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# Brain Tumor Immunotherapy

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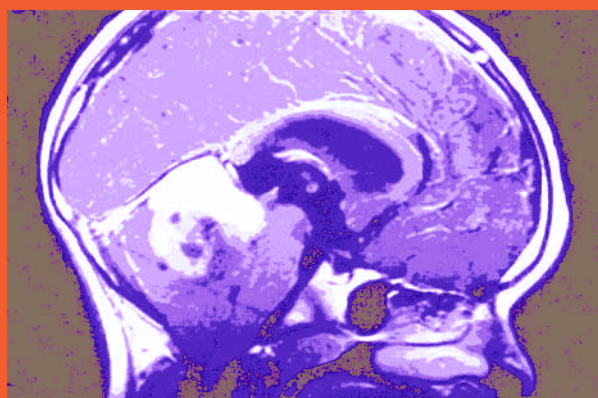
*Edited by*

Linda M. Liau, MD, PhD

Donald P. Becker, MD

Timothy F. Cloughesy, MD

Darell D. Bigner, MD, PhD



# BRAIN TUMOR IMMUNOTHERAPY



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Edited by

LINDA M. LIAU, MD, PHD

DONALD P. BECKER, MD

TIMOTHY F. CLOUGHESY, MD

*University of California at Los Angeles  
School of Medicine, CA*

and

DARELL D. BIGNER, MD, PHD

*Duke University Medical Center, NC*



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# PREFACE

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Among the new treatments currently being investigated for malignant brain tumors, none is as theoretically appealing as immunotherapy, because it offers the potential for high tumor-specific toxicity. Cancer immunotherapy is currently a rapidly developing field, and new discoveries regarding the immune susceptibility of the central nervous system have made the concept of brain tumor immunotherapy an area of active investigation. Enough information has been gained from basic research and clinical trials to allow the conclusion that immunotherapy for brain tumors is feasible, can evoke relevant biologic responses, and can provide important insights into human biology. Brain tumor immunotherapy still faces great hurdles before it becomes an established clinical therapy. However, the accomplishments in this field to date are impressive, and the intuitive logic of this treatment paradigm offers compelling hope that the immunotherapy of brain tumors may someday succeed.

The aim of *Brain Tumor Immunotherapy* is to organize a thorough critical survey of the field, with contributions from leading researchers and clinicians to help convey the many and significant recent accomplishments within this evolving discipline. We hope our book will provide both clinicians and research scientists with a reasonably comprehensive guide to modern brain tumor immunotherapy and thereby enhance future investigation in the area. The scope of this text will detail some of the laboratory experiments and clinical protocols that are currently being investigated, integrate the available information from previous and ongoing research, and help to define the current status of the field.

The feasibility of immunotherapy for central nervous system cancers is just beginning to be studied through clinical trials. Most of our current understanding of brain tumor immunotherapy has been gleaned through the use of transplantable animal brain tumor models, with the primary hope of predicting therapeutic responses in human tumors. Because of the desperate plight of patients suffering from malignant gliomas and the fact that very few treatment modalities have shown clinical efficacy against this deadly disease, it is difficult to prove that any one animal model is necessarily the most exemplary of human primary brain tumors. Nevertheless, we must caution the reader that some of the most widely used animal models of murine and rat

primary glial neoplasms are not well-suited for evaluating immunologic responses to brain tumors since they have inherent histoincompatibilities that can potentially provide misleading results in immune-competent hosts. For example, the commonly used rat C6 glioma cell line has an uncertain genetic background and therefore may not be syngeneic in the animals in which these cells are transplanted. Because of this, favorable immunotherapeutic responses using animal models must always be interpreted with caution, and extreme prudence should be exercised before basing any clinical trial decisions on information obtained solely from such models. New models developed in syngeneic backgrounds with transgenic methodology may be more useful than older models, which are often chemically induced, highly antigenic, and of questionable genetic background. Yet, these models are still far from duplicating the complexities of clinical brain tumors and the human immune system.

With this caveat in mind, *Brain Tumor Immunotherapy* may be used most effectively as a resource text for neurosurgeons, experimental neuroscientists, clinical neuro-oncologists, tumor immunologists, and others who may wish to explore further research in this field. We have attempted to provide sufficient background information about brain tumor immunotherapy strategies, while hoping to capture a contemporary glimpse of the breadth and depth of this field. This book differs from others currently available, as it is probably one of the only texts dedicated specifically to immunotherapeutic approaches for *central nervous system* malignancies.

Whether it is adoptive cellular immunotherapy, radiolabeled antibodies, cytokine gene therapy, or dendritic cell vaccines, almost every leading neuro-oncology program in the world is investigating some form of brain tumor immunotherapy. The number of clinicians and scientists interested in cancer immunotherapy is increasing. Annual meetings of multiple scientific and clinical disciplines have entire sessions dedicated to the immunobiology of brain tumors. Recent developments in our understanding of molecular microbiology and tumor immunology have resulted in increasingly clever and sophisticated immune-based treatment strategies against cancer. It is our sincere hope that dissemination of such information and further research endeavors in this field will someday translate to true therapeutic benefits for our brain tumor patients.

*Linda M. Liau, MD, PhD*  
*Donald P. Becker, MD*  
*Timothy F. Cloughesy, MD*  
*Darell D. Bigner, MD, PhD*

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# CONTRIBUTORS

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- DAVID M. ASHLEY, PHD • *Department of Hematology and Oncology, Royal Children's Hospital, Parkville, Victoria, Australia*
- DONALD P. BECKER, MD • *Division of Neurosurgery and the Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA*
- DARELL D. BIGNER, MD, PHD • *Department of Pathology and Duke Comprehensive Cancer Center, Duke University Medical Center, Durham, NC*
- NAI-KONG V. CHEUNG, MD, PHD • *Robert Steel Research Laboratory, Memorial Sloan-Kettering Cancer Center, New York, NY*
- TIMOTHY F. CLOUGHESY, MD • *Department of Neurology and the Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA*
- EDWARD P. COHEN, MD • *Department of Microbiology and Immunology, University of Illinois College of Medicine, University of Illinois at Chicago, Chicago, IL*
- FRANCES K. CONLEY, MD • *Department of Neurosurgery, Stanford University Medical Center, Stanford, CA*
- FAITH DAVIS, PHD • *Department of Epidemiology, University of Illinois, Chicago, IL*
- NICOLAS DE TRIBOLET, MD • *Department of Neurosurgery, University Hospitals of Geneva and Lausanne, Geneva, Switzerland*
- PIERRE-YVES DIETRICH, MD • *Division of Oncology, University Hospital of Geneva, Geneva, Switzerland*
- JOHN M. DUFF, MD • *Department of Neurosurgery, University Hospitals of Geneva and Lausanne, Geneva, Switzerland*
- S. ATHER ENAM, MD, PHD • *Department of Neurosurgery, Henry Ford Hospital, Detroit, MI*
- HABIB FAKHRAI, PHD • *Advanced Biotherapies, San Diego, CA*
- ROBERTA P. GLICK, MD • *Departments of Neurosurgery, Anatomy, and Cell Biology, Rush Medical College, Cook County Hospital and University of Illinois, Chicago, IL*
- SVETLANA GRAMATIKOVA, PHD • *Advanced Biotherapies, San Diego, CA*
- WALTER A. HALL, MD • *Departments of Neurosurgery, Radiation Oncology, and Radiology, University of Minnesota School of Medicine, Minneapolis, MN*

- AMY B. HEIMBERGER, MD • *Division of Neurosurgery, Duke University Medical Center, Durham, NC*
- FRANK P. HOLLADAY, MD • *Providence Medical Building, Kansas City, KS*
- CAROL A. KRUSE, PHD • *Department of Immunology, University of Colorado Health Sciences Center, Denver, CO*
- LINDA M. LIAU, MD, PHD • *Division of Neurosurgery and the Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA*
- TERRY LICHTOR, MD, PHD • *Department of Neurological Surgery, Rush Medical College, Loyola University Chicago and Cook County Hospital, Chicago, IL*
- WILLIAM H. MCBRIDE, DSC • *Department of Radiation Oncology, University of California, Los Angeles, CA*
- ROBERTA MCKEAN-COWDIN, PHD • *Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA*
- PAUL S. MISCHEL, MD • *Department of Pathology and Laboratory Medicine, Division of Neuropathology, UCLA School of Medicine, Los Angeles, CA*
- GREGORY E. PLAUTZ, MD • *Department of Pediatrics, Yale University School of Medicine, New Haven, CT*
- SUSAN PRESTON-MARTIN, PHD • *Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA*
- WILFRIED ROTH, MD • *Department of Neurology, University of Tübingen, Tübingen, Germany*
- DAVID RUBINSTEIN, MD • *Department of Radiology, University of Colorado Hospital, Denver, CO*
- ROHANGIZ SAFAEI, PHD • *Advanced Biotherapies, San Diego, CA*
- JOHN H. SAMPSON, MD, PHD • *Division of Neurosurgery, Duke University Medical Center, Durham, NC*
- SUYU SHU, PHD • *Department of Surgery, Center for Surgery Research, Cleveland Clinic Foundation, Cleveland, OH*
- VICTOR C. K. TSE, MD, PHD • *Department of Neurosurgery, Stanford University Medical Center, Stanford, CA*
- STÉPHANE VANDENABEELE, PHARM D • *Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia*
- HARRY V. VINTERS, MD • *Department of Pathology and Laboratory Medicine, Division of Neuropathology, UCLA School of Medicine, Los Angeles, CA*

---

MICHAEL WELLER, MD • *Department of Neurology, University of  
Tübingen, Tübingen, Germany*

CAROL J. WIKSTRAND, PHD • *Department of Pathology, Duke  
University Medical Center, Durham, NC*

GARY W. WOOD, PHD • *CAI Center, Kansas City, MO*

MICHAEL R. ZALUTSKY, PHD • *Departments of Pathology and  
Radiology, Duke University Medical Center, Durham, NC*

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# I INTRODUCTION TO THE DISEASE

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# 1

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## Neuropathology and Molecular Pathogenesis of Primary Brain Tumors

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*Paul S. Mischel, MD and Harry V. Vinters, MD*

### *CONTENTS*

INTRODUCTION  
ASTROCYTOMAS  
OLIGODENDROGLIOMAS  
EPENDYMOMAS  
SUMMARY  
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### 1. INTRODUCTION

The gliomas are a group of tumors that arise in the central nervous system (CNS) and share features of some degree of glial differentiation. During development, the neuroectoderm surrounding the neural tube gives rise to all of the major intrinsic cell types of the CNS, including neurons and glia. Glia are traditionally subtyped into four major categories: astrocytes, oligodendrocytes, ependymal cells, and microglia. Microglia, even though they are called glia, appear to be derived from bone marrow-derived elements, and rarely give rise to tumors; hence, they are not discussed in this chapter. Glial tumors all share some degree of differentiation along either astrocytic, oligodendroglial, or ependymal lines, or with some mixture of these features. Whether these tumors arise from glia that have differentiated along one of these lines, or from less differentiated precursors that show differentiation along glial lines, remains to be solved.

Brain tumors (BTs) have been classified mostly according to their morphologic and immunohistochemical (IHC) features. The systems of classification of BTs which are discussed, correlate relatively well with prognosis, and remain

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the current standard of diagnosis. However, fundamental work in the molecular biology of gliomas has begun to demonstrate clear patterns of molecular pathogenesis. Gliomas typically involve somatic gene alterations, which affect the cellular regulatory processes of growth response to extracellular signals, cell cycle control, and/or tumor suppressor events, often in combination. The integration of these molecular aspects of pathogenesis has already begun to identify fundamentally distinct subgroups within the current classification system. The integration of basic molecular data into the classification of gliomas will enable identification of subtypes of gliomas, based on their molecular pathogenesis, which will enable determination of prognostic differences, even among morphologically identical tumors. This integration may help enable identification of rational therapies tailored to address the fundamental molecular defects involved in the pathogenesis and progression of gliomas.

For all of these reasons, the neuropathologic classification of gliomas needs to be considered a work in progress. This chapter addresses the issue of the pathophysiology and classification of gliomas, by presenting the standard classification system used to describe and classify them. However, it will also incorporate current understanding of the molecular pathogenesis of BTs into this classification.

## 2. ASTROCYTOMAS

Astrocytomas can be subdivided into two major classes: the diffuse infiltrating astrocytomas, and a group of less common, more indolent astrocytic variant tumors. The infiltrating astrocytomas include astrocytoma (grade II), anaplastic astrocytoma (AA) (grade III), and glioblastoma (grade IV). The more indolent astrocytic variant tumors include pilocytic astrocytoma, pleomorphic xanthoastrocytoma (PXA), desmoplastic cerebral astrocytoma of infancy (DCAI), and subependymal giant-cell astrocytoma (SEGA). Although these more indolent astrocytic variants do not share many histologic features nor common molecular pathogenesis, they are grouped together, since they tend to occur in younger patients, appear to be more circumscribed and indolent, and are associated with a more favorable prognosis than the infiltrating astrocytomas. Unfortunately, the diffuse infiltrating astrocytomas are considerably more common tumors.

### *2.1. Diffuse Infiltrating Astrocytomas*

Diffuse astrocytic neoplasms are by far the most common primary BTs, and, obviously, the most frequent subtypes of gliomas. Diffuse astrocytomas can arise at any site within the CNS, but are most common in the white matter of the cerebral hemispheres. They share a number of features that separate them from the other astrocytic variants: chiefly, their tendency to progress or become more malignant over time; and their infiltrating nature, which can be appreciated both



grossly and microscopically. The diffuse astrocytomas are also characterized by resemblance of their components to fibrillary astrocytes, in that they tend to share morphologic similarities, such as the fine meshwork of fibrillary processes and the presence of glial fibrillary acidic protein (GFAP) immunopositive intermediate glial filaments. In addition to astrocytoma cells that resemble normal fibrillary astrocytes, a range of morphologic forms, including gemistocytic astrocytes and protoplasmic astrocytes, can also be seen in diffuse astrocytomas. Higher-grade tumors, particularly glioblastoma multiforme (GBM), can show a whole range of features, from cells resembling normal astrocytes to highly anaplastic, poorly differentiated cells and tumor giant cells. The separation of astrocytic tumors into diffuse astrocytomas and the more indolent astrocytic variants also appears to be borne out by recent evidence suggesting differing molecular pathogenesis in diffuse astrocytomas compared to the more indolent lesions, as is discussed in Section 2.1.2.

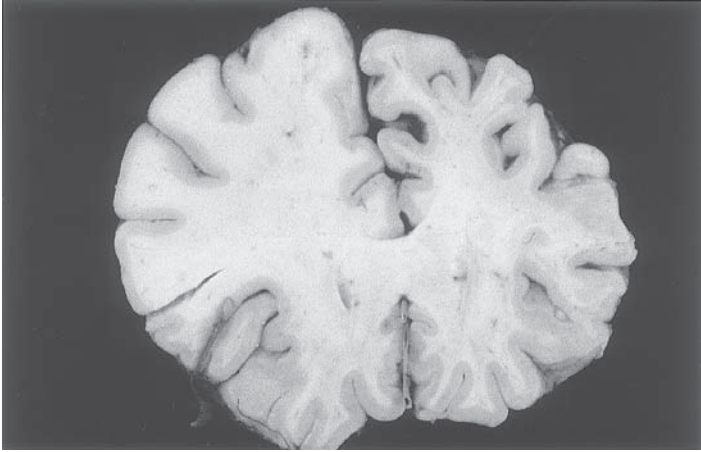
### 2.1.1. INCIDENCE

Diffuse infiltrating astrocytomas are the most frequent primary intracranial neoplasm, and account for more than 60% of all primary BTs (1). Although incidence figures vary, there are between 5 and 7 new cases/100,000 people/yr (1). Although diffuse astrocytomas can occur at any age, they are generally a tumor of adults. Low-grade diffuse astrocytomas have a peak incidence between the ages of 30 and 40 yr; AAs have a peak incidence between 40 and 50 yr, and GBM has a peak incidence between 50 and 70 yr of age.

### 2.1.2. NEUROPATHOLOGY

The diffuse astrocytomas are characterized by their infiltrating nature. Grossly, they tend to be bulky lesions that efface the normal anatomic boundaries, distorting, but not destroying, the structures being invaded (Fig. 1). Extensive invasion into surrounding brain is often seen, particularly in GBM. Extension along white matter tracts, particularly the corpus callosum, is common. Grade II infiltrating astrocytomas tend to have a solid, whitish appearance, although microcyst formation can give them a gelatinous appearance. As lesions become more malignant, particularly in evolution to GBM, large regions of necrosis and hemorrhage are often seen (Fig. 2). Cyst formation can occur in lesions of any grade.

Microscopically, neoplastic astrocytes may show significant variability, particularly in high-grade tumors (Fig. 3). Although fibrillary astrocytes, characterized by abundant fine fibrillary processes and the presence of abundant GFAP-immunopositive intermediate filaments (Fig. 4), are the most frequent, other types of astrocytes can also be seen. Gemistocytic astrocytes, which can be present in either reactive or neoplastic astrocytic lesions, are characterized by abundant glassy eosinophilic cytoplasm, with clearly discernable cell borders (Fig. 5). Protoplasmic astrocytes are cells that have far fewer fine processes

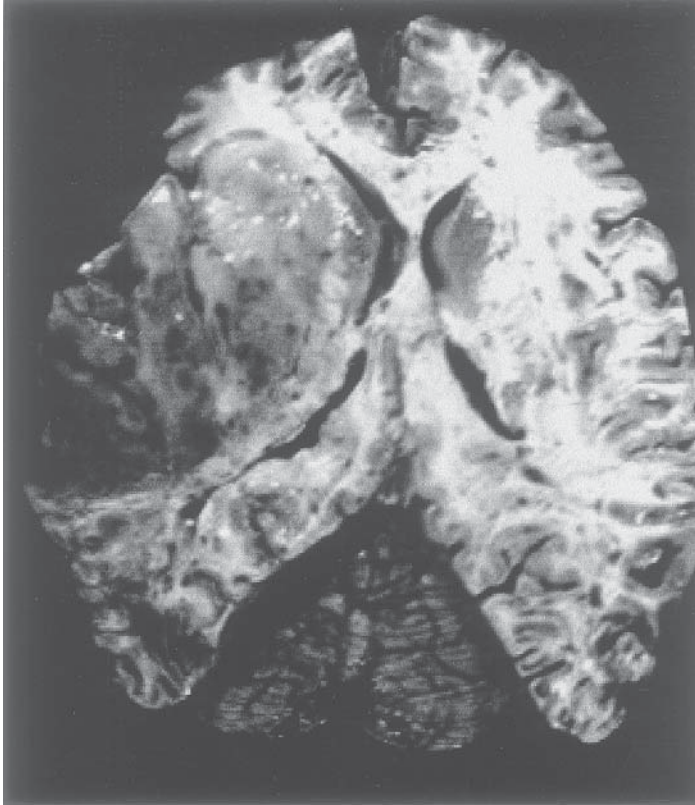


**Fig. 1.** Diffuse infiltrating astrocytoma. Grossly, astrocytomas tend to efface the normal anatomic boundaries, distorting, but not destroying, the structures being invaded. Surrounding edema with mass effect can be appreciated.

and considerably less GFAP-immunopositive intermediate filaments. These astrocytes tend to have a lacy, web-like background and prominent microcyst formation. Corresponding to these histologic subtypes, diffuse astrocytomas occur in three varieties: fibrillary astrocytoma, gemistocytic astrocytoma, and protoplasmic astrocytoma. By far, fibrillary astrocytomas are the most common type of astrocytoma, which may include scattered neoplastic or reactive gemistocytic astrocytes, and even protoplasmic astrocytes. True gemistocytic astrocytomas are tumors composed predominantly of gemistocytic astrocytes. They tend to have low proliferation indices (e.g., <4% as indicated by Ki67/MIB-1 labeling), particularly in comparison to surrounding neoplastic astrocytes (2–4). Yet, they show a high frequency of progression to AA and GBM. Astrocytomas consisting predominantly of protoplasmic astrocytes are rare tumors; some authors do not even consider them to be a true subtype.

Neoplastic astrocytes can range from cells closely resembling reactive astrocytes (in some grade II lesions) to showing bizarre anaplasia (Fig. 6), in which the cells are only recognizable as astrocytic by the presence of GFAP immunopositivity. Some tumor cells become so anaplastic that they even lose GFAP immunopositivity and can only be identified as being astrocytic within the context of the tumor.

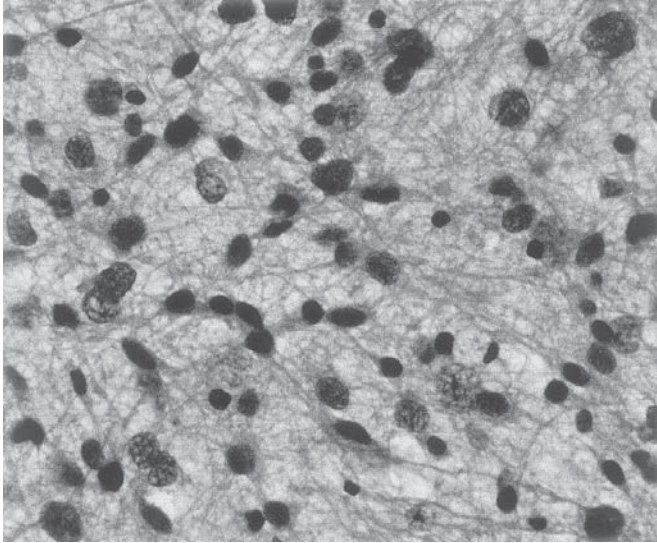
Low-grade diffuse astrocytomas show an increase in cellularity and an irregularity of spacing between tumor cells (Fig. 3). Although the differences between reactive astrogliosis and infiltrating grade II astrocytoma may be subtle, particularly at the infiltrating edge, the central region of most grade II astrocytomas has sufficient increases in cell density, irregular spacing, and nuclear/



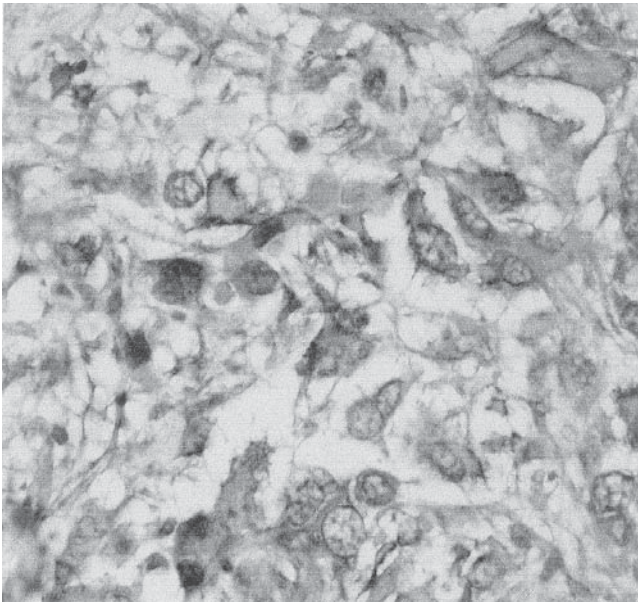
**Fig. 2.** GBM. Large regions of necrosis and hemorrhage can often be seen within glioblastomas, with extensive surrounding edema and mass effect.

cytoplasmic atypia to identify it as a tumor. Further, reactive astrogliosis demonstrates a mixture of reactive astrocytes, but the cells of a grade II astrocytoma tend to have a more monotonous appearance. Microcyst formation is common in low-grade astrocytomas, and may make the tumor initially look paucicellular. However, careful appreciation of the irregularity of spacing between cells and the nuclear/cytoplasmic atypia is usually enough to indicate the presence of a tumor.

