.<sup>30</sup> TGF- $\beta$ 2 messenger RNA (mRNA) and its protein product have been found to be greatly overexpressed in human glioblastomas and virtually absent from normal brain tissue.<sup>31,32</sup> TGF- $\beta$ 2 has been shown to have potent immunosuppressive effects, including the inhibition of T- and B-cell proliferation,<sup>33,34</sup> IL-2 receptor induction,<sup>35</sup> cytokine production,<sup>36</sup> natural killer cell activity,<sup>37</sup> cytotoxic T-lymphocyte development,<sup>36</sup> and lymphokine-activated killer cell generation.<sup>38</sup> More important, it has been shown to directly inhibit the cytotoxic response of tumor-infiltrating lymphocytes.<sup>31</sup> Furthermore, production of TGF- $\beta$ 2 by glioma cells may be a factor responsible for the low response seen in tumor-infiltrating lymphocytes isolated from brain tumors when exposed to stimulants such as lectin and Con A.<sup>39,40</sup> TGF- $\beta$ 2 also has been shown to down-regulate the expression of the class II antigen HLA-DR,<sup>41</sup> possibly another mechanism contributing to the tumor cells ability to escape immune surveillance. In addition, evidence shows that TGF- $\beta$ 2 is an important growth promoter of malignant glioma cells that exhibit TGF- $\beta$  type I and II surface receptors.<sup>42</sup> This phenomenon is believed to be caused by TGF- $\beta$ 2-induced promotion of angiogenesis and tumor stroma formation, as well as an autocrine stimulatory effect on tumor growth.<sup>43</sup>

Additional cytokines produced by the malignant glioma may also contribute to the immunosuppression associated with this tumor. These include prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)<sup>44</sup> and IL-10.<sup>45</sup> The role of IL-10 on the immune system is controversial. On the one hand, it has been suggested that IL-10 acts to inhibit the release of IFN- $\gamma$  by lymphocytes, inhibits production of tumor necrosis factor alpha (TNF- $\alpha$ ) by monocytes, partially inhibits MHC class II expression by monocytes, and functions to induce glioma proliferation and migration.<sup>45,46</sup> On the other hand, IL-10 has been shown in animal models to enhance an effective and specific antitumor immune response.<sup>47</sup> Further studies are necessary to better understand the role of IL-10 in tumor-induced immunosuppression.

PGE<sub>2</sub>, however, has profound modulatory effects on the immune system, including down-regulation of lymphokine-activated killer cell activity and suppression of T-cell proliferation. PGE<sub>2</sub> also down-regulates the expression of the class II antigen HLA-DR, possibly contributing to the ability of tumor cells to escape immune surveillance.<sup>48</sup>

## Current Strategies for Immunotherapy

Over the years, numerous attempts have been made to establish an effective form of immunotherapy for the treatment of brain tumors. Both passive and active immunotherapeutic approaches have been attempted, alone or in combination, in patients with gliomas. Historically, early trials focused on using whole tumor cells,<sup>49</sup> antibody,<sup>50-52</sup> and/or nonspecific adjuvant such as bacille Calmette-Guérin (BCG) and *Corynebacterium parvum*, and resulted in little success. Later, the discovery of T-lymphocyte growth factor (IL-2), lymphokine-activated killer cells, tumor-infiltrating lymphocytes, and tumor-sensitized cytotoxic T cells generated a new wave of enthusiasm for adoptive immunotherapy. However, to date, no trial using these new innovations has unequivocally demonstrated an improved overall survival for patients with malignant glioma.<sup>53</sup> Recent advances in basic research on antigen presentation, the use of specific cytokines, and T-cell-mediated cytotoxicity have once again given rise to a new direction for glioma immunotherapy.

### Vaccination With Dendritic Cells Pulsed With Tumor Antigens

Effective delivery of tumor antigen to T cells is a pivotal step in inducing antigen-specific immunity and is a critical challenge in tumor vaccine design. To date, dendritic cells are recognized to be the most potent APCs responsible for initiating an immune response. Dendritic cells are bone-marrow-derived cells similar to monocyte/macrophages and can be obtained from culturing peripheral blood mononuclear cells.<sup>54</sup> Upon exposure to tumor cells, isolated tumor antigen, or even tumor mRNA, dendritic cells are capable of presenting endogenous and exogenous antigens to naive T cells in an HLA-restricted manner. In addition to a high level of expression of both MHC class I and II molecules, dendritic cells possess the ability to secrete immune stimulatory cytokines and express several adhesion and costimulatory surface molecules. All of this leads to an efficient priming of the CD4<sup>+</sup> T-helper cells and the generation of potent antitumor CTLs. Potent antitumor CTLs result in a specific protective and therapeutic antitumor immune response.<sup>55-58</sup>