As astrocytomas become more malignant, a range of histologic features is noted, which correlate with increasing malignancy: increased cell density/proliferation (Fig. 3), with the presence of nuclear and cytoplasmic atypia (Fig. 6); increased mitotic activity (Fig. 7); vascular endothelial hyperplasia and/or microvascular proliferation (Fig. 8); and necrosis (Fig. 9). In more malignant astrocytomas, the cell density is greater, which is partially reflected in increased proliferation rates as measured by Ki67/MIB-1 labeling indices. Traditionally,

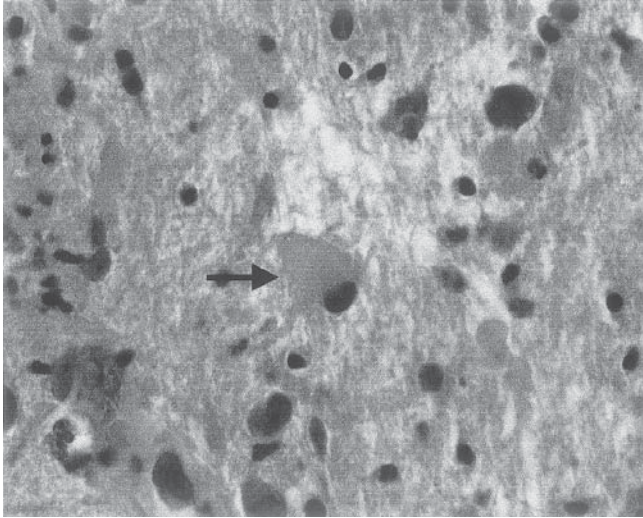


**Fig. 3.** Astrocytoma grade II. The tumor has a pattern of increased cellularity consisting of irregularly spaced astrocytes on a fibrillary background. Note the cellular atypia of the tumor cells, which is not seen in reactive astrogliosis.

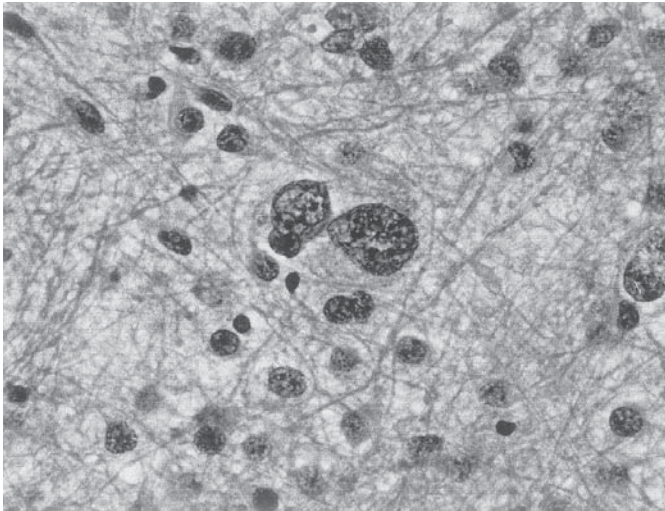


**Fig. 4.** GFAP immunopositivity within neoplastic astrocytes: Immunoperoxidase staining using an Ab against GFAP reveals extensive cellular positivity in these neoplastic astrocytes.



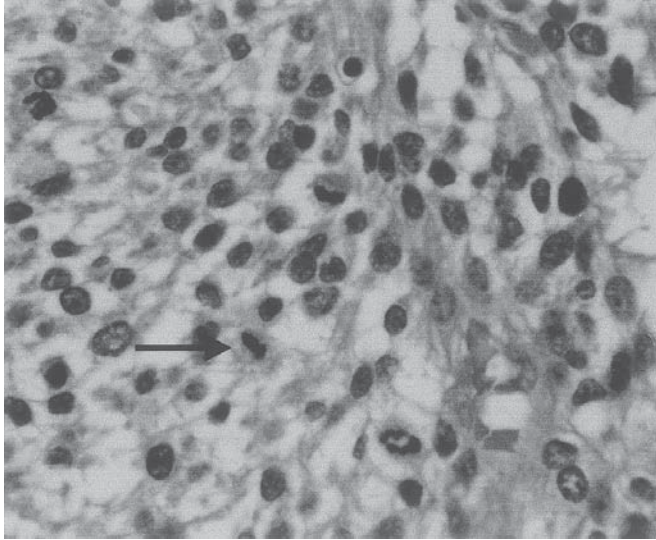


**Fig. 5.** Gemistocytic astrocyte. This gemistocytic astrocyte (arrow) is characterized by the clear cell borders and the abundant, glassy eosinophilic cytoplasm.



**Fig. 6.** Nuclear atypia. Extensive nuclear and cytoplasmic atypia is often seen in astrocytomas as they become more malignant.

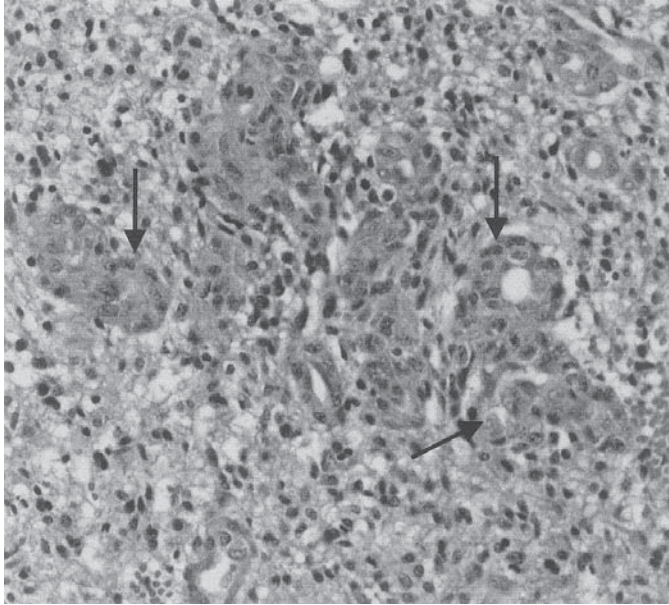
before the advent of IHC methods for measuring proliferation indices, the presence of mitotic figures was an important indicator of active cellular proliferation. Although labeling indices are a potentially more precise measure of cellular proliferation (Fig. 10), the presence of mitotic figures remains a useful micro-



**Fig. 7.** Mitotic figures (arrow) indicate increased cellular proliferation and are an important diagnostic feature of anaplastic astrocytomas and glioblastoma multiforme.

scopic feature and is, in fact, critical to the determination of tumor grade (Fig. 7). Grade II astrocytomas tend to have a labeling index of less than 5% and an absence of mitotic figures; AAs (grade III) usually have indices in the range of 5–10%; GBM (grade IV) typically shows labeling indices of 15–20% (5). Mitotic figures (Fig. 7) are identifiable in both AAs and GBM. Just as in other kinds of tumors, increased malignancy is associated with increasing nuclear and cytoplasmic atypia. This is manifest by increasing nuclear size, frequently with irregularity of nuclear shape (Fig. 6). Cytoplasmic atypia may be subtle in grade II astrocytomas and extensive in GBM.

The proliferation of microvessels, which is also known as vascular endothelial proliferation, is not seen in grade II astrocytomas, and is a common feature of GBM. Microscopically, it consists of a proliferation of endothelial and smooth muscle cells/pericytes to form small “glomeruloid” tufts (Fig. 8). This change is so characteristic that it is considered a major feature in grading systems for astrocytomas, as seen in Section 2.1.3. Microvascular proliferation consists of two components: angiogenesis with endothelial cell proliferation from pre-existing capillaries, which is regulated by vascular endothelial growth factor (VEGF) and its receptors; and smooth muscle/pericyte proliferation regulated by another set of growth factors, including platelet-derived growth factor (PDGF) and its  $\beta$ -receptor (6,7). Recent data suggests that tumors may also utilize pre-existing blood vessels, and that the balance between VEGF and the angiogenic antagonist, angiopoietin-2, may regulate this process (8).

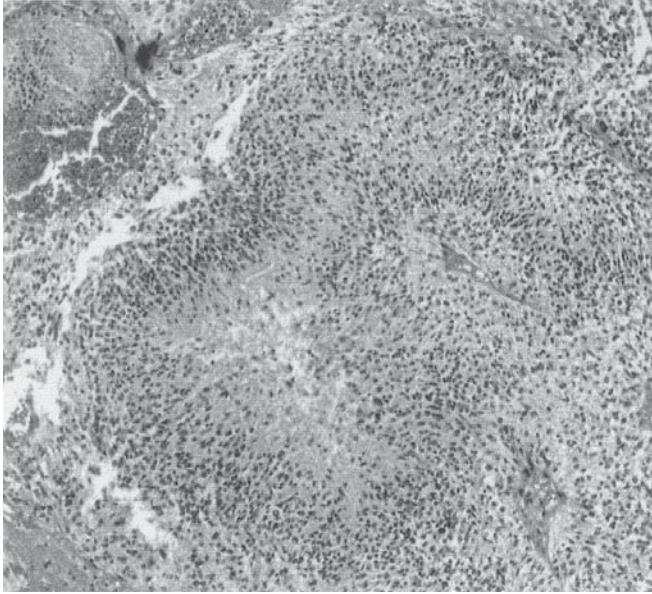


**Fig. 8.** Microvascular proliferation/vascular endothelial hyperplasia. The proliferation of microvessels, also referred to as vascular endothelial proliferation, consists of a proliferation of endothelial and smooth muscle cells/pericytes to form small glomeruloid tufts.

Necrosis is another histologic hallmark of malignancy in astrocytomas and is seen almost exclusively in GBM. Necrosis can consist of either of two types: pseudopalisading necrosis and geographic necrosis. Geographic necrosis consists of zones of coagulative necrosis in which cell outlines can still be identified, but cell staining and detail are lost. Often, the ghost outlines of large tumor vessels, including thrombosed vessels, can be identified. Pseudopalisading necrosis is characterized by a serpiginous pattern of ribboning of tumor cells around necrotic centers (Fig. 9). This type of necrosis is a hallmark of GBM.

### 2.1.3. GRADING SYSTEM

Historically, diffuse astrocytomas have been graded according to the schemes of Kernohan and Ringertz. Currently, two classification systems are commonly used. The St. Anne/Mayo grading system is based on four features: nuclear atypia, mitotic figures, microvascular proliferation, and necrosis. Starting with a baseline score of 1, all features are given one point, and an additive score is made (grade IV, GBM, is the maximum grade). Therefore, because all diffuse astrocytomas have nuclear atypia at the minimum, a grade II is the lowest grade applicable to diffuse astrocytomas. The presence of mitotic figures increases the grade to grade III (AA). The additional finding of microvascular proliferation

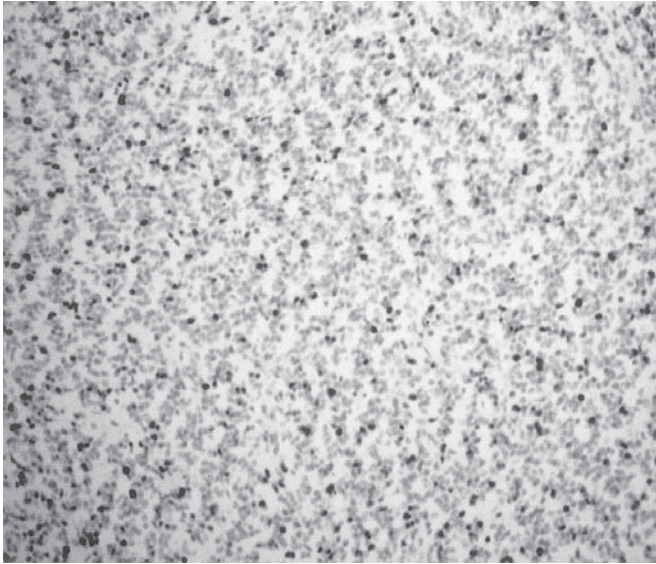


**Fig. 9.** Pseudopalisading necrosis in a GBM. Pseudopalisading necrosis is characterized by a serpiginous pattern of ribboning of tumor cells around necrotic centers. This is a hallmark of GBM.

and/or necrosis increases the grade to IV (GBM). In practice, these features tend to occur in sequence, so that mitotic figures are usually identified before either microvascular proliferation or necrosis. Therefore, AAs (grade III) are usually astrocytomas with nuclear atypia in which mitotic figures can be readily identified. The World Health Organization (WHO) proposed a four-tiered grading system, in which similar features are used, but in a more flexible fashion. These grading systems have been shown to correlate well with survival, so that survival ranges are more than 5 yr for grade II lesions, 2–5 yr for grade III tumors, and less than 1 yr for GBM. However, location of the tumor, extent of surgical resection, and response to therapy also significantly affect survival.

These grading systems have provided useful information about diagnosis and prognosis, and have remained part of the current standard of clinical care. However, purely morphologic grading of astrocytomas has a number of shortcomings. First, fundamental work in the molecular pathogenesis of astrocytomas has revealed clear molecular pathways involved in the oncogenesis and progression of these tumors. Future grading systems will need to incorporate this information. Second, astrocytomas with different molecular pathogenesis may be morphologically identical, yet have markedly different prognoses and responses to treatment. Molecular data will need to be incorporated in order to develop a more complete classification system incorporating pathogenesis, as well as





**Fig. 10.** Ki-67/MIB-1 labeling. Immunoperoxidase staining using an Ab against the cellular proliferation antigen, Ki-67, provides an index of proliferative activity. Grade II astrocytomas tend to have a labeling index of less than 5% and an absence of mitotic figures; anaplastic astrocytomas (grade III) are usually in the range of 5–10%, and GBM (grade IV) typically shows labeling indices in the range of 15–20%. Brown nuclei indicate positive cells, since Ki-67 is a nuclear antigen.

morphology. Finally, because tumors with different molecular alterations will respond differently to therapies, upfront identification of these molecular alterations in clinical tumor samples will aid neuro-oncologists, neurosurgeons, and radiation oncologists in designing treatment options and identifying potential therapeutic targets.

#### **2.1.4. MOLECULAR PATHOGENESIS OF DIFFUSE ASTROCYTOMAS**

**2.1.4.1. Molecular Pathogenesis of Gliomas.** The process of neoplasia involves a loss in the normal regulatory mechanisms of cellular proliferation and cell cycle regulation. In addition, losses of tumor suppressor pathways, which normally prevent mutated cells from replicating, have been shown to play a critical role in the oncogenesis of malignant tumors. Recent work in the molecular pathogenesis of gliomas has shown that these tumors share disruption and loss in critical regulatory pathways. In this chapter, we will concisely summarize this recent work by focusing on the loss of the *p53* tumor suppressor gene; the role of extracellular signals in gliomagenesis (particularly those transduced by the epidermal growth factor receptor [EGFR]); the loss and imbalance within the cell cycle regulatory apparatus, including the cyclins, cyclin-dependent

kinases (CDKs), their inhibitors, and the Rb effector protein; and, finally, the interaction of these multiple pathways in the development of gliomas.

**2.1.4.2. *p53/MDM2/p21/WAF-1/CIP* Pathway.** Elegant work on the pathogenesis of carcinomas has highlighted the role of the tumor suppressor gene *p53* in preventing the development of tumors (9). Patients with the Li-Fraumani syndrome, resulting from germ-line mutations within the *p53* gene, develop frequent tumors (including gliomas) at a markedly accelerated rate (10). This gene, located on chromosome (chr) 17p13.1, encodes a 53 kDa protein that plays a critical role in cell cycle regulation, in response to DNA damage, as well as being critically involved in apoptotic cell death in response to a number of stimuli (11). *p53* can directly bind to DNA and act as a transcription factor (TF) capable of functioning as either a positive or negative regulator of gene transcription. *p53* can activate the transcription of *p21/WAF1/CIP1*, which acts as an inhibitor of CDKs, thereby preventing cells from replicating (1). This activity of *p53* appears to be important for the tumor suppressor effect of *p53* in response to DNA damage (12,13). Inactivating mutations within *p53* are clustered within five highly conserved domains in the middle of the protein, and mutations within these domains involve regions where *p53* is in direct contact with DNA (14). These mutations are usually loss-of-function mutations, indicating the necessity of *p53* activity in tumor suppression.

*p53* can affect cell cycle regulation by acting as a TF. *MDM2* and *p21/WAF1/CIP* are two downstream genes whose transcription is induced by *p53* (15). The *MDM2* gene on chr 12q14.3-q15 encodes a 54 kDa TF. In an autoregulatory feedback loop, *MDM2* regulates *p53* activity by binding to *p53* and inhibiting its ability to initiate transcription (16,17), and by promoting the degradation of *p53* (18,19). Consequently, *MDM2* overexpression may mimic the effect of *p53* mutation, and has been found in approx 10% of primary glioblastomas lacking *p53* mutation (20). The *p21* gene (also known as *CDKN1A* or *WAF1/CIP1*) is on chr 6p; its gene product binds to and inhibits a range of cyclin-CDK complexes, thereby leading to G1/S phase arrest (21). *p21* is critical in *p53*-mediated G1 arrest and apoptosis in response to DNA damage (22). In glioma cells in culture, exogenous expression of *p21* suppresses growth and sensitizes tumor cells to radiation (21) and to cisplatin (23). In addition to the direct effect of *p53* on transcription, the loss of *p53* also has an indirect effect on the accumulation of mutations. *p53*-deficient cells have increased chromosomal instability (24), and therefore increased mutation rates.

Although Li-Fraumani syndrome patients demonstrate the role of germ-line *p53* mutations in patients with gliomas, considerable evidence has demonstrated the role of somatic mutations in the *p53* gene in the development of gliomas. In vitro, the expression of exogenous *p53* in glioblastoma cell lines deficient in the protein results in growth suppression (25,26). In vivo, loss or mutation of *p53* has been found in many series of astrocytomas (27–30). Loss of 17p or *p53*

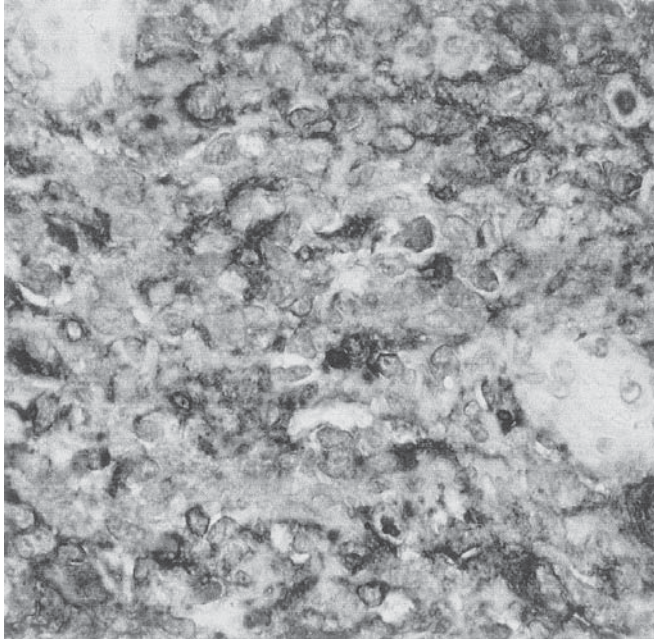
mutations are seen in approx one-third of all astrocytomas of all grades, but are more common in grade II astrocytomas that progress to GBM (31). Further, this subset of glioblastomas, which originate as grade II astrocytomas and progress, and which are associated with *p53* mutations, has helped define the molecular classification of secondary glioblastomas, which is discussed in Section 2.1.4.3.2.

#### **2.1.4.3. Extracellular Signals, Growth Factors, and Their Receptors.**

Stimuli to cells to proliferate are often provided by growth factors, and these signals are transduced by transmembrane receptors. Evidence from carcinomas has shown the prominent role of mutations, resulting in overexpression, amplification, and constitutive activation of growth factor receptors in promoting the development of tumors. Normal and neoplastic astrocytes express a wide range of cell surface receptors and are capable of responding to a wide range of growth factors (including EGF, transforming growth factor  $\alpha$  (TGF- $\alpha$ ), TGF- $\beta$ , basic fibroblast growth factor, platelet-derived growth factor (PDGF), insulin-like growth factors, hepatocyte growth factor, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor) through specific cell surface receptors. Two receptors in particular, EGFR and PDGF receptor- $\alpha$ , have been implicated in the development and progression of gliomas.

*2.1.4.3.1. Role of the EGFR.* EGFR is a 170 kDa protein encoded by a gene on chr 7. EGFR is a transmembrane tyrosine kinase receptor that binds to at least two ligands: EGF and TGF- $\alpha$ . The receptor contains an extracellular domain for ligand binding, a transmembrane domain, a kinase domain, and a carboxyl terminus. The carboxyl terminus has five tyrosine residues, which are the target sites for transphosphorylation, and also serve as motifs for ligand-induced internalization (and subsequent degradation) of the receptor. Binding of EGF or TGF- $\alpha$  results in dimerization of receptor monomers, transphosphorylation of critical tyrosine residues on each receptor of the dimeric pair, and subsequent activation of intracellular signaling pathways, through coupling with adapter proteins to activate signaling through the RAS-MAPK, STAT, PLC, and PI(3)K pathways. EGFR is the cellular homologs of the *v-erbB* oncogene (32) and can generate a transformed phenotype in a ligand-dependent manner, when exogenously expressed (33).

*EGFR* amplification is the most frequent amplification in gliomas (34) (Fig. 11). Amplification and/or overexpression of *EGFR* is present in approx one-third of all glioblastomas (35–41). The ligands, EGF and TGF- $\alpha$ , are also expressed in gliomas (35). In nearly one-half of cases with overexpression of *EGFR*, there is a gene rearrangement in which exons 2–7 are deleted, resulting in a receptor that lacks part of the extracellular ligand-binding domain (42). This mutant EGFRvIII receptor is expressed on the cell surface, and is constitutively autophosphorylated; hence, it is continually active, but at a lower level than the response of normal receptors to EGF or TGF- $\alpha$ . These rearranged



**Fig. 11.** *EGFR* expression in a primary GBM. Overexpression of *EGFR* occurs in primary glioblastomas, and can be detected with immunoperoxidase stains.

receptors, despite being activated, are internalized at the same rate as unoccupied receptors, which is much slower than the rate at which ligand-bound EGFRs are normally internalized. Therefore, the signaling stimulus remains perpetually on, even in the absence of ligand internalization (43). Although the precise molecular pathogenesis of this rearranged receptor is still being determined (regarding its ability to respond to ligand and to dimerize), it is clear that this receptor can powerfully enhance tumorigenicity *in vivo*. *EGFRvIII* is found in nearly one-half of all glioblastomas overexpressing *EGFR* (1).

*2.1.4.3.2. Primary vs Secondary Glioblastomas.* Careful study of the expression of *EGFR*, as well as *p53*, in astrocytomas has enabled researchers to identify a clear molecular classification of GBM. Although morphologically similar, and frequently identical, it has long been noticed that some glioblastomas develop from lower grade astrocytomas that progress and some present *de novo*. This observation, first published by Scherer over 50 yr ago, prompted the suggestion that this group of morphologically identical tumors may contain two different subtypes. Careful study of *p53* mutations and *EGFR* overexpression/amplification has demonstrated that primary glioblastomas, occurring as *de novo* lesions in older patients, are associated with *EGFR* overexpression/amplification, but that *p53* mutations are rare (<10%) in this subgroup (44). Secondary glioblastomas, in contrast, are associated with *p53* mutations and *EGFR* overexpression/amplification is rare.

blastomas arise as a progression from lower-grade astrocytomas. They tend to present at a younger age, and are strongly associated with *p53* mutations (44). These two types of glioblastomas account for approx two-thirds of GBM (44). In addition, giant cell glioblastomas (as discussed above), which have the distinct morphologic property of giant cells, tend to present as *de novo* glioblastomas, but occur in younger patients and show a high percentage of *p53* mutations, suggesting an intermediate molecular pathogenesis (45).

Although the dramatic role of EGFR in gliomas has not been demonstrated for other growth factors, the clear role of PDGFR- $\alpha$  overexpression in a percentage of progressing low-grade astrocytomas (46) suggests that it may play a role in BT progression. Future research into other growth factors and their receptors, as well as new receptors and ligands that have yet to be identified, will probably provide insight into the role of growth factor and cell surface receptor signaling in the development and progression of gliomas.

**2.1.4.3.3. Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN) and its Role in Gliomagenesis.** Many growth factors activate the PI(3)K pathway and its downstream effector, Akt. Activation of this pathway appears to be important for cell survival. PTEN is a phosphatase that converges on this pathway to act as a negative regulator of PI(3)K and Akt activity (47). It has been observed in cytogenetic studies that loss of heterozygosity (LOH) on chr 10 is the most frequent cytogenetic alteration in glioblastomas, occurring in more than 80% (48–53). *PTEN* is located on chr 10, and therefore, great interest has focused on the role of *PTEN* in gliomas. Although it appears that *PTEN* mutations cannot account for all of the lesions associated with LOH on chr 10 (49), *PTEN* is mutated in 30–40% of glioblastomas (53,54). In vitro, *PTEN* can mediate growth suppression when exogenously expressed in *PTEN*-deficient glioblastoma cell lines (55), and can induce G1/S-phase arrest. All of these studies demonstrate that *PTEN* regulates the level of cellular activation transduced by growth factors and extracellular matrix signals (56), suggesting that its loss contributes to the development of some gliomas.

**2.1.4.4. Disruptions of Cell Cycle Regulatory Proteins in the Development of Gliomas: the *p16/CDKN2a*, *CDK4*, *RB* Pathway.** As part of the normal regulation of cell cycle events, proliferation stimuli (such as growth factors) induce downstream mitogenic signals, which result in the association of cyclins (such as cyclin D) with CDKs (such as CDK4 and CDK6). These complexes interact with the Rb protein, phosphorylating it, and allowing release of the *E2F* TF. *E2F* initiates downstream transcription of genes required for cellular proliferation (57). These events are required for the G1/S-phase transition in response to mitogenic signals. These cell cycle regulators are counterbalanced by a set of inhibitors. The role of *p53* (and *p21*) in the inhibition of cell cycle progression has already been discussed. In addition to *p53* and *p21*, CDK inhibitors (CDKNs) play a critical role in balancing the system. *p16* (also known



as *CDKN2a* and *INK4a*), *p15* (*CDKN2b* and *INK4b*), and *p27* inhibit CDK4 and CDK6 by preventing their ability to phosphorylate Rb in conjunction with cyclin D. Disruptions of this exquisitely balanced cell regulatory cycle are clearly implicated in oncogenic events in BTs.

CDK4 and CDK6 both complex with cyclin D and catalyze the phosphorylation of Rb, allowing release of the *E2F* TF. Overexpression and/or amplification of these CDKs could therefore result in unregulated cell proliferation. Studies evaluating *CDK4* overexpression in gliomas have shown a range of findings, with *CDK4* amplification in up to 15% of high grade gliomas (58,59).

*p15* and *p16* are both encoded on chr 9p21, and function as inhibitors of CDKs. They therefore block the phosphorylation of Rb and progression through the cell cycle. Losses of *p15* and *p16* activity lead to unregulated growth. In vitro, *p15* and *p16* are often deleted in glioma cell lines. Exogenous *p15* and *p16* can induce cellular senescence and G1 growth arrest in glioblastoma cells in culture (60,61). Deletion of *p16* is seen in nearly one-half of all cases of GBM (62,63), and with a lower frequency in AAs. In a recent series of 42 oligodendrogliomas (ODGs) and 36 astrocytomas, *p16* was decreased in 17/42 ODGs and in 9/10 glioblastomas, 5/9 AAs (grade III), and 3/10 grade II diffuse astrocytomas (64). Furthermore, *p16* expression was inversely correlated with cellular proliferation in the tumors, and loss of *p16* was associated with a poor prognosis (64).

*p27* (also known as *kip-1*) inhibits the association of CDKs with cyclins, thereby acting as a negative regulator of cell cycle progression (65). It is widely expressed in well-differentiated gliomas (65,66), but its expression appears to be lost in high-grade gliomas (65). Furthermore, its expression in gliomas is associated with a better prognosis (67).

The Rb protein is a 107 kDa protein that complexes to the *E2F* TF. After phosphorylation by CDK4 (or CDK6) in conjunction with cyclin D, *E2F* is released to allow transcription of genes required for cellular proliferation. Rb protein is lost or mutated in approx 10–15% of glioblastomas (68,69). In vitro, adenoviral transfer of *Rb* suppresses the growth of glioma cells in culture (70). From this cell cycle data, it is clear that alterations within CDK4, the cyclin kinase inhibitor, *p16*, and/or the Rb protein play critical roles in the development of gliomas. An analysis of 120 glioblastomas demonstrated that only 6% of the cases studied did not have an abnormality of *p16*, *CDK4*, or *Rb* (69).

**2.1.4.5. Converging Molecular Events: Concurrent Overexpression of *EGFR* and Mutations in the Cell Cycle Regulatory Apparatus.** The above sections have outlined how overexpression, amplification, and/or rearrangement of the *EGFR* results in signals to induce proliferation that are processed by the cell cycle regulatory apparatus. The alteration of these signals by mutations in the *PTEN* phosphatase has also been discussed. Mutations in *p53*, by abrogating the normal cell cycle checkpoints in response to damaged DNA (some of

which are regulated by *p21*), release the cell cycle apparatus when it should be in check. Finally, mutations within the cell cycle processes can themselves result in the development of gliomas. Because these separate molecular pathways seem to converge along the same points, one would expect that multiple mutations along these pathways would synergize in the oncogenesis of gliomas. The finding of simultaneous mutations in many of these pathways in glioma cell lines (71), and in a number of series of human gliomas (69,72–76), suggests this kind of synergy.

More directly, a system to explore the cooperation of multiple mutational events in the genesis of gliomas has recently been developed (77). *EGFR* was expressed as a transgene in mice, in association with either disruption of the *p16/INK4a/Arf* locus, overexpression of *CDK4*, or in association with *p53* mutations. There was no cooperativity between *p53* mutations and *EGFR* overexpression, which goes along with the molecular classification of primary and secondary glioblastomas. However, the investigators did notice cooperative oncogenesis between *EGFR* overexpression and either *p16/Ink4a/ARF* loss or *CDK4* overexpression (77). This observed experimental cooperativity is supported by studies of human gliomas, in which there was an association between *p16* mutations and *EGFR* overexpression in tumor biopsies (39,68,78).

## 2.1.5. NEUROPATHOLOGY OF DIFFUSE ASTROCYTOMAS

**2.1.5.1. Low-Grade Astrocytoma (Astrocytoma Grade II).** Grossly, diffuse grade II astrocytomas appear as poorly defined masses that infiltrate and expand surrounding anatomical structures. They typically blur the anatomical boundaries, but do not show destruction of the invaded tissue (Fig. 1). Diffuse grade II astrocytomas usually have a whitish appearance and a firm texture, although microcystic changes can cause a gelatinous appearance. Occasionally, larger cysts may be observed. These tumors are usually surrounded by edema and may exhibit considerable mass effect.

Microscopically, grade II diffuse astrocytomas show an increase in cellularity, with irregular spacing of the cells (Fig. 3). Most fibrillary astrocytomas contain a rich, dense network of fine filamentous processes, so that they give the appearance of irregular nuclei floating in a fibrillary matrix. Occasional scattered gemistocytic astrocytes, with more distinct cytoplasmic borders and glassy eosinophilic cytoplasm, can usually be appreciated admixed within fibrillary astrocytomas. The tumor cells usually show a modest degree of nuclear atypia, characterized by enlargement of the nuclei (relative to nonneoplastic astrocytes), with subtle to marked alterations in the regularity of nuclear shape. Mitotic figures, microvascular proliferation, and necrosis are not seen in grade II diffuse astrocytomas, by definition. Infiltrated cells, such as neurons, are initially preserved as the tumor invades through underlying anatomical structures.

The background pattern in grade II diffuse fibrillary astrocytomas can range from a relatively solid fibrillary background to a looser matrix, with faintly

basophilic (on hematoxylin and eosin staining) extracellular material. A microcystic pattern can also be seen, in which small islands of tumor cells float among abundant microcysts.

Gemistocytic astrocytomas are tumors consisting predominantly of gemistocytic astrocytes. Gemistocytic astrocytes are characterized by abundant glassy eosinophilic cytoplasm with clearly discernible cell borders. They tend to have low proliferation indices (e.g., <4%, as indicated by Ki67/MIB-1 labeling index), particularly in comparison to surrounding neoplastic astrocytes (2,3). Gemistocytic astrocytomas are graded according to the same St. Anne/Mayo and WHO classification systems as other astrocytomas. Therefore, grade II lesions do not contain mitotic figures, microvascular proliferation, or necrosis. However, low-grade gemistocytic astrocytomas appear to have a high rate of more rapid progression to AA and GBM.

Immunocytochemistry using an antibody (Ab) against the intermediate filament, GFAP, usually demonstrates abundant GFAP immunoreactivity in glial tumor cells. S100 and vimentin, both intermediate filaments expressed by astrocytes, are also present in most grade II astrocytomas. The proliferation marker, Ki67/MIB-1, is a useful marker for determining labeling index. This provides a measure of a tumor's proliferation rate, which is highly useful, because elevated labeling indices have been associated with worse prognosis (79). It is critical to determine labeling index by rigorously counting MIB-1-positive cells and total cells. Quick visual estimates are unreliable.

The importance of the molecular alterations in astrocytomas and their prognostic importance have been described above. Since Abs to assess the protein level of p53, CDK4, p16, Rb, and EGFR, as well as other receptors and cell cycle regulatory proteins, are now available, their incorporation into diagnostic neuropathology will eventually be warranted and easily justified. Although their utility in clinical practice is currently being validated by studies assessing staining patterns and prognosis, they will clearly soon be integrated into the clinical setting.

Immunocytochemical analysis of some proteins, such as p53, will not supplant mutational analysis by single-strand confirmation polymorphism analysis or density gradient gel electrophoresis (which may be followed by sequencing). For example, because mutant p53 is less subject to degradation than wild-type p53, the presence of prominent and persistent p53 immunopositivity has been used as evidence of mutation. Direct comparison of p53 immunostaining has suggested that, although p53 immunostaining is sensitive, it is not entirely specific for detecting p53 mutations, because p53 accumulation by immunocytochemistry may be seen in the absence of mutations (at least within the major hot-spots examined) (80,81).

**2.1.5.2. AA (Astrocytoma Grade III).** Grade III AAs usually appear grossly similar to grade II diffuse astrocytomas. Microscopically, grade III AAs tend to

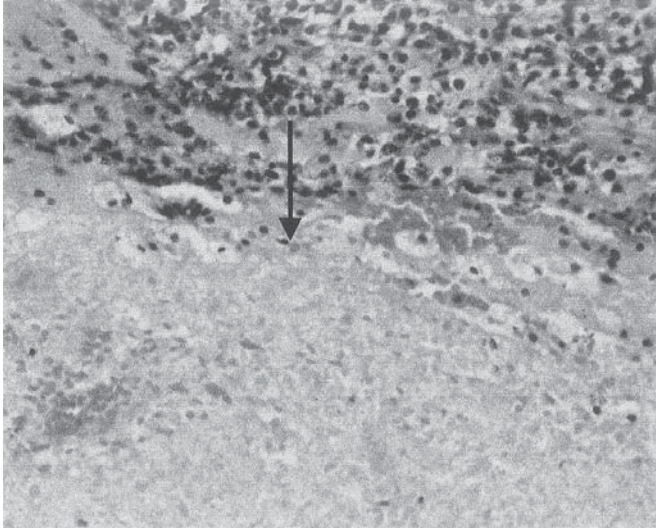


be more cellular than grade II tumors. This increase in cellularity is usually readily appreciable, at least focally. In addition to the increase in cellularity, increased nuclear/cytoplasmic atypia is also usually seen (Fig. 6). Nuclei become larger, and are more irregular and varied in shape. Nucleoli may become prominent. Cytoplasmic atypia may also become significant, especially in tumor cells with more apparent borders. In addition to the increased cellularity and atypia, AAs have a higher proliferation rate, and mitotic figures are apparent, particularly on careful examination of the specimen. In addition, the finding of atypical mitotic figures, such as tripolar mitotic spindles, is a useful diagnostic feature. Microvascular proliferation and necrosis are not normally seen, since they would increase the grade if seen in conjunction with mitotic figures. Rarely, microvascular proliferation or necrosis may be seen in the absence of mitotic figures, which creates a diagnostic problem in using the St. Anne/Mayo grading scheme. Fortunately, this situation is rare, and often represents a GBM that has been incompletely sampled.

GFAP immunopositivity is usually seen in grade III astrocytomas, although immunopositivity may be decreased or lost with increasing anaplasia. S100 and vimentin, as well as  $\alpha\beta$ -crystallin are usually present. Ki-67/MIB-1 immunostaining, with appropriate performance of a labeling index, is useful and usually demonstrates an elevated labeling index between 5 and 10% (82,83). Immunostaining using Abs against regulatory and cell cycle proteins may be of additional benefit, as described above.

**2.1.5.3. GBM (Astrocytoma Grade IV).** Grossly, glioblastomas are usually poorly circumscribed, highly infiltrating tumors that creep along white matter tracts, such as the corpus callosum. Occasionally, glioblastomas (particularly giant-cell glioblastomas) may have a much more circumscribed appearance. On gross inspection, glioblastomas tend to have enormous heterogeneity, with regions of yellowish softening (necrotic foci), reddish zones of hemorrhage, and even cystic regions, along with solid areas (Fig. 2). Considerable edema and mass effect usually surround glioblastomas, and cerebral herniations may be seen. Although most lesions are solitary, incidences of multifocal gliomas, ranging from approx 2.5 (84) to 7.5% (85), have been described. Furthermore, careful examination of multicentric lesions may reveal subtle finger-like connections between the lesions. Extension of GBM into the subarachnoid space is not frequent, but certainly can occur. "Drop metastasis" to other parts of the CNS, such as the spinal cord, may also occur. Systemic metastasis of glioblastomas is a rare event, but has been described (5).

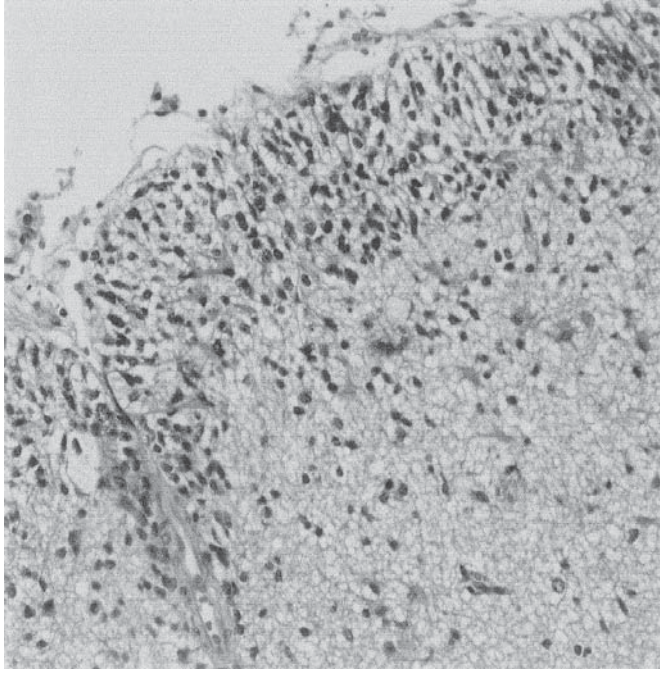
Microscopically, glioblastomas may show extensive heterogeneity, with regions of solid, dense cellular growth, and other areas with lower cell density and different cellular morphology. Although the genetic heterogeneity underlying the phenotypic heterogeneity is only beginning to be explored, recent work



**Fig. 12.** Geographic necrosis, which is seen within glioblastomas and after radiation therapy, is characterized by large zones of coagulative necrosis in which cell outlines can still be identified, but cell staining and detail are lost.

suggests that there is extensive intratumoral molecular heterogeneity (86,87). The individual tumor cells themselves may show a staggering range of morphologies, from small cells to giant cells, with many variants in between. Multinucleated tumor giant cells may be present. If they are the predominant cell type, the tumor may fall into the giant-cell glioblastoma subtype, which is discussed below.

Glioblastomas are characterized by dense cellularity (at least focally), with extensive nuclear and cytoplasmic atypia. Mitotic figures are readily identifiable (Fig. 7), and atypical mitotic figures can often be seen. Microvascular proliferation is a common accompaniment (Fig. 8), and necrosis is also usually present. Pseudopalisading necrosis is a hallmark of glioblastomas and is seen in both primary and secondary glioblastomas (Fig. 9). Geographic necrosis, with its preserved ghost outlines, appears to be more common in primary glioblastomas (Fig. 12). Although necrosis is an important feature in the grading of astrocytomas, geographic necrosis also occurs in response to radiation therapy. Therefore, the neuropathologist needs to be aware of whether the patient has already been irradiated at the time of a given biopsy. Other features that may be seen in glioblastomas include satellitosis of tumor cells around neurons and BVs; subpial spread of tumor cells with the accumulation of subpial cushions of tumor (Fig. 13), so-called “Scherer’s secondary structures”; lipidization of tumor cells; and perivascular lymphocyte cuffing.



**Fig. 13.** Subpial tumor spread of a GBM (Scherer's secondary structures). Subpial spread of tumor cells, with the accumulation of cushions of tumor beneath the subarachnoid space, may be seen in high-grade gliomas.

As discussed in Section 2.1.5.3, molecular analysis has identified the presence of at least two distinct subtypes of glioblastoma: primary and secondary. The primary glioblastomas present as *de novo* lesions, tend to occur in older patients, and have a more aggressive course. They are associated with overexpression/amplification and gene rearrangement (in approx 50% of cases) of *EGFR*. They also tend to have co-existing abnormalities of the cell cycle regulatory proteins, such as loss of p16/INK4a/ARF and overexpression of CDK4 (78). Secondary glioblastomas occur as a progression from lower-grade astrocytomas, which originate in younger patients, and tend to have a more protracted course. Secondary glioblastomas are associated with *p53* mutations. Approximately two-thirds of glioblastomas can be characterized as primary or secondary glioblastomas (44,78).