The possibility of utilizing dendritic cells as a vaccine for glioma has been addressed in several animal experiments with promising results. First, Siesjo et al<sup>55</sup> showed that immunization with tumor cells mixed with syngeneic spleen-derived dendritic cells resulted in a significantly prolonged mean survival time for rats harboring established intracranial gliomas. Later, Liau et al<sup>58</sup> reported prolonged survival for rats harboring pre-established intracranial 9L gliomas after vaccination with bone-marrow-derived dendritic cells pulsed *ex vivo* with acid-eluted protein from 9L glioma cells. Vaccination with antigen-loaded dendritic cells resulted in increased CD8<sup>+</sup> T-cell infiltration into the tumor and increased 9L-specific CTLs. Similar results have also been reported when vaccinating mice with dendritic cells pulsed with Semliki Forest virus-mediated glioma complementary DNA<sup>59</sup> and dendritic cells fused to glioma cells.<sup>60</sup>

These promising results from dendritic cell vaccinations in the animal glioma models have prompted a number of clinical trials in patients with glioma. Liau et al<sup>61</sup> published a case report on a patient with glioblastoma who was immunized with autologous dendritic cells pulsed with allogeneic MHC class I-matched tumor peptides. The tumor progressed 2 months after dendritic cell vaccination but on histologic analysis showed increased CD3<sup>+</sup> T-cell infiltration with no signs of experimental allergic encephalomyelitis. Later, the first phase I clinical trial using a dendritic cell-based vaccine for glioma patients was reported by Yu and colleagues.<sup>56</sup> In this trial, patients received autologous peripheral blood dendritic cells, which were prepared with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and pulsed with peptides eluted from the surface of autologous glioma cells. Patients undergoing the dendritic cell vaccinations showed a prolonged survival time, possibly related to the production of an enhanced systemic cytotoxicity response and an increased intratumoral cytotoxic and memory T-cell infiltration. Kikuchi et al<sup>62</sup> recently reported on a phase I clinical trial in which glioma patients were vaccinated with a novel fusion product of autologous dendritic and glioma cells. Although no statistically significant treatment-associated response was observed, vaccination with the fusion cells increased CD16<sup>+</sup> and CD56<sup>+</sup> cells in peripheral blood lymphocytes and IFN- $\gamma$  production in peripheral blood mononuclear cells. Again, this therapy proved to be safe, and no serious adverse autoimmune responses occurred.

Immunotherapy with dendritic cells seems to be capable of generating a glioma-specific antitumor immune response and results in an improved survival in animals with pre-established glioma. Its benefit in humans remains to be demonstrated.<sup>54-56,58,60,62,63</sup> In

most of the studies reported, an array of tumor-associated antigens rather than single peptides were used because of the inability to identify a specific universal glioma antigen. Dendritic cell immunotherapy appears to be biologically safe with no serious side effects noted, and no evidence of autoimmune toxicity has occurred in either the animal or the clinical trials. Further research on the use of dendritic cell therapy as an adjuvant to our current therapy for the treatment of patients with malignant glioma is warranted.

### *Gene Technique-Based Immunotherapy*

Advances in recombinant gene technology provide new tools for the development of tumor-specific therapies. Genetic constructs can be modified by viral vectors or plasmid DNA to express a variety of genes in vitro and in vivo that encode tumor antigens, cytokines, or accessory molecules. Genetic modification of tumor cells can increase their immunogenicity and potentially enhance the systemic immune response generated against an intracranial tumor. Numerous cytokines such as IFN- $\gamma$ , GM-CSF, or IL-12 have been tested. Vaccination with cytokine-producing tumor cells has been shown to stimulate a potent immunity against tumors within the brain and provides a basis for gene-based immunotherapy.<sup>8</sup> Vaccination with allogeneic pre-B cells transduced with a glioma-related mutant antigen, epidermal growth factor receptor variant III (EGFRvIII), has been shown to produce a systemic immune response against autologous intracranial tumor expressing the same antigen.<sup>64</sup>

Gene-based glioma immunotherapy is not limited to the expression of new genes. Vaccination with genetically engineered glioma cells expressing anti-sense molecules that block specific gene expression, such as glioma-derived immunosuppressive factor TGF- $\beta$ 2 and insulin-like growth factor (IGF)-1, have also been shown to suppress intracranial tumor growth.<sup>42,65</sup>

In addition to the subcutaneous vaccination of genetically engineered tumor cells, the effect of local cytokine production on intracranial glioma growth has also been examined. Two approaches have been used to introduce cytokines intracranially. The first approach involves the direct intracranial implantation of tumor cells genetically engineered to secrete specific cytokines. IL-2, IL-4, GM-CSF, TNF- $\alpha$ , and IFN- $\gamma$  have all been shown to demonstrate a significant survival advantage in the animal model.<sup>66-68</sup> This approach, however, has yet to be used clinically. The second approach involves direct in situ cytokine gene transfection. Genetically engineered adenovirus and herpes simplex virus expressing various cytokines have been tested, and again a definite survival advantage was obtained when used in experimental brain tumor models.<sup>69-71</sup>