The giant-cell glioblastoma constitutes a variant subtype of glioblastoma that has a distinct gross and microscopic appearance, and may have a slightly different molecular pathogenesis. Giant-cell glioblastomas constitute approx 5% of glioblastomas (88). Grossly, they are well circumscribed, compared to the usually infiltrative pattern of other glioblastomas. Microscopically, they have

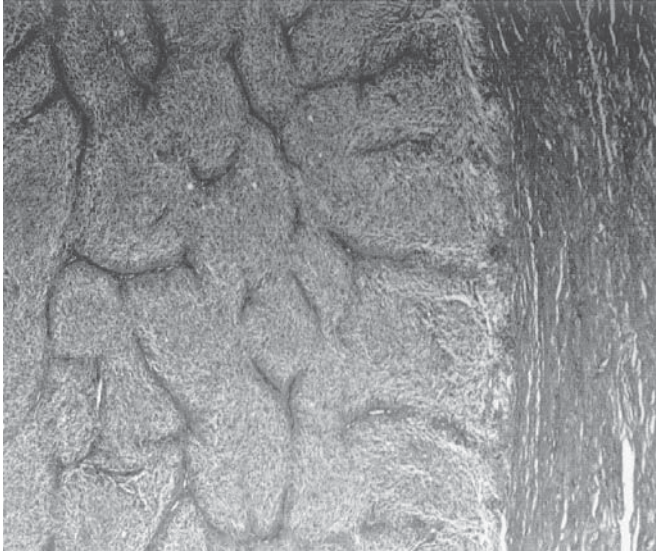
numerous multinucleated tumor giant cells admixed with smaller neoplastic astrocytes. The giant cells are extremely bizarre, and may be huge (up to 500  $\mu\text{m}$  for a single cell). They have abundant eosinophilic cytoplasm, which may stain variably with GFAP, although S100 immunopositivity is usually preserved. Mitotic figures, microvascular proliferation, and necrosis may be seen, just as in other glioblastomas. A dense, reticulin-rich background may also be present. A number of reports have suggested that patients with giant-cell glioblastomas may have a slightly longer survival than with other glioblastomas, even though they are highly malignant lesions. Giant-cell glioblastomas present like *de novo* glioblastomas, yet they do not have overexpression of *EGFR*, amplification of *CDK4*, or loss of *p16* (45,89), as do primary glioblastomas. Rather, they have a high incidence of mutations in the *p53* gene (45,89). Therefore, they share the molecular pathology of secondary glioblastomas, but tend to present like primary glioblastomas.

Approximately 2–8% (90,91) of all glioblastomas are associated with a sarcomatous element. These tumors, known as gliosarcomas, are usually superficially located, firm masses that appear relatively circumscribed. Microscopically, the tumors have a biphasic pattern, containing elements of glioblastomas and elements that usually resemble fibrosarcomas, with a herringbone pattern of densely packed atypical spindle cells. Usually, these elements are mixed, so that islands of glioblastoma are seen interrupted by a herringbone fibrosarcoma-like proliferation, which is most prominent around BVs. A reticulin stain often dramatically demonstrates the sarcomatous area, in which individual spindle cells are surrounded by a dense deposition of reticulin. Reticulin also outlines the glioblastomatous islands (as opposed to individual tumor cells). GFAP immunostaining is useful to demonstrate immunopositivity in the glioblastomatous portion and lack of GFAP staining in the sarcomatous region. Two important caveats need to be considered before making the diagnosis of gliosarcoma. First, exuberant fibrous proliferation can occur around regions of microvascular proliferation in glioblastomas. Unequivocal evidence of malignancy, such as severe atypia and mitotic figures, must be seen in fibrous cell population in order to diagnose a gliosarcoma, as opposed to a glioblastoma with prominent desmoplasia. Second, glioblastomas are highly malignant tumors that can show a range of morphology, including highly spindled cells. The diagnosis of gliosarcoma requires that the sarcomatous portion must not show any glial differentiation, as assessed by GFAP immunohistochemistry. The sarcomatous component of gliosarcomas is thought to arise from zones of microvascular proliferation.

## **2.2. Astrocytic Variants: Pilocytic Astrocytoma, PXA, SEGA, DCAI**

### **2.2.1. PILOCYTIC ASTROCYTOMA**

Pilocytic astrocytomas generally occur during childhood and early adulthood, with a peak incidence in the second decade of life (92). Pilocytic astrocytomas



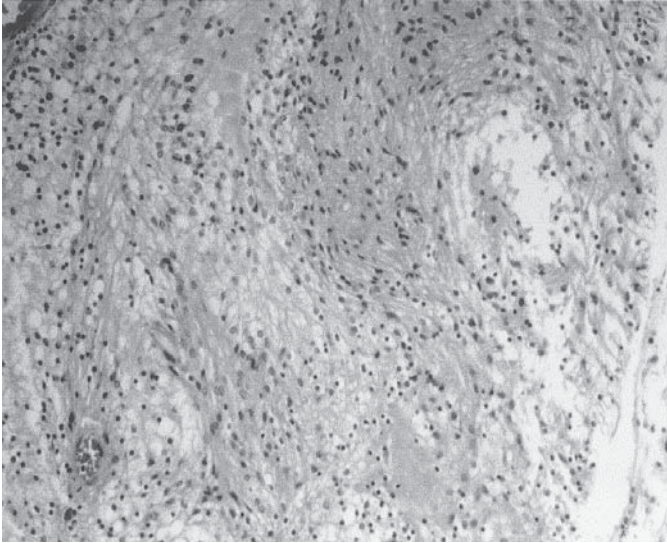
**Fig. 14.** Pilocytic optic nerve glioma with expansion of the nerve. Pilocytic astrocytomas may involve and grossly expand the optic nerve.

can occur anywhere in the CNS, but show a predilection for midline structures, including the cerebellum, third ventricular region, optic nerves, brain stem, and deep central gray matter. Spinal cord pilocytic astrocytomas are less common.

**2.2.1.1. Neuropathology.** Pilocytic astrocytomas are discrete masses, often with a cystic component. Extension into the subarachnoid space is a relatively common occurrence, particularly in cerebellar pilocytic astrocytomas. Pilocytic astrocytomas of the optic nerves tend to produce a diffuse enlargement of the nerve as a result of direct extension of tumor into the subarachnoid space (Fig. 14).

Microscopically, pilocytic astrocytomas have a biphasic pattern (Fig. 15). Dense areas of compact, elongated piloid (hairlike) cells, with abundant Rosenthal fibers and eosinophilic granular bodies, are interspersed with microcystic zones of lower cellularity. Rosenthal fibers are corkscrew-shaped eosinophilic masses that are often present in pilocytic astrocytomas (Fig. 16), although they may also be seen in reactive gliosis. Ultrastructurally, they are dense masses within cellular processes filled with intermediate filaments. Eosinophilic granular bodies are aggregates of proteinaceous material, which are common in a range of low-grade (WHO grade I) tumors, including pilocytic astrocytoma and PXA (as is discussed in Section 2.2.2.). The looser regions may contain astrocytes with relatively few processes and more rounded morphology, resembling protoplasmic astrocytes. Clusters of ODG-like cells may also be appreciated within pilocytic astrocytomas and do not necessarily indicate a mixed tumor.

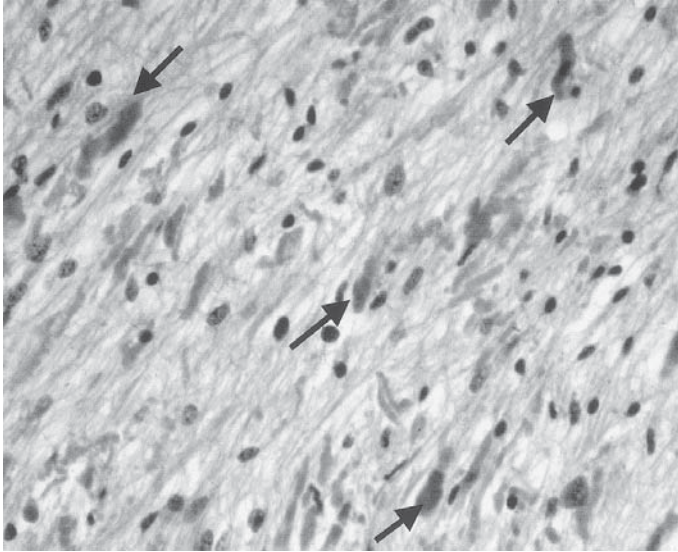




**Fig. 15.** Pilocytic astrocytoma. Microscopically, pilocytic astrocytomas have a biphasic pattern, consisting of compact areas of elongated piloid (hair-like) cells interspersed with microcystic zones of lower cellularity.

Neoplastic pilocytic astrocytes are GFAP-immunopositive and have delicate fibrillary processes. A significant degree of nuclear atypia may be seen, although this usually represents degenerative atypia and is not a sign of aggressive behavior. Mitotic figures are usually inconspicuous. Ki67/MIB-1 immunostaining shows a generally low proliferation index (often less than 1%), although focal areas of increased labeling, up to 5%, may be seen (92). Microvascular proliferation and extension into the subarachnoid space, both of which portend a grim prognosis in diffuse astrocytomas, may be present. However, these features do not indicate a worse prognosis in the context of a pilocytic astrocytoma. Pilocytic astrocytomas are typically relatively indolent grade I lesions, although rare examples can transform into more rapidly growing tumors (93).

**2.2.1.2. Molecular Pathogenesis.** Patients with type 1 neurofibromatosis (NF1) have a high rate of developing pilocytic astrocytomas, particularly of the optic nerve (94). In this neurocutaneous syndrome, these tumors may be bilateral. Somatic mutation of the *NF1* gene does not appear to play a major role in the genesis of sporadic pilocytic astrocytoma (95). Analysis of *p53* mutations in pilocytic astrocytomas has shown contradictory data. Most studies have shown *p53* mutations to be vanishingly rare in pilocytic astrocytomas (96–98). However, a recent study of 20 pilocytic astrocytomas demonstrated *p53* mutations by density gradient gel electrophoresis in seven cases, 35% of the tumors tested (99).



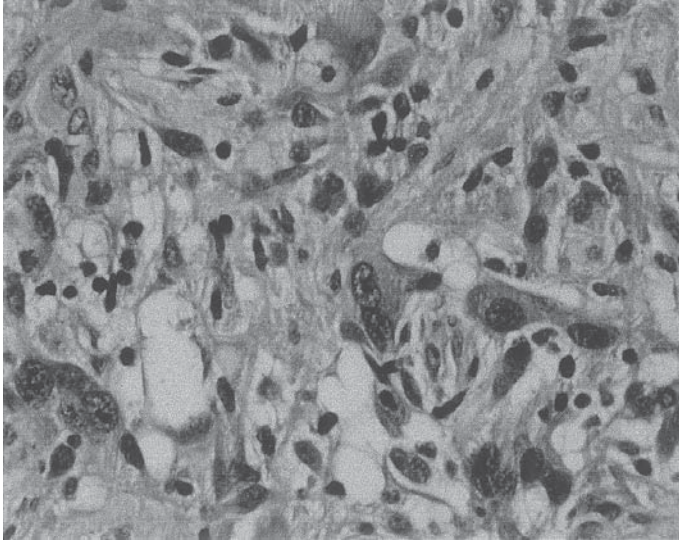
**Fig. 16.** Pilocytic astrocytoma. Rosenthal fibers (arrows) are corkscrew-shaped eosinophilic masses that are often present in pilocytic astrocytomas, although they may also be seen in reactive gliosis. Ultrastructurally, they are dense masses filled with intermediate filaments.

## 2.2.2. PLEOMORPHIC XANTHOASTROCYTOMA

**2.2.2.1. Incidence.** PXA is a relatively rare tumor of children and young adults. It accounts for less than 1% of all astrocytic neoplasms (100), and usually presents with seizures. Most PXAs arise in the cerebrum, particularly the temporal lobe. They tend to have a superficial location with involvement of the meninges.

**2.2.2.2. Neuropathology and Molecular Pathogenesis.** Grossly, PXAs involve the cerebral cortex, and are attached to the leptomeninges, but not the dura. They are often cystic, and may appear as a cyst with a mural nodule. Microscopically, PXAs are characterized by marked pleomorphism, abundant reticulin fibers, and scattered lipidization of tumor cells. The tumor cells are highly pleomorphic, ranging from polygonal cells to spindle cells, in a fusiform pattern, to large multinucleated giant cells (Fig. 17). The reticulin-rich nature of this tumor is shown clearly by reticulin stains, which demonstrate the dense reticulin network surrounding individual tumor cells (Fig. 18). Some of the atypical astrocytes may have a vacuolated (lipidized) appearance. Scattered perivascular lymphocyte cuffing is also a common feature, and eosinophilic granular bodies (as described in Section 2.2.1.1.) may be seen. Atypical ganglion cells have been described in a small number of cases (101).

Immunohistochemistry analysis for GFAP usually demonstrates the astrocytic nature of the pleomorphic cells. Although cytoplasmic atypia is prominent,



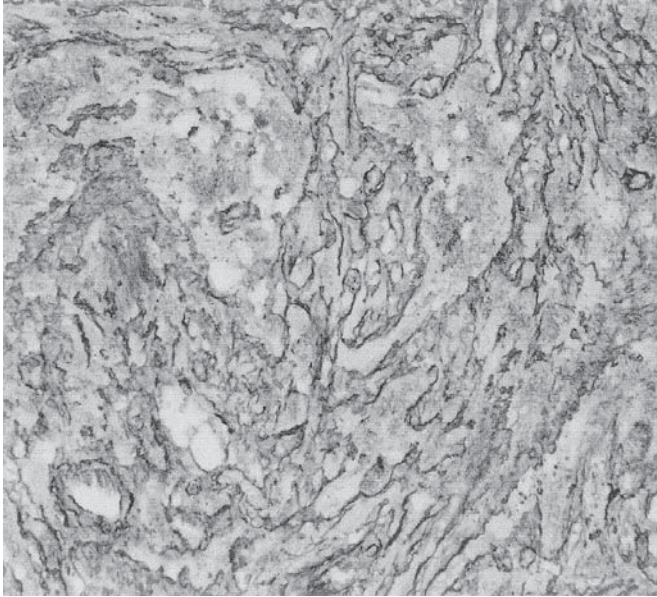
**Fig. 17.** PXA. Microscopically, these tumors are characterized by marked pleomorphism, with tumor cells that range from polygonal to spindle to fusiform shapes. Large multinucleated tumor giant cells may also be seen.

mitotic activity, vascular proliferation, and necrosis are usually absent. Ki67/MIB-1 immunostaining usually demonstrates a low labeling index (often less than 1%), although increasing rates of proliferation usually indicate a worse prognosis (102). PXAs are considered to be grade II lesions in the WHO scheme. However, a subset of tumors tends to recur and show increasingly malignant features, such as mitotic activity, necrosis, and vascular proliferation. The molecular pathogenesis of PXA seems to be different than that seen in diffuse astrocytomas. The common alterations seen in diffuse astrocytomas (e.g., *p53* mutations, *EGFR* overexpression, and LOH on chr 10q or 19q) occur in only a minority of PXA cases (103).

### 2.2.3. SUBEPENDYMAL GIANT CELL ASTROCYTOMA

SEGAs are rare tumors that occur primarily in patients with tuberous sclerosis complex (TSC), a genetic disease associated with mutations in the *TSC2* gene, which encodes for tuberin, and the *TSC1* gene, which encodes the hamartin. TSC is characterized by a variety of lesions, including cortical tubers, retinal hamartomas, facial angiofibromas, and angiomyolipomas (among other findings) (104,105). SEGAs occur in up to 14–16% of patients with TSC (100,106). Whether SEGAs ever occur in non-TSC patients is unclear, particularly because TSC may have extremely variable presentations and manifestations. Indeed, some authors consider SEGAs to be a TSC-defining lesion.



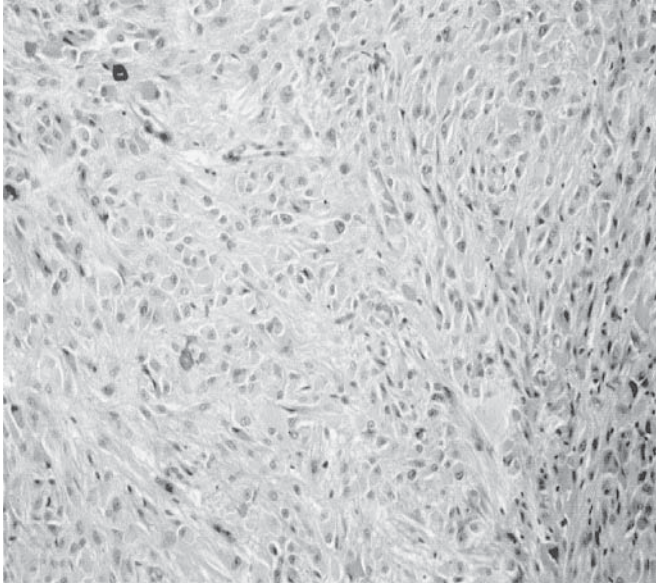


**Fig. 18.** PXA. The reticulin-rich nature of this tumor is shown clearly by a reticulin stain, which demonstrates the dense reticulin network surrounding individual tumor cells.

**2.2.3.1. Neuropathology.** SEGAs generally develop in the walls of the lateral ventricles, usually adjacent to the foramen of Monro. Grossly, they tend to be nodular, well-demarcated lesions. Microscopically, SEGAs consist of heterogeneous collections of tumor cells, ranging from large polygonal strap-like cells, with abundant glassy eosinophilic cytoplasm, to small cells on a fibrillary background (Fig. 19). The elongated strap-like cells may bear a resemblance to the balloon cells seen in cortical tubers and the hamartomatous lesions of cortical dysplasia (107). Although the cells may show atypia, mitotic activity is rare. True vascular endothelial hyperplasia and necrosis are not seen. Immunohistochemistry shows variable reactivity of tumor cells with GFAP and S100 Abs, and some of the strap cells may co-express neuronal markers, such as neurofilament or class III  $\beta$ -tubulin (108). The co-expression of neuronal and glial markers in some of these tumor cells is similar to a phenomenon noted in the balloon cells seen in the cortical tubers of TSC and in cortical dysplasia (104). The strong association with TSC indicates the role of mutations within the tuberin (*TSC2*) and hamartin (*TSC1*) genes in the pathogenesis of this tumor.

#### **2.2.4. DESMOPLASTIC CEREBRAL ASTROCYTOMA OF INFANCY**

These rare tumors arise in infants and present as huge (nearly hemispheric) tumors involving the superficial cerebral cortex and leptomeninges. They are



**Fig. 19.** SEGA. This tumor consists of a heterogeneous collection of cells, ranging from large polygonal strap-like cells, with abundant glassy eosinophilic cytoplasm, to small cells on a fibrillary background. Mitotic activity, microvascular proliferation, and necrosis are seldom present.

frequently cystic, with a solid portion in the leptomeninges that may be attached to the dura. Microscopically, they contain a variable mesenchymal component with dense desmoplasia and a GFAP-positive component. Mitotic figures are usually not seen, except rarely in undifferentiated areas of the tumor. Necrosis and vascular proliferation are also not seen. Frequently, atypical ganglion cells or cells expressing neuronal markers are admixed. In these mixed tumors, the diagnosis of desmoplastic infantile ganglioglioma (DIG) is usually more appropriate. DCAI and DIG are considered to be WHO grade I tumors. The children with these neoplasms usually do well, even with incomplete tumor resection (109–111). The molecular pathogenesis of these rare tumors is unknown, although a number of studies have suggested that *p53* mutations are not implicated (110,112).

### ***2.3. Gliomatosis Cerebri: A Rare Neoplasm with Astrocytic Differentiation but Unknown Histogenesis***

Gliomatosis is an unusual, diffuse, highly infiltrative tumor that often shows extensive astrocytic differentiation, although it has been difficult to classify. In the WHO system, it is classified as a tumor of unknown origin, but its extensive astrocytic differentiation and its infiltrative growth pattern merit its inclusion in

this subheading. Clinically, gliomatosis cerebri is a rare tumor, with less than 200 cases having been described in the literature. The peak incidence is between 40 and 50 years of age, but may occur at any age. The prognosis is poor, because the lesions are (by definition) unresectable and largely unresponsive to chemotherapy or radiation therapy.

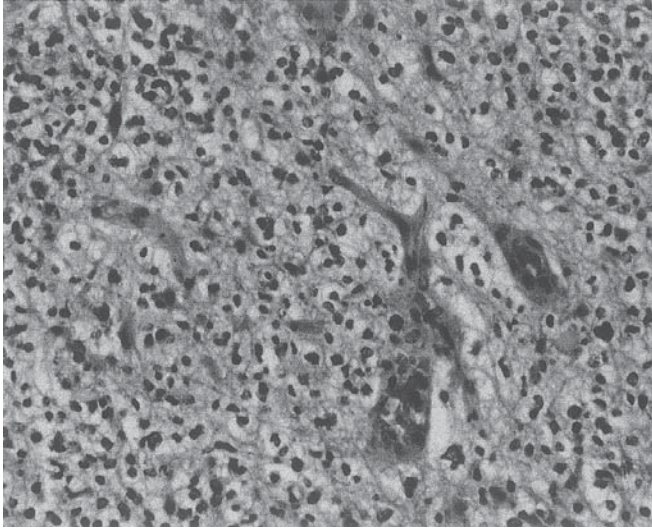
Grossly, these tumors present more as diffuse enlargement, rather than as a discrete mass (although a discrete mass can occasionally be seen in association with the diffuse enlargement). Gliomatosis cerebri is usually so massively infiltrative that it causes invasion and enlargement of large portions of the cerebrum, including the cerebral cortex, subcortical white matter, deep central gray matter, and even the brain stem. The tumors usually involve nearly an entire lobe or even a cerebral hemisphere. Microscopically, gliomatosis cerebri is characterized by a proliferation of elongated glial cells, which infiltrate brain structures and can be seen extending along white matter tracts. The cells have elongated nuclei, some with even a rod-like appearance. The cytoplasm is fibrillary and appears astrocytic. GFAP immunostaining is often, but not invariably, positive. The molecular genetic changes in gliomatosis cerebri have not been characterized. However, the chromosomal abnormalities found in one case of gliomatosis cerebri did not match those usually seen in diffuse astrocytomas. Further work to characterize the molecular pathogenesis of this rare tumor is needed.

### 3. OLIGODENDROGLIOMAS

ODGs are a group of gliomas arising from oligodendrocytes. They account for approx 10–17% of intracranial gliomas (*113*). Most ODGs arise between the fourth and fifth decades (*114*), but can arise at any age. ODGs tend to preferentially affect the white matter of the cerebral hemispheres, particularly the frontal and temporal lobes (*100*).

#### 3.1. Neuropathology

Grossly, ODGs tend to be well-circumscribed lesions (although they are often infiltrative on microscopic examination). They may appear gelatinous or cystic, and calcification (either in or adjacent to the tumor) is frequent. Microscopically, ODGs consist of rounded cells that appear morphologically similar to oligodendrocytes (Fig. 20). They may be arranged in a variety of patterns, ranging from patternless sheets to nodules or lobules of tumor cells. Occasionally, parallel rows of tumor cells are seen in a pattern referred to as “rhythmic palisading” (Fig. 21). ODGs frequently contain a “chicken-wire” vascular pattern, consisting of abundant delicate thin-walled vessels (Fig. 20). Microcystic areas are also common. In addition, neoplastic astrocytes tend to infiltrate the overlying cerebral cortex and surround neurons.



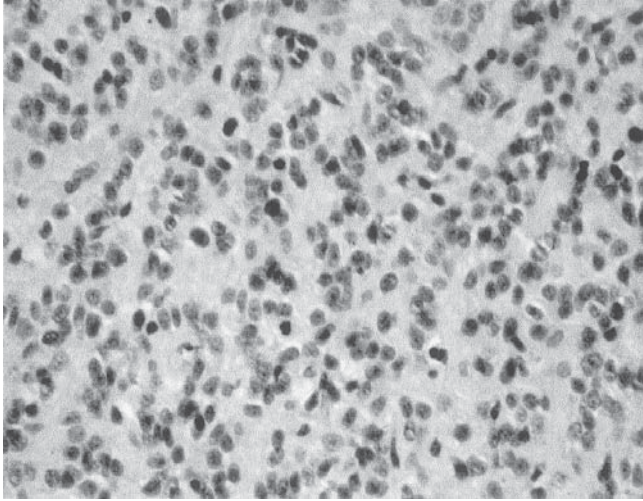
**Fig. 20.** ODG. Microscopically, oligodendrogliomas consist of rounded cells that appear morphologically similar to oligodendrocytes. The clear halo “fried-egg” appearance is a useful artifact of fixation. ODGs frequently contain a “chicken-wire” vascular pattern, consisting of abundant delicate thin-walled vessels, as seen here.

Oligodendroglial cells, both normal and neoplastic, tend to have a rounded morphology, with only a scant fibrillary matrix. In addition, in response to fixation, these cells develop a characteristic “fried egg” appearance, with rounded cells surrounded by a clear halo (Fig. 20). Reactive fibrillary astrocytes are commonly seen within ODGs. However, an admixed astrocytic component is often also seen, as is discussed below. In addition, small gemistocytic astrocyte-like cells that are strongly GFAP-positive may be seen. These minigemistocytes are morphologically similar to gemistocytic astrocytes, but are noticeably smaller.

Distinct immunohistochemical markers to specifically identify oligodendrocytes have been difficult to identify, with most distinctive antigens being either poorly or inconsistently expressed in ODGs. Therefore, identification of oligodendroglial tumors has mostly been based on morphology, and on the absence of strong GFAP immunoreactivity.

ODGs are classified as WHO grade II lesions. The presence of high cellularity, nuclear atypia and pleomorphism, brisk mitotic activity, microvascular proliferation, and necrosis are all features seen with increasing malignancy. Anaplastic oligoastrocytomas (WHO grade III) show a constellation of these features (Fig. 22). With increasing malignancy, oligodendroglial cells may appear more astrocytic, so that the differentiation between an anaplastic oligoastrocytoma with vascular proliferation/necrosis and a GBM may be difficult.





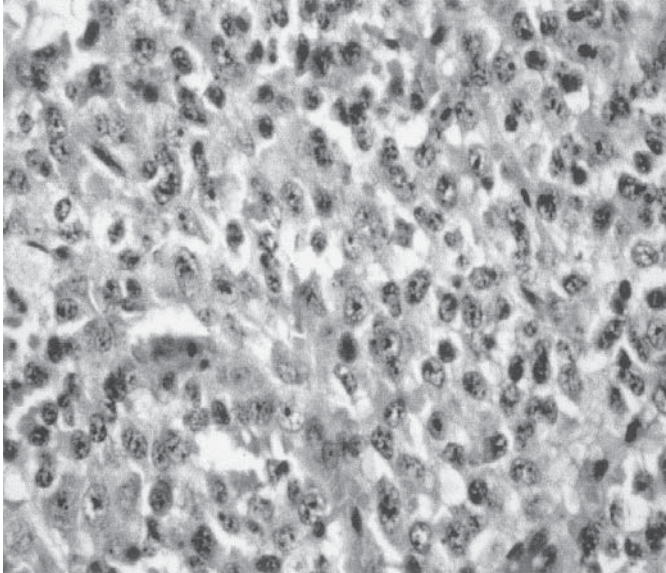
**Fig. 21.** ODG. Occasionally, parallel rows of tumor cells are seen in a pattern referred to as “rhythmic palisading.”

Reactive astrocytes may be admixed within ODGs, but a significant number of ODGs contain intimately intermingled neoplastic astrocytes. Traditionally, it has been assumed that astrocytomas and ODGs arise from mature astrocytes and oligodendrocytes, respectively. However, recent work has suggested that some astrocytic and oligodendroglial tumors arise from oligodendrocyte precursor cells that express the NG2 proteoglycan and the  $\alpha$ -receptor of PDGF (115). These findings suggest that a number of glial tumors may arise from precursor cells that may be capable of astrocytic or oligodendroglial differentiation, or both.

Mixed oligoastrocytomas may be either predominantly astrocytic or predominantly oligodendroglial. The components may be admixed or each subtype may be focally prominent within different areas of the tumor. Just as astrocytomas are thought to be more aggressive than ODGs, the most aggressive areas of the astrocytic component usually determine the malignancy of mixed oligoastrocytomas. Hence, oligoastrocytomas are usually considered grade II lesions, anaplastic oligoastrocytomas are considered WHO grade III tumors, and mixed oligoastrocytomas tend to be graded according to the often more malignant astrocytic component. In addition, GBM may contain a considerable oligodendroglial component, along with a clearly astrocytic component.

### ***3.2. Molecular Pathogenesis of ODG, Anaplastic ODG, and Mixed Oligoastrocytomas***

Recent elegant work has shed light on the molecular alterations associated with ODGs and mixed ODGs, and also upon critical gene losses associated with



**Fig. 22.** AAs (WHO grade III). With increasing malignancy, ODG cells may appear more astrocytic, so the differentiation between an AA with vascular proliferation and necrosis vs a GBM may be difficult.

progression to anaplastic tumors. Specifically, this work has focused on the role of LOH on chr 1p and 19q, *CDKN2A* (*p16*) loss, and loss of *p53*.

### 3.2.1. LOSS OF 1P AND 19Q

LOH on chr 1p and chr 19p, suggesting the presence of tumor suppressor genes at these loci, are the most frequent molecular events associated with ODGs. In one series, loss of 1p36 or 19q13 was found in 73% of ODGs, with simultaneous loss of both regions in 64% of tumors tested (116). In a well-characterized series by Bigner et al. (117), the authors used LOH analysis and comparative genomic hybridization to determine the molecular alterations associated with low-grade ODGs, anaplastic ODGs, and mixed oligoastrocytomas. They found that 1p or 19q were lost in 74% of well-differentiated ODGs, 83% of anaplastic ODGs, and 38% of mixed oligoastrocytomas. Additional evidence has suggested that losses on chr 1p and 19q are significantly associated with increased sensitivity to chemotherapy and longer recurrence-free survival after chemotherapy (118).

### 3.2.2. *CDKN2A* (*p16*)

In their series, Bigner et al. (117) observed that deletion of *CDKN2A* (*p16*) or LOH on chr 9p (the locus associated with the *p16* gene) was highly correlated with progression to anaplastic ODG. This observation is in agreement with data

from another group that found loss of *CDKN2A* associated with poor prognoses in ODGs (116). This finding has been further supported in another recent series (64), in which the authors demonstrated that loss of *p16* expression (as determined by immunohistochemical analysis) is associated with clinical progression in ODGs.

### 3.2.3. *p53* Loss

In Bigner et al.'s (117) molecular analysis of ODGs and mixed oligoastrocytomas, 14/55 tumors showed *p53* loss, the vast majority of which were mixed gliomas. There were no associated losses of 1p or 19q in 13/14 tumors. These findings suggest an important role for *p53* mutations in mixed oligoastrocytomas, but not in ODGs.

### 3.2.4. SUMMARY OF MOLECULAR ALTERATIONS IN ODGs, ANAPLASTIC ODGs, AND MIXED OLIGOASTROCYTOMAS

From these elegant studies, one can conclude that loss of some tumor suppressor genes on chr 1p and 19q are implicated in the development and chemosensitivity of ODGs. One can also conclude that loss of the region of chr 9p containing the *CDKN2A* gene locus and loss of p16 expression (the protein encoded by *CDKN2A*) are strongly associated with progression to anaplastic ODG. Furthermore, the current data suggest that *p53* mutations may not play a role in ODGs, but may play an important role in mixed oligoastrocytomas. Other molecular alterations seen in association with ODGs include strong expression of the *EGFR* gene in the absence of its amplification (119). The importance of this observation remains to be further studied.

## 4. EPENDYMOMAS

Ependymomas are tumors composed of ependymal cells. They arise from the ependymal linings around the ventricles, the cerebral aqueduct, and the central canal of the spinal cord. They give rise to tumors of the cerebral hemispheres, cerebellum, brain stem, and spinal cord, usually in the vicinity of the ventricles or spinal cord. In children and adolescents, ependymomas tend to be intracranial. In adults, they are frequently spinal, and are the most common type of spinal glioma encountered. Ependymomas and their variants are WHO grade II tumors, except for the myxopapillary ependymoma, which is considered a grade I tumor. As ependymomas become more malignant, they may become grade III, or anaplastic ependymomas, as is discussed in section 4.2.

### 4.1. Neuropathology

In the posterior fossa, ependymomas arise from the roof or the floor of the fourth ventricle. They grow as exophytic masses extending into, and often fill-

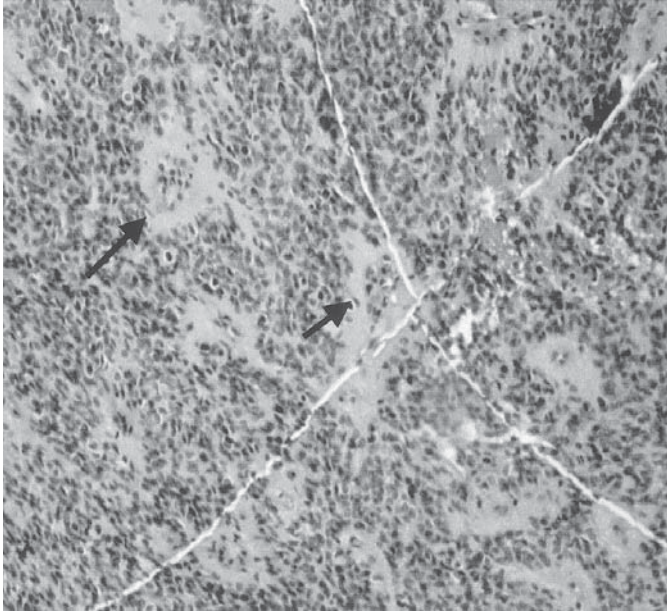
ing, the ventricle. Ependymomas arising from the lateral ventricles or the spinal cord tend to invade adjacent brain or spinal cord parenchyma. They tend to have pushing (as opposed to infiltrating) borders on gross examination.

Microscopically, ependymomas are moderately cellular tumors that are characterized by sheets or lobules of cells with characteristic perivascular pseudorosettes and occasional true ependymal rosettes. Perivascular rosettes are BVs surrounded by tumor cells, with a relatively clear space between the BV and the nuclei of the ependymal cells (Fig. 23). These clear zones consist of the radially aligned processes of the ependymal tumor cells surrounding the BVs. True ependymal rosettes consist of ependymal cells surrounding a central lumen. Immunohistochemical examination shows focal GFAP positivity, particularly in the clear zone around perivascular pseudorosettes. Ependymomas usually stain positively for S100 and variably for cytokeratin and epithelial membrane antigen. Ki67/MIB-1 labeling of ependymomas shows a relatively low labeling index of 2.6%, on average (120).

A number of well-recognized histologic variants of ependymomas have been described: cellular ependymoma, clear cell ependymoma, papillary ependymoma, and tanycytic ependymoma. Although these variants are morphologically distinct, they all have the same prognosis, and all need to be distinguished from anaplastic ependymomas. The cellular ependymoma is characterized by a relatively solid architecture, with few perivascular pseudorosettes or ependymal rosettes. The clear cell ependymoma has a prominent round cell component, with perinuclear halos. These tumors, which arise around the foramen of Monro, tend to be confused with ODGs or central neurocytomas. Papillary ependymomas are a variant that morphologically mimics choroid plexus tumors, because of the prominent pseudoepithelial papillary growth pattern. These tumors tend to have tumor cells surrounding the BVs, but in a papillary pattern. Immunohistochemistry for GFAP, cytokeratin, and vimentin is useful, because choroid plexus tumors are markedly cytokeratin positive, while papillary ependymomas are focally GFAP and vimentin positive with minimal cytokeratin staining. Tanycytic ependymomas have long processes that are strongly GFAP positive. This finding, in combination with the relative lack of perivascular pseudorosettes, may give the appearance of an astrocytoma.

Another distinct variant of ependymoma is the myxopapillary ependymoma. This tumor, which arises in the conus medullaris, cauda equina, and filum terminale region of the spinal cord, has unique histologic features and relatively indolent behavior. The myxopapillary ependymoma consists of columnar or cuboidal cells surrounding BVs with hyaline and mucoid degeneration. They tend to be highly papillary and strongly GFAP and vimentin immunopositive. Myxopapillary ependymomas have a very good prognosis, even when subtotally resected (121).





**Fig. 23.** Ependymoma. Perivascular pseudorosettes are important diagnostic features of ependymomas. They consist of BVs surrounded by tumor cells, with a relatively clear space between the BV and the nuclei of the ependymal cells. These clear zones of radially aligned ependymal processes are usually GFAP immunopositive.

#### ***4.2. Anaplastic Ependymomas***

Ependymomas and their variants, except for the grade I myxopapillary ependymomas, are WHO grade II tumors. They may contain areas of dense cellularity, myxoid degeneration, and even foci of necrosis. Some posterior fossa ependymomas may even spread into the overlying subarachnoid space. However, with increasing malignancy, they may become anaplastic ependymomas (WHO grade III), which are characterized by increased cellularity, increased atypia, increased mitotic activity, and frequent microvascular endothelial proliferation. An increased Ki67/MIB-1 labeling index has also been shown to correlate with early tumor recurrence (122).

#### ***4.3. Molecular Pathogenesis of Ependymomas***

The molecular pathogenesis of ependymomas remains unknown. Mutations in *p53* have only rarely been observed. Analyses for mutations in the other genes associated with gliomas (e.g., *EGFR*, *p15*, *p16*, *CDK4*) have failed to reveal any consistent alterations in ependymomas.

#### 4.4. Subependymoma

Subependymomas are usually asymptomatic tumors found around the fourth and lateral ventricles. They are small intraventricular masses of tumor cells in a fibrillary matrix. They are indolent WHO grade I tumors that are usually clinically insignificant, unless they cause hydrocephalus. Their importance lies in the awareness of their existence and an understanding of their benign nature. Furthermore, mixed ependymoma/subependymoma tumors may occasionally be encountered.

### 5. SUMMARY

In this chapter, we have summarized the neuropathology and the molecular pathogenesis of the gliomas. To do this, we have presented the current understanding of the classification system used to categorize them. We have included references to fundamental work on the molecular biology of gliomas, that has begun to demonstrate clear patterns of molecular pathogenesis and progression, particularly with reference to the fundamental processes that affect the cellular regulatory pathways of growth response to extracellular signals, cell cycle control, and/or tumor suppressor events, often in combination. This integration of basic molecular data into the classification of gliomas will enable identification of subtypes of gliomas based on their molecular pathogenesis. This may help to determine prognostic differences, even among morphologically identical tumors. This integration may help in identifying rational immunotherapies tailored to recognize the fundamental molecular defects involved in the pathogenesis and progression of primary BTs.

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## Epidemiology of Primary Brain Tumors

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*Susan Preston-Martin, PhD, Faith Davis, PhD,  
and Roberta McKean-Cowdin, PhD*

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### 1. INTRODUCTION

In 1999, it is estimated that 16,800 individuals in the United States were diagnosed with a malignant primary nervous system tumor and that 13,100 of these would die from the disease (1). When benign as well as malignant brain tumors (BTs) are included, the incidence is over twice that for malignant BTs alone; 34,345 individuals were newly diagnosed with a benign or malignant nervous system tumor in 1998 (2). Only about one-half of patients with malignant BTs are still alive one year after diagnosis (3). Controversy continues to surround the issue of whether or not the incidence of BTs, particularly the more lethal subtypes, increased in recent decades (4–6). It appears that trends in childhood (7,8) and adult BTs increased because of the introduction of diagnostic improvements, including CT scans in the mid-1970s and magnetic resonance imaging (MRI) scans in the mid-1980s. This issue, and the recent explosion of epidemiological and molecular genetic studies of BTs, has focused attention on this important human cancer, which, up until only a few decades ago, was relatively little studied. Despite this surge of interest, the etiology of the majority of

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nervous system tumors remains unknown. Inherited syndromes that predispose affected individuals to BT development and/or the presence of nervous system tumors in other family members appear to be present in fewer than 5% of BT patients. Some environmental agents, particularly ionizing radiation, are clearly implicated in the etiology of BTs, but also appear to account for few cases. Numerous other physical, chemical, and infectious agents that have long been suspected risk factors have not yet been established as etiologically relevant.

This review focuses on tumors of the brain, cranial nerves, and cranial meninges, which account for 95% of all central nervous system (CNS) tumors. These tumors are unique because of their location within the bony structure of the cranium. Symptoms depend on location of the tumor. Further, histologically benign tumors can result in similar symptomatology and outcome as malignant tumors, because growth of both normal and tumor tissue is confined to the cranial space. For this reason, some cancer registries voluntarily include both benign and malignant intracranial tumors. For simplicity, this group of tumors will be called “brain tumors,” or, when benign tumors are excluded, “brain cancer.” The term “central nervous system tumors” (or CNS cancer) indicates that tumors of the spinal cord and spinal meninges are included along with BTs, and “nervous system tumors” indicates that tumors of the peripheral nerves are included as well. This review first discusses the descriptive epidemiology of CNS tumors, including the change of incidence rates in different age groups over time; patterns of occurrence by gender, race, geography, and social class; and median survival. Evidence relating to a number of other suggested risk factors is summarized, and prospects for future research explored. For each topic, reference is made only to a few of the numerous relevant papers, but includes a recent paper with a comprehensive bibliography.

## **2. DESCRIPTIVE EPIDEMIOLOGY**

### ***2.1. Variation in Inclusion Criteria***

The descriptive epidemiology of CNS tumors has been difficult to study, because of the wide variation in specific tumors included in published rates. Quantitatively, the most important variation is estimated to be approximately 50% and relates to the inclusion or exclusion of benign tumors (9). This critical difference has often been ignored in comparisons across geographic areas. Although reporting of malignant tumors alone eases geographical comparisons, it is unfortunate that incidence rates for benign nervous system tumors are not also reported. For this reason, benign tumors will not be excluded from descriptive data shown here for Los Angeles County. It should be noted that pineal and pituitary tumors, included in some standard definitions of BT and CNS tumors, are not included. In fact, it becomes clear from discussions of analytic studies below, more is known about the etiology of benign histologic types, such as

meningiomas, than about the etiology of neuroepithelial tumors, which are more common than meningiomas and are usually malignant.

Another variation relates to whether or not clinically diagnosed tumors are included. The microscopic confirmation rate of brain and nervous system cancers included in the latest edition of *Cancer Incidence in Five Continents (10)* varies widely across geographic areas, from a high of 99% (e.g., in Los Angeles County Japanese and Koreans) to a low of 0% (e.g., in Setifi, Algeria). Rates vary considerably among registries, as well as among specific population groups within a country. For example, the rates of histologic verification range from 76 to 95% in Switzerland, 27 to 91% in Canada, 45 to 87% in Brazil, 52 to 98% in Japan, and 63 to 99% in the United States (10). Such wide variation suggests that caution in the interpretation of these rates is warranted. In general, for relatively inaccessible cancer sites, a higher rate of microscopic confirmation increases the likelihood that a neoplasm actually existed, and that it was correctly classified. In some registries, however, a high rate of microscopic confirmation of BTs may indicate that clinically or radiologically diagnosed tumors may have been missed. With the advent of radiosurgery, this is an increasing limitation.

## 2.2. Pathologic Classification

The histologic groups of tumors that occur within the CNS and their corresponding ICD-O codes, are shown in Table 1. A modification of this scheme is proposed for classification of pediatric BTs (11). In both children and adults, neuroepithelial tumors (still more commonly called gliomas) are the most common major histologic type. These are predominantly malignant tumors that arise in the glial cells that comprise the supporting structure of the brain. In Los Angeles, neuroepithelial tumors account for 59% of primary tumors of the brain and cranial meninges, among men and 42% among women. Over 80% of neuroepithelial tumors are astrocytic gliomas (i.e., astrocytomas and glioblastoma multiforme [GBM]). Astrocytic tumors that are grades 1 and 2 are generally classified as astrocytomas, those that are grade 3 are classified as anaplastic astrocytomas (AA), and those with grade 4 are classified as glioblastomas. However, the possibility that this practice is not followed consistently is suggested by the considerable geographic variation in the relative proportions of astrocytic tumors that are classified as glioblastomas. This variation is seen, for example, among the various U.S. registries in the Surveillance, Epidemiology, and End Results (SEER) Program. In comparison with the other SEER registries, Connecticut has a considerably higher proportion of tumors classified as glioblastomas and a correspondingly lower proportion of astrocytomas (12).