Despite its promise, gene-based immunotherapy presents several potential barriers to clinical application. The first of these barriers is the need to modify individual glioma cells taken from each patient prior to treatment. The modification of these tumor cells may be difficult and time consuming, often involving the establishment of individual cell lines from each patient. Even when possible, the established cell lines, due to their inherent nature of selecting out cells for their capacity to grow in vitro, may no longer express the same antigens as the primary tumor. The second concern is the ability, in vivo, of effectively transfecting tumor cells with the viral gene product. This is of particular concern to tumor cells along the edge of a glioma, which have infiltrated well into the "normal" brain. In addition, reimplanting into a patient genetically engineered tumor cells with their potential to grow being unknown is a concern.

Several solutions have been suggested to overcome these barriers. One of the simplest methods is to avoid using genetic manipulation. Stimulation of an antiglioma immune response by local cytokines injection, with vaccination, has been reported by us<sup>20</sup> and others.<sup>72-74</sup> The subcutaneous implantation of irradiated 9L tumor cells, along with IFN- $\gamma$ , IL-2, or GM-CSF, resulted in the inhibition of intracranial 9L tumor growth. Another method to overcome the need for autologous tumor cell gene modification is to use a nontumor carrier cell to deliver immunotherapeutic gene products. Glick et al<sup>75</sup> demonstrated that mice implanted subcutaneously or intracranially with autologous glioma cells mixed with allogeneic fibroblasts, genetically engineered to secrete IL-2 or IL-2/IFN- $\gamma$ , developed systemic antiglioma cytotoxic immune responses resulting in prolonged survival. This approach avoids the need to transduce glioma cells from individual patients and allows for gene-based immunotherapy to be administered immediately after surgical intervention.

Despite these exciting preclinical results, few clinical trials have been reported. Sobol et al<sup>76</sup> reported on a single case of a patient with glioblastoma multiforme treated with repeated immunizations using autologous tumor cells and genetically modified fibroblasts using retroviral gene transfer to secrete IL-2. An antitumor immune response, mediated in part by CD8<sup>+</sup> cytotoxic T cells, was demonstrated in the patient's peripheral blood mononuclear cells. The patient survived for 10 months after the initiation of immunization. Recently, Schneider et al<sup>77</sup> reported a phase I clinical trial in which 11 patients underwent immunization with autologous tumor cells modified with Newcastle disease virus. Noticeable peripheral immune responses after immunization were noted, but survival was not significantly longer compared with patients who received the combined treatment of surgery, radiotherapy, and chemotherapy.

## Reversal of Tumor-Induced Immunosuppression

To date, efforts to reliably manipulate the immune system to promote tumor regression in the brain have been universally disappointing. This poor response is partly due to a direct modulatory effect of the tumor on the immune system. The ability of malignant gliomas to affect the systemic immune system has been long recognized and demonstrated.<sup>20,78</sup> Over-expression of TGF- $\beta$ 2 is believed to contribute to the immunosuppressed state seen in patients with gliomas and to be a major factor responsible for failure of current immunotherapy strategies.<sup>65,78</sup> Gorelik et al<sup>79</sup> recently demonstrated that with T-cell-specific blockade of TGF- $\beta$  signaling, an effective immune response capable of eradicating tumors in mice could be generated. Therefore, the ability to inhibit this immunosuppressive factor and reverse tumor-induced immunosuppression could have significant beneficial therapeutic consequences. Ruffini et al<sup>80</sup> demonstrated that by effectively antagonizing TGF- $\beta$ 2 secretion through use of a neutralizing antibody, the *in vitro* proliferative capacity and antitumor cytotoxicity of adherent lymphocyte activated killers cells was enhanced.

Because glioma cells infiltrate widely through the normal parenchyma, current therapy, in general, fails to completely eradicate these infiltrating tumor cells. These residual cells, from which recurrent tumors arise, undoubtedly maintain the ability to secrete immunosuppressive factors, including TGF- $\beta$ 2. In cell culture, the ability of glioma cells to secrete TGF- $\beta$ 2 even after irradiation has been demonstrated.<sup>81</sup> Since TGF- $\beta$ 2 not only inhibits immune cell proliferation, but also directly hampers the cytotoxicity of tumor-infiltrated lymphocytes,<sup>31</sup> this implies that high concentrations of TGF- $\beta$ 2 locally will inhibit the function of cytotoxic T cells that may have been activated systemically through a vaccine therapy strategy. This local immunosuppressive environment of gliomas is only now being appreciated and explored. It is believed, however, that it is a major obstacle in the development of effective immunotherapy strategies. Therefore, a therapeutic reduction in local TGF- $\beta$ 2 protein production would be expected to reverse TGF- $\beta$ 2-derived immunosuppression both systemically and locally, with the later possibly being more important for tumor eradication.