The other two most common major histologic types are both predominantly benign. Meningiomas arise in the cranial meninges and account for 20% of all primary BTs in men and for 36% in women. Nerve sheath tumors, called neuro-

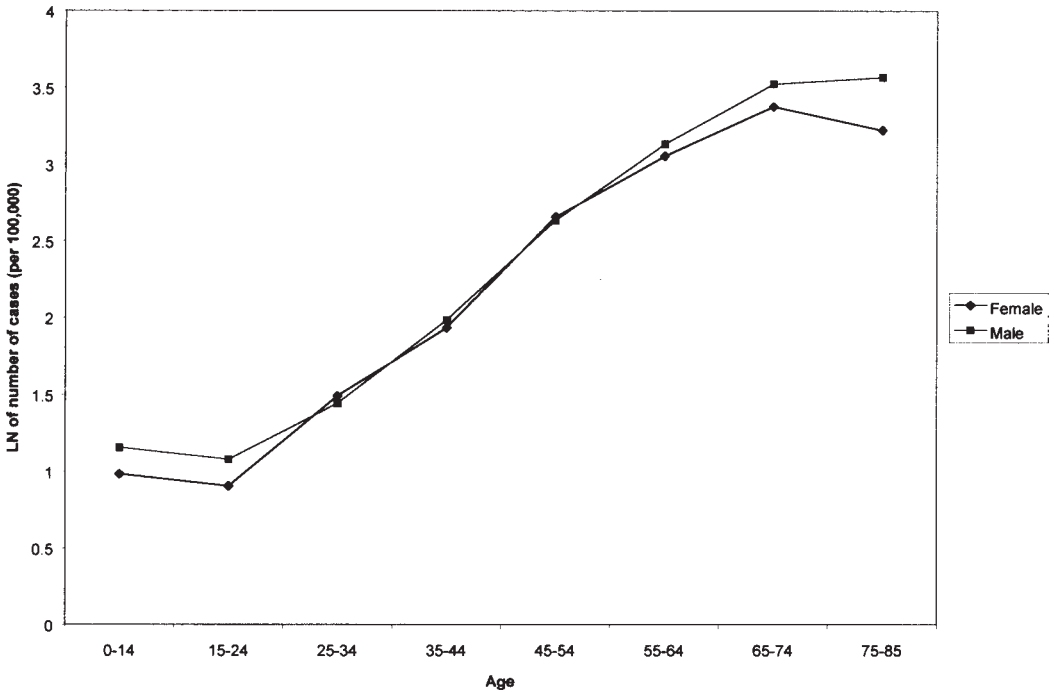
**Table 1**  
**Anatomic and Pathologic Classification of Tumors**  
**of the Central Nervous System**

	<i>ICD-O Codes, 1976</i>	<i>ICD-O Codes, 1991</i>
<b>Subsite</b>		
Brain	191.0–191.9	C 71.1 – C 71.9
Cranial nerve	192.0	C-72.2 – C72.5
Cerebral meninges	192.1	C-70.0
Spinal cord	192.2	C-72.0
Spinal meninges	192.3	C-70.1
<b>Histologic Type</b>		
Neuroepithelial tumors	9380–9481	
Astrocytoma	9384, 9400–21	
Glioblastoma multiforme	9440–42	
Ependymoma	9391–94	
Primitive neuroectodermal tumor (PNET)	9470–73	
Oligodendroglioma	9450–60	
Other neuroepithelial tumors	9380–83, 9390, 9422–30, 9443, 9472–81	
Meningioma	9530–39	
Nerve sheath tumors	9540–60	
Other	9120–61	
Unspecified	8000–02	
No microscopic confirmation	9990	

mas, neurilemmomas, or schwannomas, arise in the Schwann cells of the nerve sheath. About 8% of BTs in both men and women are nerve sheath tumors. It is curious that about 90% arise in the eighth cranial nerve; these tumors are also known as acoustic neuromas.

Now that improved diagnostic technology is available in many general hospitals in the United States and other industrialized countries, the differential diagnosis of intracranial masses is often made by physicians who are not specialists in neurological diseases. The heterogeneous nature of many CNS tumors makes the assignment of histologic class difficult. In a recent survey in the UK, fewer than one-half of the patients with CT diagnoses were referred to neurosurgeons for histologic confirmation by surgery or biopsy. The positive predictive value of the CT diagnosis was around 90% for neuroepithelial tumors and meningiomas, but only 50% for metastatic tumors (13). The introduction of CT-imaging technology in the United States in the mid-1970s, and MRI in the mid-1980s, appears to have resulted in increases in BT incidence rates, without parallel increases in mortality (7,8). Accuracy of clinical diagnosis of primary BTs will





**Fig. 1.** Average annual age-specific incidence of tumors of the brain, cranial nerves, and cranial meninges (benign and malignant combined) in males and females, Los Angeles County, 1972–1997, whites (excluding Spanish-surnamed). Total cases = 5724 in males and 6180 in females.

continue to vary by geographical region and hospital, even though CT and MRI imaging is now available to a greater proportion of regions in the United States. This may result from variations in how the equipment is used and the degree of training of individuals who interpret the films.

### ***2.3. Distribution by Age and Change in Age Curves and Rates over Time***

The average annual age-specific incidence of BTs is shown in Fig. 1. In both males and females, rates decline after a peak in childhood (under age 10), increase after age 25, and level off after age 75. Comparisons of data from different areas of the United States have shown that the shape of the age–incidence curve after age 60 is highly dependent on the autopsy rate (14). Prior to 1955, rates among those over age 55 increased steeply with age in data from Rochester, MN (location of the Mayo Clinic), but decreased after age 55 in data from other areas (e.g., the Second National Cancer Survey, Connecticut and Iowa). Subsequent analyses showed that the proportion of cases first diagnosed at death

**Table 2**  
**Average Annual Age-adjusted Incidence Rates (per 100,000)**  
**by Major Histologic Type of Primary Brain Tumor by Sex**  
**and Ethnic Group, Los Angeles County, 1972–1997**

	<i>Neuroepithelial</i>	<i>Meningiomas</i>	<i>Nerve sheath tumors</i>	<i>All histologic types</i>	<i>(No.)</i>
<b>Males</b>					
Black	3.9	2.1	0.4	7.0	(679)
Spanish surnamed	4.3	1.3	0.4	6.6	(1200)
Other whites	6.4	1.7	1.0	9.8	(5724)
Chinese	2.6	0.7	0.3	4.3	(85)
Japanese	1.7	0.8	0.9	3.6	(59)
Filipino	2.0	1.6	0.7	4.9	(81)
Korean	2.4	0.4	0.1	3.2	(39)
Other races	1.6	1.2	0.7	3.8	(111)
All races	5.3	1.6	0.8	8.4	(7978)
<b>Females</b>					
Black	2.6	3.0	0.4	6.7	(789)
Spanish surnamed	3.4	2.4	0.4	6.7	(1295)
Other whites	4.3	2.9	1.0	8.9	(6180)
Chinese	1.5	1.6	0.4	3.9	(77)
Japanese	1.1	1.4	0.7	3.5	(68)
Filipino	1.9	1.9	0.6	4.7	(100)
Korean	1.7	1.1	0.3	3.7	(47)
Other races	1.5	1.9	0.8	4.3	(146)
All races	3.6	2.7	0.8	7.8	(8702)

was considerably higher in Rochester than in Connecticut; and, when these cases were excluded from the Rochester data, rates declined after age 65 rather than continuing to rise sharply (15,16). These comparisons suggest that BT incidence continues to increase with age throughout life, but that there is often a significant under-ascertainment of cases in the oldest age groups. Therefore, comparisons of BT rates from different registries may be more meaningful if restricted to age groups under age 65.

#### **2.4. Distribution by Gender, Race, and Geography**

In Fig. 1, for all types of BTs combined, rates are higher in males than in females. Table 2 shows the age-adjusted annual incidence rates for the major histologic groups of primary BTs by sex and ethnic group in Los Angeles County, 1972–1997. For all histologic types and races combined, the rate is higher in men than in women. For most ethnic groups, male rates for all histologic types

combined are higher than female rates. The male:female sex ratio (SR) varies considerably, however, by histologic type. In each ethnic group, neuroepithelial rates are higher in males than in females (SR for all races combined = 1.5), and meningioma rates are higher in women (SR = 0.6). The SR in children under age 15 is 1.2 for all tumor types combined. In contrast, primitive neuroectodermal tumors (PNET, formerly called medulloblastomas), which occur almost exclusively in children, have a SR of approx 2 (17,18), but no male excess is seen among U.S. black children (19).

SRs for specific histologic types also vary by anatomic subsite and age group. One of the most interesting examples of this relates to meningiomas. Among non-Spanish surnamed whites in Los Angeles County, spinal meningiomas are 3.5× more common in women than in men (SR = 0.3); cerebral meningiomas are only 1.5× more common in women (SR = 0.7). Similar patterns are seen for meningiomas in Norway (20). Also, the female: male ratio for spinal meningiomas increases with age, but, for cerebral meningiomas, the female excess is greatest during the female reproductive years and declines after age 55. The sex differential for spinal meningiomas suggests the etiologic relevance of some factor related to aging in women. The authors hypothesized that this factor may be vertebral osteoporosis, and a series of three epidemiological studies designed to test this hypothesis do provide it with some, although limited, support (21).

Incidence rates in the Central Brain Tumor Registry of the United States are 12.07 and 10.97/100,000 for males and females, respectively. Overall rates are lower among blacks than among whites (7.72 and 11.6/100,000, respectively) (22). Some of these racial differences vary, however, from one histologic type of BT to another. For example, the rate of neuroepithelial tumors is lower among black males and females than among whites, but the reverse is true for meningiomas (Table 2).

In general, BT rates among whites in Canada, the United States, Europe, the UK, and Australia are relatively similar, although rates are lower in certain Eastern European countries and former Soviet republics (e.g., Russia, Belarus, Krygystan). Rates are lowest in Asian populations in Japan, India, and among Chinese in Singapore. Rates are also lower in Puerto Rico, Costa Rica, and Brazil. Among each racial group, rates are usually higher in migrant populations than in native populations that remain in their country of origin. These differences between migrant and native populations suggest that some change in lifestyle may be occurring in migrant populations that places them at higher risk for BTs, although an increase in diagnostic efficiency may partially explain some of these differences.

## 2.5. Social Class

Table 3 shows the proportional incidence ratios (PIRs) for primary tumors of the brain and cranial meninges by social class (as determined by census track of

**Table 3**  
**Proportional Incidence Ratios (PIRs) for Primary Brain Tumors**  
**by Social Class and Total Number of Cases,**  
**Los Angeles County, 1972–1997, Non-Latino Whites**

<i>Socioeconomic Status</i>	<i>Proportional Incidence Ratios</i>		
	<i>Neuroepithelial</i>	<i>Meningiomas</i>	<i>Nerve sheath tumors</i>
<b>Males</b>			
1 (high)	104.5	93.5	154.1
2	110.6	96.1	113.2
3	106.9	99.2	87.0
4	103.7	100.4	72.9
5 (low)	68.7	114.5	58.8
<b>Females</b>			
1	92.7	96.4	119.0
2	112.7	87.7	121.7
3	108.8	99.1	90.5
4	106.8	114.7	87.0
5	73.7	106.1	70.3

residence) for Los Angeles County non-Spanish surnamed whites. These age-adjusted PIRs represent the ratio of the number of cases observed to that expected in a subgroup. A PIR of 100 indicates that the number observed is the same as the expected number, which was calculated for each 5-yr age group by assuming that the distribution of BTs by social class was the same as that for all other cancer sites combined. There is a clear trend of increasing incidence with increasing social class. For males, this trend is evident for neuroepithelial tumors and nerve sheath tumors. For females, this trend is only clearly evident for nerve sheath tumors. The exception to this is meningiomas, which show the inverse relationship among both males and females. A similar trend of increasing overall brain cancer rates with increasing social class (as determined by occupation) was reported for men in England and Wales a few decades ago (23), and more recently in Washington State (24) and New Zealand (25). Because this trend occurs more strikingly among males than among females, it seems unlikely that it may relate to factors such as diagnostic efficiency or exposure to diagnostic radiography of the head (e.g., dental X-rays), both of which might be expected to be greater among those in higher social classes.

## *2.6. Survival*

Recent relative 5-yr survival rates for brain and nervous system cancers are around 25% (24% for whites and 32% for blacks among U.S. cases diagnosed from 1981 to 1986), compared to just under 20% 20 yr earlier (18% for whites

and 19% for blacks diagnosed in 1960–1963) (26). Survival rates for all tumors vary considerably by location, behavior, histologic type, and age (27). For example, astrocytomas that occur in the deep cerebrum have a much lower 5-yr survival rate than those that occur in the frontal lobe (13.3 vs 28.1%, respectively); and survival is lower for malignant vs nonmalignant tumors (21.6 and 72.4%, respectively) (27). The relative 5-yr survival rate in children, ages 0–14 yr, is now 59%, compared to 35% 20 yr ago (27,28). Clinically significant improvements in survival rates are not apparent in patients over the age of 65 yr (29).

In a recent study in Victoria, Australia, 52% of female (compared to 37% of male) BT patients were living 5 yr after diagnosis (30). As might be expected, nerve sheath tumors, tumors in the “other” category (mostly hemangiomas and gangliogliomas not classified as malignant), and meningiomas, all of which are predominantly benign, have the best prognosis; 100, 96, and 92% of patients with tumors of these three types survive 5 years, respectively. Patients with GBM have the poorest prognosis (5% survive 5 years), and the proportion who survive 5 years is also low for patients with unspecified tumors (20%) and those whose tumors were not confirmed microscopically (28%). Survival for the other subtypes of neuroepithelial tumors varies considerably. The proportion of BT patients who are living 5 yr after diagnosis is considerably greater for patients with ependymoma (65%) and oligodendroglioma (61%) than for those with medulloblastoma (43%) or astrocytoma (44%). For most individual histologic types, survival curves for the two sexes are similar. However, females who develop meningiomas are more likely to have benign tumors and survive significantly longer than males with meningiomas (94 vs 87% 5-yr survival in women and men, respectively).

### ***2.7. Summary of Descriptive Epidemiology***

Perhaps the most important finding from this review of the descriptive epidemiology of brain tumors is that the patterns of occurrence and survival both vary considerably by histologic type, age, and tumor location. For neuroepithelial tumors: the SR (male:female) is greater than one; incidence declines after an early peak under age 10 and continues to rise again after age 25; rates are higher in whites than nonwhites, and are lowest in Asians; and incidence increases with increasing social class, particularly in males. For meningiomas: the SR is less than one (females > males); the female excess is greatest from ages 25 to 54; and rates in U.S. populations are commonly higher in blacks than in whites.

## **3. SUGGESTED CAUSES OF HUMAN BT**

### ***3.1. Ionizing Radiation***

The occurrence of excess BTs after high-dose exposure to ionizing radiation is well established. An updated follow-up of the Israeli cohort who received

scalp irradiation as a treatment for ringworm showed the relative risk (RR) is greatest for nerve sheath tumors of the head and neck (RR = 33.1), intermediate for meningiomas (RR = 9.5), and lowest for neuroepithelial tumors (RR = 2.6) (31). Case-control studies of meningiomas and nerve sheath tumors in adults have found elevated risks associated with exposure to full-mouth dental X-rays decades ago (when doses were relatively high), as well as with prior radiation treatment to the head (32–35). The association with low-dose exposure is more controversial. Prenatal exposure to diagnostic radiography has been related to excess pediatric BTs in several studies, since this association was first reported in 1958 (36), including a study of Swedish twins that found abdominal X-rays of the mother during pregnancy were associated with increased CNS tumor incidence. The findings appeared not to be confounded by mother's age, obstetrical complications, or other factors (37). Exposure to low levels of ionizing radiation during infancy was associated with an elevated risk of intracranial tumors (Standardized Incidence Ratio (SIR) 1.42; Confidence Interval (CI) = 1.13 to 1.75) in a pooled analysis of two Swedish hemangioma cohorts and was highest among infants exposed before 5 mo of age (38).

### ***3.2. Nonionizing Radiation***

#### **3.2.1. ELECTROMAGNETIC FIELDS**

Much controversy in the last 15 years has surrounded the suggestion that exposure to nonionizing electromagnetic radiation, such as power frequency (50–60 Hz) magnetic fields, may contribute to the development of CNS tumors. These fields have not been shown experimentally to be either genotoxic (39) or carcinogenic (40), but there is some suggestion that they may act as a tumor promoter (40). Epidemiological evidence is puzzlingly inconsistent, both in studies of residential exposures and pediatric CNS tumors (41,42) and in studies of CNS cancer in relation to high levels of job exposure (43–45). Two recent studies (42,46) showed no evidence of a link between residential exposure and BTs in children, and a recent review article (47) concluded that, overall, there is little evidence for an association.

#### **3.2.2. RADIOFREQUENCY RADIATION**

Studies of the effect of radiofrequency (RF) exposure in humans have included microwave exposures, the use of radar equipment (occupational and handheld), and direct occupational exposures (such as RF heaters, sealers, plastic welders, medical exposures, amateur radio operator exposures, and telecommunication worker exposures). Although some studies suggest a possible effect of RF on all cancers, brain cancer, or specific other types of cancers, the data are equivocal. An association with cellular telephone usage and the development of BTs has been raised in the legal arena. The rapid increase in the use of cellular tele-



phones, combined with their direct exposure to selected regions of the brain, has stimulated ongoing epidemiological studies (48).

### ***3.3. Occupational Exposures***

Numerous epidemiological studies have investigated the variation in BT occurrence as it relates to employment, which have been summarized previously (49). Repeated studies in various geographic areas have been completed for only a few groups of workers, including those employed in agricultural (25,50,51) or health professions (52,53), and for rubber (54), petrochemical (55,56), and electrical (44,45) workers. Various studies of each occupational group have shown conflicting findings. For the most part, no specific chemical or other exposure has been implicated. Even when a particular chemical exposure was investigated, results have been inconclusive, as in studies of job exposure to vinyl chloride, which were prompted by experimental findings (57,58).

Similarly, several studies have investigated possible associations between occupational exposures of parents and the development of BTs in their children, but many associations have been suggested by only single studies. Multiple studies have suggested an increase in pediatric BT risk among children with a parent employed in paint-related, aircraft, electricity-related, agricultural, metal, and construction industries, although these studies have also failed to implicate any particular exposure (59).

The marked inconsistency of these occupational studies may be partially attributed to: the often small sample sizes (leading to imprecise risk estimates); the different geographic areas (with different major industries) studied; the variation across studies in tumor types, ages at diagnosis, years of diagnosis; and the presumed latent period from exposure to tumor diagnosis. The exposure periods of interest also vary, and, thus, for many industries in which procedures changed over time, the exposures vary as well. Variation also relates to the sources of data about and the criteria used for classifying occupations and nervous system tumors. All studies are limited by the fact that occupation is acting as a surrogate for often unidentified specific environmental agents, the true exposures of interest.

### ***3.4. Pesticides***

Several epidemiological studies have investigated home and occupational use of pesticides, insecticides, or herbicides as possible etiologic factors for BTs, which have been reviewed (60,61). Excess risk of brain cancer was found in a study of licensed pesticide applicators (Standardized Mortality Ratio SMR = 200) (62) and occupational exposure to pesticides (RR = 1.8; 95% CI = 0.6-5.1) (43). Some case-control studies have linked household use and pest exterminations to the development of childhood BTs (63), but few associa-

tions were seen in a recent study of pesticide exposure during gestation and pediatric BTs (64). Associations of CNS tumors with either household or occupational exposures to pesticides are not well established and require further confirmation.

### 3.5. Nitroso Compounds

Although various chemical, physical, and biological agents can cause nervous system tumors in experimental animals, *N*-nitroso compounds (NOCs), particularly the nitrosoureas, are by far the most effective and the most studied (65). These carcinogens show striking nervous system selectivity in some species, including various primates, and tumors can be produced by relatively low levels of NOC precursors in the animals' food and drinking water. If exposure is transplacental, only one-fiftieth (1/50) the dose of ethyl nitrosourea (ENU) required in adult animals is sufficient to cause 100% tumor induction (66). However, no tumors develop if ascorbate (Vitamin C) is also added to the pregnant dam's diet (67). Because there is no reason to think that man is less susceptible to these compounds, it is likely that NOCs cause cancer in humans as well. Although NOC exposure in some occupational settings (e.g., machine shops, tire and rubber factories) can be substantial, most people have low-level, but virtually continuous, exposure to NOC throughout life. However, because NOCs are the most potent of carcinogens in animals (and probably in humans as well), only small doses are needed to cause cancer.

#### 3.5.1. POPULATION EXPOSURE TO NOC

Human exposure to NOC is estimated to derive half from exogenously made and half from endogenously formed compounds (68). Only levels of nitrosamines (not nitrosamides) have been widely measured in human environments and consumer products, even though many of these exposures probably involve both nitrosamines and nitrosamides. Endogenous formation in the stomach or bladder, when both an amino compound and a nitrosating agent are present simultaneously, is likely to be the primary source of human exposure to nitrosamides. Food is a primary source of both highly concentrated nitrite solutions (e.g., from cured meats) and amino compounds (e.g., in fish and other foods, but also in many drugs). Another source of nitrite is reduction (e.g., in the saliva) from nitrate, which comes predominantly from vegetables in the diet. This source is likely to be a far less important contributor to the NOC formed endogenously, because it is highly diluted (and, therefore, less readily reactive), and because vegetables also contain vitamins that inhibit the nitrosation reaction. Drinking water also contains nitrate (in the absence of vitamins), but this is a minor source unless levels are extraordinarily high (69). The level of NOC in the human body is also influenced by other factors, such as the amino compounds present, presence of nitration inhibitors (e.g., vitamins C or E), presence of bacteria or other nitration catalysts, gastric pH, and other physiologic factors. Uncertainties as to

the simultaneous presence of NOC precursors and of inhibitors and/or catalysts of nitration make this hypothesis difficult to study epidemiologically. This difficulty is compounded by further uncertainty about what exposure period during a person's life is most likely to be etiologically relevant.

### 3.5.2. EPIDEMIOLOGIC EVIDENCE

Epidemiological studies of pediatric (70–73) and adult (74–78) BT patients have provided limited support for the hypothesis that NOC exposure is related to the development of CNS tumors. Findings that the use of vitamin supplements and/or high intake of fresh fruit or vegetables protect against BT development may also be interpreted as supportive of the *N*-nitroso hypothesis, although this effect may result from another mechanism (71). The experimental model and its potential relevance to humans are sufficiently compelling to encourage further investigation of this hypothesis, despite the fact that it is a difficult one to test epidemiologically. Future studies must include more complete dietary histories, if they hope to differentiate between findings supportive of the NOC/BT hypothesis and those suggestive of other mechanisms of dietary effects.

### 3.6. Other Dietary Factors

Most dietary investigations among CNS tumor patients have only collected data on dietary sources of NOC exposures, rather than complete dietary histories. Nonetheless, these studies have attempted to evaluate the association between certain dietary micronutrients and BT risk. Adequate evaluation of micronutrient intake will require investigation of complete dietary histories. Use of vitamin supplements (particularly vitamins C, E, and multivitamins), has been found to reduce BT risk in adults in some studies (77), but not others (78,79). In children, risk may be reduced by the child's personal vitamin use (80,81), by the mother's vitamin use during pregnancy (73,82,83), or by her intake of fruit, fruit juice, and vegetables (71,73,81).

Although the findings of reduced risk of BTs in children and adults associated with increased intake of vitamin supplements, fruits, and vegetables may be related to the *N*-nitroso hypothesis by inhibiting endogenous formation of nitrosamines, it is important to consider other potential mechanisms of this effect. In this respect, it is interesting that a study of childhood BTs reported higher RRs associated with the child's consumption of cured meats when the child did not take multivitamins than when they did take multivitamins (84).

Recent studies have investigated the possible associations of BTs with other dietary micronutrients (71,73). In particular, a case-control study of childhood PNET (73) found significant protective trends with increasing levels of dietary vitamins A and C,  $\beta$ -carotene, and folate taken by the mother during pregnancy. In a related study of childhood astrocytoma, reduced risks were evident for

dietary vitamins A and C, but these trends were not significant (71). There was no relationship between childhood astrocytoma and dietary  $\beta$ -carotene and/or folate. Although these preliminary results suggest exciting prospects for the possible prevention of childhood BTs, interpretation is difficult, because both studies were primarily focused on the evaluation of dietary NOCs. Thus, evaluation of other micronutrients was limited to the micronutrient composition of NOC-related food items. These results highlight the need to incorporate complete dietary evaluations into future epidemiological studies.

### ***3.7. Prior Head Trauma, Infection, or Other Medical Conditions***

#### **3.7.1. HEAD TRAUMA**

The epidemiological evidence associating head trauma and BTs is strongest for meningiomas. Numerous case reports have presented convincing circumstantial evidence. Case-control studies have found an excess risk of meningiomas in women with histories of head trauma treated medically, in men who boxed as a sport, and in men with histories of serious head injuries (32-34,83). Limited experimental evidence suggests that trauma may act as a co-carcinogen in the induction of neuroepithelial tumors, as well as meningiomas (85). Childhood BTs, which are predominantly neuroepithelial tumors, have sometimes been associated with birth trauma, such as prolonged labor, forceps delivery, and Caesarean section (46,81,82). Because trauma is often regarded by lay persons as related to tumor development, an attempt must be made to limit the reporting of trauma to injuries of a certain minimum severity (such as those requiring medical attention or hospitalization), and thereby limit recall bias.

#### **3.7.2. ACOUSTIC TRAUMA AND ACOUSTIC NEUROMAS**

The observation that over 90% of all nerve sheath tumors arise in the eighth cranial nerve (the acoustic nerve) suggests an exposure unique to this nerve. A case-control study of acoustic neuromas in Los Angeles County residents supports the hypothesis that acoustic trauma may be related to the development of these tumors (86). A dose-response analysis showed an increase in risk related to the number of years of job exposure to extremely loud noise ( $p$  for trend = 0.02), with an overall risk of 13.2 (CI = 2.01 to 86.98) for exposure of 20 yr or more, accumulated up to 10 yr before diagnosis. These findings may support the more general hypothesis that mechanical trauma could contribute to tumorigenesis (84).

#### **3.7.3. VIRUSES AND INFECTIOUS AGENTS**

Astrocytomas, but not other histologic types of BTs, were previously associated with positive antibody titers to *Toxoplasma gondii*, but a recent study failed to confirm this (87). There are numerous reports in the literature of the isolation of viruses or virus-like particles from human cerebral tumors or tumor cell lines,

but whether these findings may have etiologic implications is uncertain (88). Excess BTs have not been found among those who received polio vaccines contaminated with SV40 or those with mothers who had influenza or various other infections while they were *in utero* (89). Recently, a reduced risk has been reported between patients with neuroepithelial tumors and chickenpox, shingles, or the associated immunoglobulin G antibodies to the *Varicella zoster* virus. This is a novel finding requiring replication (90).

### 3.7.4. CHRONIC DISEASES

BTs have been associated with various chronic diseases, but none of these associations have been investigated in more than one or two studies. Neuroepithelial tumors, but not meningiomas, occur much less frequently in diabetics (91), who have a lower frequency of all cancers at autopsy (92). Excesses of BTs reported in various cohorts of epileptics probably occur because seizures are a common early BT symptom (93), and studies have found no increase in risk related to *in utero* or childhood exposure to barbiturates after a history of epilepsy was considered (94). Serum cholesterol has been positively related to brain cancer in some, but not all, studies (95); but, because none have evaluated dietary intake, the possibility that an existing BT may cause a spurious rise in serum cholesterol has not been excluded. A deficit of allergic conditions has been found in case-control studies of neuroepithelial tumors alone (79), and of all BTs (91,96).

Clinicians should be aware that an association between meningiomas and breast cancer has been observed (97), so that they will not assume that CNS lesions that are discovered after breast cancer diagnoses are necessarily metastatic. Tissues from meningiomas have been shown to contain hormone receptors, but it is unclear whether or not this finding has etiologic implications (98).

### 3.8. Predisposing Genetic Syndromes and Familial Occurrence

Some CNS tumors have a relatively clear genetic character, particularly those that occur in association with neurofibromatosis and other phakomatoses, which often display an autosomal dominant pattern of inheritance with varying degrees of penetrance. The occurrence of multiple primary BTs of either similar or different histologic types are associated with the phakomatoses, but also occur in the absence of such syndromes (99).

There are few population-based studies of familial aggregation of CNS tumors. One study found that Connecticut children with CNS tumors more often had relatives with nervous system tumors than did control children. However, this familial occurrence, although statistically significant, was observed for fewer than 2% of the children with CNS tumors (100). Medulloblastomas and glioblastomas were overrepresented among children whose relatives had nervous system tumors (100). Population-based studies that have investigated associa-

tions of BTs with recognized predisposing genetic syndromes, and/or with familial aggregations, have suggested that the proportion of BTs attributable to inheritance is no more than 4% (101,102).

### ***3.9. Other Suggested Risk Factors***

A number of other factors have been suggested as being related to BT risk, including barbiturates and other drugs, alcohol, tobacco smoke, and reproductive/hormonal factors (89), but these possible associations have not been studied often or very thoroughly. The few BT studies that have investigated some factors (e.g., alcohol and tobacco) have had contradictory findings. The best one can do in attempting to evaluate their etiologic relevance is to keep them in mind, and hope that future BT studies will also investigate possible associations with these factors.

## **4. PATHOGENESIS OF NERVOUS SYSTEM TUMORS**

Various physical, infectious, and chemical agents appear to relate to the development of cancer because they increase cell proliferation (103). For example, this may explain why acoustic trauma can lead to the development of acoustic neuromas (86). Replication may perpetuate a DNA mutation before it can be corrected in the cell in which it arises. Apparently, various genetic pathways can be involved in the pathogenesis of CNS tumors (as reviewed in Chapter 1 of this volume), and this may be true even for tumors of the same phenotype (104,105). Although many of the inherited syndromes that predispose to CNS tumors were described decades ago, the chromosomal loci of the affected genes have now been identified for most. In the past decade, hundreds of investigators have described molecular events that they have observed in tumor tissue from patients with various types of CNS tumors. A few of the most common of these mutations, which may interact with the environmental epidemiology of brain tumors, are summarized below.

### ***4.1. Molecular Genetic Characteristics***

Studies of the molecular biology and cytogenetics of CNS tumors suggest that specific types of tumors have characteristic genetic abnormalities, which have been summarized in review papers (106–108). Such characterization contributes to understanding the pathogenesis of CNS tumors. Glioblastomas, for example, commonly show losses of chromosomes 9p, 10, or 17p, and gains of chromosome 7. Losses of alleles at 17p appear to be the earliest abnormalities that occur in the genesis of these tumors. Most of these tumors express the *c-sis* oncogene, and some express other oncogenes as well. Related characteristics



include the synthesis and secretion of growth factors and/or their receptors that influence mitotic activity (109).

Various CNS tumors (but particularly those of astrocytic origin) have been associated with loss or mutation of the *p53* gene located on the short arm of chromosome 17 (110). *p53* is a tumor suppressor gene, and mutations in this gene appear to play a role in the development of a number of human cancers (111). *p53* mutations have been observed in GBM, as noted (111), in neurofibrosarcoma occurring in association with neurofibromatosis 1 (111,112), and in patients with Li-Fraumeni syndrome (113), which is a rare autosomal dominant genetic syndrome that predisposes those affected to cancers of the brain and other sites (114). This predisposition may relate to germ cell mutations in the tumor suppressor gene, *p53* (113). Because benign tumors from patients with neurofibromatosis 1 appear not to have *p53* mutations, it is thought that inactivation of this gene may be associated with the malignant transformation of these tumors (112).

Other tumor types show distinct pathophysiologic features. For example, loss of regions on chr 22 is the characteristic feature of meningiomas. Also, pediatric CNS tumors show different genetic patterns than adult tumors (28,115). For example, astrocytomas (World Health Organization grades II–IV) in children/young adults may demonstrate loss of heterozygosity for chr 17p and/or mutations in *p53* (115–118); astrocytomas in older adults often have mutations in chr 10, amplification of the epidermal growth factor receptor, and no mutations in *p53* (116,117). The characterization of the various tumor types is still in progress. The etiologic, prognostic, and other implications of specific characteristics remain to be defined. It is anticipated that molecular markers may aid in reducing the known misclassification in the diagnosis of some tumor subtypes.

#### **4.2. Possible Interactions of Genetic and Environmental Factors**

For a number of other reasons, epidemiological studies of the hypothesis that nitrosamide exposures relate to BTs are very difficult. For this reason, it is appealing to be able to rely on some biomarker of exposure. Unfortunately, finding a biomarker of *N*-nitroso exposure for use in BT patients (or their mothers), when the relevant exposures occurred years earlier, has not proved easy. Adduct formation by *N*-nitrosoureas *in vivo* is beginning to be studied, but the extent of damage induced in various tissues does not seem to correlate well with tumorigenicity (119). It may be more promising to identify a genetic polymorphism (one that could easily be assayed in epidemiological studies) for an enzyme or other system that regulates *N*-nitroso metabolism, or that repairs the molecular damage caused by nitroso compounds. One interesting candidate may be alkyltransferase, an enzyme involved in the repair of O<sup>6</sup>-alkylguanine, which is

formed and persists in brain DNA after exposure to alkylating agents, such as the nitrosoureas (120). Nitrosoureas produce different types of nervous system tumors in different species. Identifying those histologic types in humans will also make future studies of nitrosamide exposures more efficient.

Many of the problems confronted by epidemiological studies of BTs and nitrosamides also apply to studies of other suspected brain carcinogens, such as the several agents investigated in occupational studies. Although a number of industries have long been noted to have an apparent excess of BTs among workers, it has proved difficult to implicate specific exposures. Simultaneous evaluation, both of exposures to specific chemicals and of individual susceptibility to insult from those chemicals, may be the direction of the future.

## 5. PROSPECTS

We simply have no idea what causes most nervous system tumors. Certain inherited syndromes may predispose individuals to the development of BT and other nervous system tumors. However, at most, only a few percent of patients with nervous system tumors have one of these rare phakomatoses, or a family member with a nervous system tumor. Studies of such patients and their families have described genetic events that are correlates of nervous system tumor pathogenesis, but the etiologic implications of these findings are unclear.

Ionizing radiation, the only well-established environmental risk factor for nervous system tumors, can cause all three major histologic types of BTs: neuroepithelial tumors, meningiomas, and nerve sheath tumors. However, only a few percent of incident CNS tumors are likely to relate to such exposure, and the association appears weakest for gliomas. Nonetheless, minimizing population exposure to X-rays of the head is, at this point, the best prospect for prevention of all three types of tumors. Beyond this, the environmental etiology of neuroepithelial tumors remains largely unknown. More is known about the etiology of meningiomas and nerve sheath tumors. Ionizing radiation and trauma appear to be important risk factors for both.

Because nitrosamides, especially the nitrosoureas, are the most potent nervous system carcinogens used experimentally, it seems likely that these compounds may also cause nervous system tumors in humans. To date, most epidemiological studies of a possible association of BTs with *N*-nitroso exposures have focused on the other major group of these compounds, nitrosamines. Nitrosamines are easier to study, because reliable assays exist for nitrosamines, unlike nitrosamides, and monitoring of human environments and consumer products for levels of nitrosamines has been done. However, nitrosamines have not caused nervous system tumors in any of the many experimental species tested. Field and laboratory investigations of potential environmental sources of human exposure to nitrosamides and of their precursors (such as alkylamides) are needed.

Given that assays of relevant genetic polymorphism are not yet incorporated into epidemiological studies of nervous system tumors, what further work seems indicated? Diet will be an important focus of the next generation of epidemiological studies of neuroepithelial tumors. Studies to date have included some questions about a limited number of dietary variables, such as the several studies that looked at foods thought likely to be relevant to the *N*-nitroso hypothesis. A number of intriguing associations are emerging from these and other studies, including the suggestion that intake of cured meats, fruit, and vitamin supplements all relate to neuroepithelial tumor risk, with fruit and vitamins being protective. Future studies must include relatively complete dietary surveys, to adequately evaluate associations with various micronutrients, cholesterol, nitrite from cured meats, and other suggested associations.

Are there additional etiologic clues to be gleaned from the descriptive epidemiology of BT? The increase in incidence and mortality rates in recent decades was initially thought by some to suggest the effect of an environmental exposure, but on further consideration appeared to be largely an artifact of improved diagnosis. Compared to other cancer sites, BT rates show relatively little international variation. This suggests that either the relevant environmental exposures are ubiquitous or that endogenous factors are important. The gender differences in distribution by histologic type of BT, namely, the male predominance of neuroepithelial tumors and the female predominance of meningiomas, have long been noted. Although evidence suggesting the importance of hormonal factors is weak, any compelling hypothesis related to this difference would be worth investigating. Most BTs in children are neuroepithelial tumors, and some types such as PNET, occur predominantly in children under age 5 yr. The observation that PNET rates, unlike rates of other pediatric BTs that are similar in the two genders, are up to 2× higher in boys than in girls, also remains unexplained. For neuroepithelial tumors as a major group, as well as for specific glioma subtypes, some of the crucial etiologic questions have not yet been posed.

The etiology of the majority of nervous system tumors remains unexplained. Genetic predisposition, ionizing radiation, and other suggested risk factors each seem to account for only a small proportion of total cases. It may be that there are numerous nervous system carcinogens, such as known animal neurocarcinogens, which have not been fully evaluated in human studies, each with low attributable risk. Continued investigation of suspected brain carcinogens needs to identify and focus on histology-specific associations, and to use improved methods of exposure assessment.

In addition, host factors that influence susceptibility need to be simultaneously considered. In particular, detectable polymorphisms and host immune responses may play important roles in the etiology, progression, and potential treatment of CNS cancers. The relationship of immunological variables and BT develop-

ment has not been well studied in epidemiological investigations. Future studies of such variables are warranted, and need to be considered when interpreting the potential successes and failures of BT immunotherapy trials.

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## Current Therapy for Primary Brain Tumors

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*John M. Duff, MD, Pierre-Yves Dietrich, MD,  
and Nicolas de Tribolet, MD*

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### 1. INTRODUCTION

Despite surgical and technological progress in the treatment of neurological diseases over the past 50 years, advances regarding to the treatment of malignant brain tumors (BTs) have not kept abreast. Malignant astrocytoma is the most frequent primary BT in adults, and accounts for 2% of all cancers in this population (1). The incidence of malignant astrocytoma varies considerably with age. For glioblastoma multiforme (GBM), it ranges from 0.2/100,000 population in the under 14 yr age group to 4.5/100,000 population in the over 45 yr age group. There is a similar but less dramatic increase in the incidence of anaplastic astrocytoma (AA) with age, ranging from 0.5 to 1.7/100,000 population between the same age groups (2). This steady rise in the incidence of malignant astrocytoma, initially thought to peak in the 55–60-yr-old age group, has more recently been shown to continue to increase into the eighth decade of life (3,4). The average annual increase in the incidence of primary BTs in elderly patients in the United States, between 1974 and 1985, was 7.0% for the 75–79-yr-old

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age group, 20.4% in the 80–84 yr age group, and 23.4% in those 85 yr and older. These diagnoses were predominantly GBM and AA (4). The precise reason for this rise is uncertain. It may result from either the increased availability of computed tomography and magnetic resonance imaging (MRI) or, as suggested in one study (5), a true independent increase in incidence. Overall, there is a slight male predominance with both GBM and AA (3,6–9). With the incidence of malignant gliomas starting to increase in the fifth decade (7), this fatal disease inflicts an enormous social and economic impact, often striking during the most productive period in a patient's life.

## 2. SURGERY

### 2.1. General Principles

Once the presumptive radiological diagnosis of malignant glioma has been made, the onus lies with the neurosurgeon to obtain a tissue sample for histological confirmation. Prior to any surgery, corticosteroids can often alleviate headaches and transiently improve the neurological status of the patient. This effect is short-lived and typically lasts for 6–8 wk. An important goal of surgery is to provide the means for a histological diagnosis through biopsy or resection. Theoretical advantages of resection are to provide immediate palliation from the mass effect of the tumor, to reduce the tumor cell burden for future adjuvant therapies, and, in the event of mechanical obstruction of the ventricular system by the tumor, to restore normal cerebrospinal fluid flow. With superior multiplanar imaging, contemporary microsurgical techniques, and the use of stereotaxis when necessary (10), the present generation of neurosurgeons are able to keep patients in better neurological condition postoperatively than their predecessors.

The goals of open surgery are to perform a craniotomy with adequate exposure of the cortex overlying the tumor, without unnecessarily exposing adjacent cortex that would thereby be put at risk of injury. The scalp incision should be planned to maintain a vascular pedicle, and particular attention should be paid to tissue handling of the scalp in patients who anticipate radiation and possibly chemotherapy. The exposed cortex may show changes, such as abnormal pial vascularity, arterialized veins caused by shunting of tumor vessels, and swollen pale gyri. A corticectomy may be made in an overlying abnormal gyrus, with dissection through the direct subcortical white matter track to reach the tumor for tumorectomy. Occasionally, the preoperative MRI scan will show a sulcus that extends down to the tumor; in such cases, the sulcus should be used to access the underlying mass. Great care should be taken to preserve arteries passing through the tumor mass, while coagulating blood supply directly to the tumor. The principle is to radically resect the contrast-enhancing tumor mass, while avoiding indiscriminate resection of adjacent white matter. However, one must be aware that radiographic images of a necrotic tumor does not necessarily



allow one to resect overlying or involved cortex indiscriminately in eloquent regions of the brain where function may be preserved (11). Techniques of cortical mapping, and even resection under local anesthesia, are useful in these cases (12,13). Finally, every effort should be made to preserve the dura for closure at the end of the procedure, which forms a remarkably effective barrier to tumor spread.

## 2.2. *Biopsy vs Resection*

The past two decades have seen an increased overall incidence of malignant glioma, with a concomitant increase in tumor-related disability and death. Surgery remains at the forefront of the management of BT patients. Since the first reported successful resection of a glial tumor over a century ago (14), surgical management of these tumors has taken a quantum leap in terms of technological advancements in microsurgery, contemporary anatomic and functional imaging, and stereotaxy. Despite this, questions still remain as to what has been gained in tangible terms for patients stricken with malignant gliomas.

What is the advantage of aggressive surgery over biopsy for these lesions? Although there are proponents of both approaches, this question has never been answered in a truly scientific fashion with a prospective randomized study (15). Biopsy provides a histological diagnosis and grading of the tumor, but must be seen as a nontherapeutic, though important, step in the management of patients. It is indicated in deep or midline lesions not amenable to resection. Craniotomy adds cytoreduction by decreasing the tumor burden and relieves mass effect (at least temporarily) by creating room for normal brain, peritumoral edema, and recurrent tumor. In fact, improved postoperative cognitive function on neuropsychological testing has been attributed to a reduction in mass effect after tumor resection (16). However, confirmation of the presence of neoplastic cells distant to the radiographic tumor site (17) highlights the unresectability of malignant gliomas, as well as the futility of intraoperative frozen sections looking for distinct tumor margins. Without losing sight of the primary surgical goals as stated, surgical adjuncts for malignant glioma resection include stereotaxy involving frameless neuronavigation systems and intraoperative MRI scanning to minimize the violation of normal surrounding brain (18).

Despite these advances, the prognosis for patients with malignant astrocytomas remains poor. Because of the infiltrative nature of these tumors, a cure is currently not possible with present modalities of treatment. The current surgical goal is to optimize local tumor control, and to maximize quality of survival. A review by Salzman (19) of the available retrospective data of a large number of patients who underwent surgery for malignant glioma in the form of simple biopsy, limited resection, or radical resection, suggested that extensive surgery prolonged survival. This and other studies (20–25) have laid the foundation for the observation that the degree of resection of malignant gliomas correlates with survival. One prospective study (26) used postoperative MRI to predict survival,

and found that residual tumor enhancement postoperatively was the most predictive prognostic factor for patients with GBM. This was manifested by a six-fold increase in the risk of death in those with residual tumor, compared to those without residual tumor (26). In the same study, it was observed that approx 80% of tumor recurrences occurred from definitely enhancing remnants of tumor (26). Similar conclusions were reached in a study of patients in the Brain Tumor Study Group (27), with objective measures of surgical resection on postoperative computed tomography scans. A survival benefit after radical resection followed by radiation (mean survival 50.6 wk) vs radiation therapy (RT) following a stereotactic biopsy (mean survival 30.8 wk) has been demonstrated for patients with grade IV gliomas in one study (28). The studies mentioned above provide some rationale for radical surgery for malignant gliomas, with the caveat that the more recently observed increased survival from the time of diagnosis may be a factor of earlier diagnosis, rather than the benefits of modern surgical treatment.

### ***2.3. Reoperation for Recurrent Disease***

Regarding surgical therapy for recurrent malignant glioma, a modest survival benefit has been suggested in a recent retrospective study (29). The most important prognostic factors in this study were the age of the patient and the Karnofsky performance scale score (30) at the time of recurrence, with younger patients and patients with higher scores doing better. However, those authors were careful to point out that a true estimate of increased survival is only possible with a prospective trial. In this study, the median survival after a second surgical procedure was 36 wk (29), which is essentially a replication of results after reoperation in prior series (31–33). This is consistent with the observation that, in general, younger patients with malignant gliomas have a better outcome, which is highlighted by a diminished survival benefit after surgical resection in the elderly (34,35).

The authors' own view is that reoperation should be reserved for patients whose symptoms may be directly attributed to the mass effect of recurrent tumor growth, and who have a good performance score following the first round of treatments. These are more likely to be the younger patients, and this inherent selection bias is mirrored in one of the studies mentioned above (29).