## Immune Monitoring

With the initiation of various vaccination trials aimed at inducing tumor-specific CD8<sup>+</sup> T cells, accurate and reliable assays for testing T-cell function is crucial for the evaluation, comparison, and further development of these approaches. Until recently, the analysis of antigen-

specific CD8<sup>+</sup> T-lymphocyte responses depended on the standard chromium release assay, in which CTLs are detected by measuring their ability to lyse MHC-matched antigen-expressing target cells. This technique is useful in determining the basic rules governing the behavior of T cells and still remains one of the most widely used assays. However, the need to first propagate the effector cells *in vitro* prior to performing the lysis assay may once again influence the results. A selection bias is automatically introduced with culturing of the effector cells, and the results subsequently obtained from this assay may not reflect *in vivo* CTL function.

Several sensitive, new, and easy to perform techniques have been developed to detect and quantify antigen-specific T cells without the necessity for *in vitro* expansion. These assays ensure a more accurate analysis of the tumor-specific immune responses. One such technique is the ELISPOT assay,<sup>82</sup> which is based on the principle of the enzyme-linked immunosorbent assay. To quantify the presence of tumor-specific T lymphocytes, lymphocytes harvested from the patient are incubated in antibody-coated wells together with tumor cells or a tumor-specific antigen. Cytokine secreted from the individual lymphocytes, in response to recognition of the antigen, is captured and visualized with an enzyme-labeled secondary antibody with a corresponding chromogenic substrate. Each "spot" represents the cytokine of interest secreted from a single lymphocyte cell. IFN- $\gamma$  and TNF- $\alpha$  are currently used by most groups to quantify antigen-specific CD8<sup>+</sup> T cells.

Two techniques based on flow cytometry have also been developed to detect and quantify antigen-specific T cells. The first technique is an intracellular cytokine staining assay for quantitative and qualitative assessment of antigen-specific cytokine-producing T lymphocytes.<sup>83-85</sup> T cells are stimulated *in vitro* with antigen for several hours and then stained with a fluorochrome-labeled antibody specific for the intracellular cytokine of interest. Cytokine producing cells are identified and counted by flow cytometry. The second technique is based on the use of tetrameric HLA class I/peptide complexes.<sup>86,87</sup> This tetramer method involves the engineering of a biotinylation signal sequence onto the C terminus of a recombinant MHC class I or II molecule, which then complexes with a specific peptide. Fluorochrome-labeled peptide-MHC tetramers are produced by mixing the biotinylated peptide-MHC complex with avidin at a 4:1 ratio and are able to recognize TCRs on lymphocytes specific for the particular epitope. The stained antigen-specific T cells can then be subsequently analyzed by flow cytometry. This technique has proven to be a powerful tool in enumerating and characterizing specific T cells without the need for *in vitro* expansion of lymphocytes.

Lastly, a new technique called quantitative real-time polymerase chain reaction (PCR) assay is now available for directly measuring the antitumor immune status of selected individuals.<sup>88,89</sup> In this technique, blood and spleen cells are stimulated *in vitro* with either a peptide or tumor cells for 2 hours. The cells are then harvested for RNA isolation and cDNA transcription. Quantitative real-time PCR is then performed for IFN- $\gamma$  mRNA expression. This technique has the ability to quantitatively measure the cytokine gene message from lymphocytes harvested from patients or animals without the need for any *in vitro* cell amplification.

## Conclusions

Immunotherapy for the treatment of cancer has reached an exciting phase in its evolution due to the enormous progress in tumor immunology. Recent advances in the manipulation of APCs and in our knowledge of tumor-induced immunosuppression have renewed hope in our ability to induce tumor-specific cell-mediated immunity through systemic vaccination. However, these hopes continue to be hampered by the absence of well-defined glioma-specific antigens, the heterogeneity of tumor cells seen in human gliomas, and our limited understanding of tumor-induced immunosuppression. The challenge remains to effectively apply what we are learning in the research laboratory to patient care. Although an effective immunotherapeutic regimen has yet to be demonstrated in the clinical setting and the ultimate role of immunotherapy in the treatment of glioma is still unknown, the potential for immunotherapy as an adjunct to our current treatment of gliomas is now based on solid technical and conceptual footing. Novel technologies for characterization and monitoring of specific tumor responses will also provide an opportunity to greatly accelerate the development of new anticancer immune strategies.

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