## **3. RADIATION THERAPY**

### ***3.1. Conventional RT***

There have been many retrospective studies and clinical trials outlining the efficacy of RT in the treatment of malignant gliomas (36–38). In a comprehensive literature review by Salzman (39) of over 1561 patients who underwent radical resection of malignant astrocytomas, the median survival with surgery alone was 4 mo, which compared unfavorably with that of 9.25 mo after surgery and RT. Further evidence for the survival benefit of radiation at 6 mo was shown

in a prior study (36), which excluded early mortality (less than 2 mo) post-treatment. A multicenter randomized trial, carried out by the Brain Tumor Study Group (37) in the process of evaluating 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) chemotherapy, also confirmed the survival benefits of postoperative irradiation. In this latter study, the median survival of patients treated with surgery alone was 17 wk, compared to 37.5 wk for those treated with surgery plus RT. The addition of chemotherapy did not confer any survival benefit in this trial. Although it has been shown that survival correlates with the total dose of radiation up to 70 Gy (40), the results of the Radiation Therapy Oncology Group (RTOG) regarding dosing regimen failed to demonstrate any survival benefit with increased doses over 70 Gy given over 8–9 wk (41).

Tumor progression during RT is invariably an ominous sign, and tumor mass on posttreatment CT scan has been shown to correlate strongly with decreased survival in a large series of 510 patients (42). Tumors not displaying progression, or even showing regression on interval computed tomography scans, fared considerably better. This biological behavior is also predictive of chemoresistance, because of the similar mechanisms of action of some chemotherapy agents with ionizing radiation (1). Neurological deterioration during RT is not always a harbinger of tumor progression, however. Up to 28% of patients may improve after an initial decline in general neurological condition during the post-irradiation period (43), which is consistent with acute radiation-induced changes, some of which are reversed by remyelination, restoration of the blood–brain barrier (BBB), and decreased peritumoral edema (44). This observation would appear to justify the use of steroids during the course of RT (1).

### ***3.2. Interstitial Brachytherapy***

A theoretically more refined method of administering high-dose radiation to malignant gliomas is with the use of interstitial brachytherapy (1). Continuous radiation of 0.4–1.0 Gy/h allows tumor cells to reoxygenate, and also permits more cells to divide during prolonged therapy, thus making them more susceptible to the effects of irradiation. The usual total dose is in the range of 50–75 Gy, delivered to the tumor bed. Interstitial brachytherapy can be used as rescue therapy, and it has been shown, in one series (45), to be associated with a median survival of 81 wk from the time of recurrence in patients with grade III tumors and 54 wk in patients with grade IV disease. In a study utilizing Iridium-192, a median survival of 34 wk was noted in a group of patients with recurrent malignant astrocytoma (46). These studies clearly demonstrate the efficacy of interstitial brachytherapy in selected patients with recurrent tumor. In fact, selection bias has been cited as an important factor in patient outcome with brachytherapy (47). Using different selection criteria, one study (48) showed no survival advantage of brachytherapy over cytoreductive surgery.

Thus, the conventional form of treatment for malignant gliomas remains cytoreductive surgery followed by external-beam RT, with doses in the order of 40 Gy to the tumor bed and a 3-cm margin. This has become a standard by which to judge other treatment regimens.

## 4. CHEMOTHERAPY

At present, there is a limited role for chemotherapy in the treatment of malignant gliomas. Many potential obstacles lie in the path of chemotherapeutic agents for central nervous system neoplasms, notwithstanding the intrinsic chemoresistant nature of these tumors (49). Difficult physical access through the BBB, although not entirely normal in tumor tissue, favors the passage of compounds of low mol wt with high lipid solubility and low protein binding. The prospective compound then encounters potential enzyme degradation, recrossing the BBB in a retrograde fashion. Current chemotherapeutic agents also have poor target specificity, entering nontumoral cells as well as tumor.

### 4.1. Mono-Chemotherapy

The most frequently used drugs in chemotherapy are the nitrosoureas. The use of BCNU/(*N*-(2-chloreethyl)-*N'*=cyclohexyl-*N*-nitrosourea (CCNU) as single agents has been reported, with response rates from 10–40% (50) up to 64% in a subgroup of patients with AAs (51). Much of this data, however, originated from the pre-CT/MRI era, and results were based on clinical patient profiles without radiological evaluation.

Vincristine, as a single agent, was studied over 30 yr ago with some clinical benefits noted and response rates reported between 20 and 50% (50), again without radiological correlation (52). This formed the basis for inclusion of vincristine in subsequent poly-chemotherapeutic regimens for glial tumors.

Much recent interest has focused on temozolomide, because of its low toxicity and ease of use. Based on earlier encouraging results, temozolomide was further studied by the Charing Cross Group, with a reported response in 25% of patients (53). Recent data available from a rigorously designed multicenter phase II trial (54) reported a response rate of 42% for AAs. In this latter study, it is noteworthy that a significant number of patients (41%) were unable to be evaluated for a variety of reasons, mostly because of inaccurate histological diagnosis. This occurrence in a very well-designed study clearly raises doubts regarding the validity of data derived from other multicenter trials that lack centralized diagnosis reviews.

Use of procarbazine has been reported to have response rates between 6 and 26% in trials, with a median time to tumor progression (MTTP) between 7 and 12 mo in pretreated patients (50). Interest in this agent has re-emerged, because

of its depleting activity against methylguanine-DNA methyltransferase, which is involved in malignant glioma resistance to nitrosureas (49).

#### **4.2. Poly-Chemotherapy**

The most commonly used multi-agent regimen is procarbazine, CCNU, and vincristine (PCV), which was originally described in 1975 and subsequently modified in 1978 and 1980 (55). The final regimen was studied in 46 patients, for 40% of whom this was the first-line chemotherapy. The overall response rate was 26%, reaching 42% in the subgroup of patients who were not previously treated, and a mere 17% in the subgroup of GBM patients (pretreated or not). Response was considered to be an improvement in at least 2/3 parameters (neurological examination, radionuclide scan, and computed tomography scan). The overall MTTP in this study was 26 wk. The results of this study formed the basis for the subsequent use of PCV as a regimen in newly diagnosed glioma patients, or in those with recurrent disease.

Procarbazine in combination with vincristine and mechlorethamine (MOP) had been initially used in the pediatric BT population with good efficacy (50). A series of 31 adult patients with recurrent malignant glioma were treated with MOP in a phase II study reported in 1990 (56). The response rate was 37% for glioblastomas and 100% for AAs. The median survival was 30 wk for all patients who could be assessed, and 60 wk for all patients who responded (56). A separate study demonstrated the efficacy of a combination of cyclophosphamide and vincristine without procarbazine (57). Considering these studies together, the results suggest that alkylating agents exert a potent antiglioma effect, despite their limited diffusion across the BBB, and deserve further study.

A phase II trial, looking at the combination of ifosfamide, carboplatin, and etoposide was recently reported in 36 patients with recurrent malignant glioma, who had all been pretreated with surgery, irradiation, and nitrosureas (50). The overall response rate was 28%. In this series, glioblastomas appeared to be as sensitive as AA; and responders seemed to benefit, with a median survival of 44 wk, compared to the overall median survival of 29 wk. This benefit, however, was achieved at the price of severe hematological toxicity.

A complex association of six agents (thioguanine, procarbazine, dibromodulcitol, CCNU, 5-fluorouracil, and hydroxyurea) was studied with the objective of overcoming a presumed resistance to nitrosureas, because most of the drugs were administered at subtherapeutic levels (58). A series of 75 patients with malignant gliomas were studied, with a radiological response rate of 32%.

A common feature in most studies of both mono- and poly-chemotherapeutic is that therapeutic benefit probably occurs in young patients with the AA sub-

type and good pretreatment performance status. This was supported in a retrospective study specifically designed to address this question (59).

### ***4.3. Adjuvant Chemotherapy***

The first randomized study testing adjuvant chemotherapy was performed between 1972 and 1975 by the Brain Tumor Cooperative Group (38). Patients treated with BCNU + RT had a mean survival of 51 wk, compared to 36 wk for patients treated with RT alone. However, this difference was not statistically significant, and the difference in outcome between GBM and AA was not addressed.

A further study was carried out by the European Organization for Research on Treatment of Cancer (EORTC) comparing postoperative RT alone vs BCNU, dibromodulcitol + RT (60). Chemotherapy induced a slight but significant improvement in median survival (13 vs 10.4 mo) and MTTP (8.1 vs 6.7 mo) in 255 patients. This benefit was more pronounced for AA than for GBM. In this study, the real effect of dibromodulcitol was questionable, in view of the limited role of this drug in the treatment of gliomas. There was very limited increase in survival and minimal benefit to the individual patients, despite the statistical significance of the results.

Adjuvant therapy using PCV was studied, and was shown to prolong MTTP (126 vs 63 wk) and median survival (157 vs 82 wk), compared to BCNU for AA patients (61). These patients had good prognostic factors, with a mean age of less than 46 yr. In contrast, no difference between PCV and BCNU treatment appeared among GBM patients.

All of these studies included a small number of patients. To better analyze these results, a meta-analysis of 16 studies was carried out (62). In such an analysis, the increase in survival with RT and chemotherapy was 10.1% at 1 yr and 8.6% at 2 yr. The maximal survival advantage with chemotherapy was seen later (18–24 mo) for GBM patients than for AA patients (12–18 mo), suggesting that mostly long-surviving GBM patients may benefit from adjuvant chemotherapy. This probably represents a subgroup of patients with initially favorable prognostic factors (i.e., young age, minimal residual tumor, and good performance status).

### ***4.4. Chemotherapy for Anaplastic Oligodendroglioma***

Anaplastic oligodendrogliomas (ODGs) are a special case of gliomas, in that they are relatively chemosensitive (63), as demonstrated by the success of the PCV treatment regimen for these tumors. This represents one of the most important advances in recent years in the treatment of malignant gliomas. In marked contrast, glioblastoma patients do not benefit from chemotherapy, in terms of survival, and their quality of life has rarely been evaluated. ODGs are generally slower-growing tumors with a better prognosis than AA or GBM (50).



In a multicenter phase II study, 33 patients with anaplastic ODGs were treated after initial diagnosis or with recurrent disease (63), most of whom had been preirradiated. Of the 24 patients who could be evaluated, the overall (complete and partial) response rate with PCV treatment was 75%, with a complete response noted in 38% of patients. Patients with pre-existing low-grade ODG had a response rate of 90%, and patients with necrotic tumors had a 67% response rate. The MTTP was 25.2 mo for patients with a complete response and 14.2 mo for those with partial response. There was no significant difference in response rate between irradiated and nonirradiated patients.

Despite this unexpected efficacy, it is still not known whether PCV, administered as an adjuvant therapy, prolongs tumor control and/or survival. This is currently being addressed in an RTOG randomized trial comparing RT alone with PCV followed by RT. Also, whether PCV should be given initially or at the time of relapse, and whether PCV has the potential to delay RT, remain unanswered.

Treatment of aggressive ODG with high-dose thio-tepa and stem cell rescue is also currently under investigation (64), with the goal of deferring RT and its deleterious effects. This regimen can also be considered a promising salvage treatment (50).

The answer as to whether the oligodendroglial component of a mixed anaplastic oligoastrocytoma confers these tumors with an added degree of sensitivity to PCV remains unknown. This is primarily the result of the lack of well-defined histological criteria. Nevertheless, there are some reports of these oligoastrocytoma tumors having a favorable response to PCV (50).

#### **4.5. Polymers and Interstitial Chemotherapy**

Local control of malignant gliomas remains a goal of treatment for neurosurgeons, radiation oncologists, and medical neuro-oncologists, because an estimated 90% of recurrences occur within 2 cm of the original resection site (65). Administration of chemotherapy in the tumor bed or in the tumor itself confers the potential advantages of bypassing the BBB, reducing chemotherapy-induced systemic toxicity, increasing drug concentration at the target site, and lengthening the duration of tumor exposure to drug. This can be achieved using Ommaya reservoirs, implantable pumps, or implantable polymer matrices impregnated with drug. The polymers have certain advantages, in that they are not subject to mechanical malfunction and blockage like other devices, or to the risk of infection by multiple percutaneous injections. However, they do require more invasive surgery for *in situ* placement (66).

In a phase I–II study assessing BCNU wafers, there was neither local nor systemic toxicity (67). A multicenter randomized trial with 222 patients compared implantation of BCNU wafers vs placebo-containing wafers (68). Median survival was prolonged (31 vs 23 wk) with the drug-containing polymers. In patients with GBM treated with the BCNU wafers, 6-mo survival was 50%

greater; but survival curves converged afterwards to be almost identical. No unexpected toxicity was observed. Although the benefit to the individual patient was minimal in this trial, this technique does provide a means to deliver drugs that do not penetrate the BBB.

#### ***4.6. Intra-Arterial and High-Dose Chemotherapy***

Alternative modes of drug delivery have been extensively studied over the past decade, and have included intra-arterial infusion, as well as very-high-dose intravenous delivery necessitating bone marrow autograft or peripheral stem cell rescue (50). The agents most commonly used are the nitrosureas. These therapies have not been shown to improve definitively outcome.

### **5. IMMUNOTHERAPY**

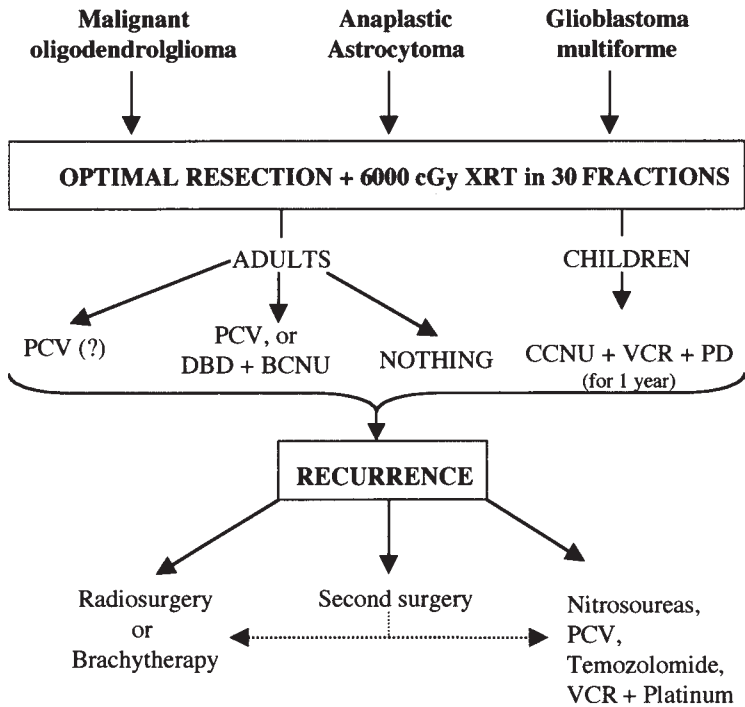
Glial tumor progression results from a complex interaction of biological mechanisms, including loss of cell cycle control, neoangiogenesis, and evasion of immune control. Advances in these areas have created possibilities for new therapeutic modalities for malignant gliomas by targeting cell proliferation and death, neoangiogenesis, or appropriate immune responses.

Vascular endothelial growth factor (VEGF) plays a key role in glioma angiogenesis. Several approaches have been developed to inhibit the effects mediated by VEGF, including antisense technology and the use of monoclonal antibodies (69).

It is known that immune cells in a complex network of intercellular interactions and cytokine-mediated signals can sometimes protect and eradicate the host of cancer. There is also evidence that T-lymphocytes play a critical role in antitumor responses within the central nervous system. A specific T-cell response requires antigen-specific interactions between tumor cells, antigen-presenting cells, and T-lymphocytes. Recent progress in the manipulation of these cellular interactions holds promise for the future of immunotherapy for brain tumors. Further detailed discussion of these and related topics in the treatment of malignant gliomas are beyond the scope of this chapter and are addressed elsewhere in this book.

### **6. CONCLUSIONS**

Despite the improvements in the treatment of malignant BTs, the authors must conclude with the grim prognosis that still exists for afflicted patients with brain cancer. Current modalities of therapy (surgery, RT, and chemotherapy) are inadequate. The present treatment algorithm that the authors currently adopt is detailed in Fig. 1, which illustrates the multimodality nature of such interventions. The limited efficacy of current therapies (70) indicates the need for innovative treatments to be explored in the form of experimental strategies for glioblastomas and for AAs that have not responded to first-line chemotherapy.



**Abbreviations:**

- XRT = radiation therapy
- PCV = procarbazine, CCNU, vincristine (combination)
- DBD = dibromodulcitol
- VCR = vincristine
- PD = prednisolone

**Fig. 1.** Treatment algorithm for malignant gliomas.

Advances in molecular biology and immunology provide novel means with which to treat these malignancies. The inherent difficulties lie in the heterogeneity of these neoplasms, which are associated with multiple genetic events and constant modification of such genetic alterations. However, clinical observation and biological data suggest that tumor proliferation results from an imbalance between factors promoting cell proliferation vs cell death, angiogenesis stimulation vs inhibition, and immune evasion vs immune response. Correction of one or more key biological factors, such as immune responsiveness, could tilt the balance toward tumor regression. It is likely that several components contributing to carcinogenesis should be targeted to maximize tumor control. Therefore, therapies based on immune enhancement could theoretically be used with other approaches, such as those designed to inhibit neoangiogenesis, to restore

cell cycle control, or to induce apoptosis. Such an integrated treatment strategy may ultimately provide some means of improving the poor prognosis currently facing patients with malignant gliomas.

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# II

## INTRODUCTION TO IMMUNOTHERAPY OF BRAIN TUMORS

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# 4

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## Immunostimulation and Immunomodulation of Brain Tumors

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*Victor C. K. Tse, MD, PhD  
and Frances K. Conley, MD*

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### 1. INTRODUCTION

Initially, the brain was thought to possess no immunosurveillance activity, and thus existed as an “immunologically privileged” site (1,2). However, recent experimental evidence suggests that, despite the anatomical segregation of the brain from the general circulation by the blood–brain barrier (BBB) and lack of discrete lymphoid tissue, there are immunocompetent cells within the brain parenchyma. These cells do provide surveillance and also permit the brain to mount an immunologic response in concert with peripheral mechanisms of immunity, which include both cellular and humoral components (3).

Further, it is now known that an immunologic response in the brain is essentially the same as that which occurs in the periphery. An antigen-presenting cell (APC) in the presence of class II major histocompatibility (MHC-II) antigens (Ags) first processes foreign Ags. These Ags, in turn, activate the production of a number of cytokines, as well as T-lymphocytes and other effector cells, such

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as natural killer (NK) cells. In the brain, microglia, endothelial cells, capillary pericytes, and, occasionally, astrocytes, all can act as APCs (4). Of these, the microglial cell has received the most attention. Microglia express both MHC-I and -II Ags, and secrete several lymphocyte-activating factors, such as interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-1 (IL-1), interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor (GM-CSF). They express the macrophage marker, OX-42, on their surfaces, and demonstrate phagocytic activity in culture. Microglia have been shown to lyse tumor cells that are expressing epidermal growth factor receptor (EGFR) in the presence of MAb 425, a monoclonal antibody (MAb) against EGFR (5). The identification of tumor-infiltrating lymphocytes (TIL) in malignant glioma favorably argues for the existence of glioma-specific and tumor-associated Ags. The infiltrate is prominent in the perivascular spaces and at the advancing edge of the tumor. Many of these cells are T-lymphocytes, which proliferate with stimulation, and are cytotoxic to tumor cells in vitro.

## 2. WHAT ARE IMMUNOSTIMULANTS?

Gliomas do process Ags, but there is continued controversy as to whether the Ags are tumor-specific. Some Ag genes found in gliomas are the melanoma antigen- encoding genes (*MAGE1* and -3). The mRNA of these genes are expressed in glioblastoma multiforma (GBM). Melanocytes and glial cells may share a common origin, since they are both neural ectodermal cells, but *MAGE1* and -3 are not expressed in normal brain (6,7). Moreover, many of the proteins related to melanogenesis are antigenic and capable of eliciting a cytotoxic T-cell response in humans (8). Other Ags, which may be tumor-specific, are extracellular matrix-associated tenascin and proteoglycan chondroitin sulfate associated antigen (GP 240), along with membrane-associated ganglioside 3',6'-isoLD1. Tenascin is associated with the basal membrane of proliferating vessels in high-grade glioma, and a bispecific MAb (anti-CD3 coupled with antitenascin) has been used as an adjunct therapy for gliomas (9). Other less-common Ags, such as chondroitin sulfate proteoglycan Ag, have been reported in glioma (10). Lymphocyte function-associated antigen 3 (LFA-3) and CD44, which play an important role in receptor-mediated T-cell proliferation, are also expressed in GBM (11,12). Glioma has also been shown to express lymphoid differentiation Ags, such as CD10 and CD57. Other groups of tumor Ags are related to mutated gene products, such as alterations of the *p53* tumor suppressor gene and the epidermal growth factor receptor variant (*EGFRvIII*) (13). Mutated *p53* was identified in glioma by MAbs (14), and circulating Abs against mutant *p53* have been found within patients with lung cancer. Over 30% of GBMs exhibit amplification of *EGFR*, and specific Abs have detected a deletion variant of this receptor. Experimentally, there is some evidence of tumor-specific Ags found in cell lines such as the RT-2 and 9L gliomas (15).



### 3. WHY DO TUMORS ESCAPE IMMUNOSURVEILLANCE?

Clinically, it has been well documented that patients with brain tumors (BTs) have lower humoral and cell-mediated immune responsiveness. They generally have abnormal, delayed hypersensitivity to such Ags as *Mycobacterium tuberculosis* and *Candida albicans*. Their total count of circulating T-cells is low; and T-cell responsiveness to mitogens, such as phytohemagglutinin, concanavalin A, and phorbol ester, is suppressed. It is also well known that immunosuppressed patients have a higher-than-expected incidence of central nervous system neoplastic disease. However, in vitro, human glioblastoma cells have been shown to act as accessory immunologic cells, with the ability to secrete cytokines such as IL-1, IL-6, tumor necrosis factor, and IFN- $\gamma$ . They can also present Ag to cytotoxic T-cells, resulting in the induction of cytotoxic effector function that is MHC-restricted.

This paradox of tumor-induced cytotoxicity and the apparent lack of effective immunologic control of tumor growth may, in part, result from an imbalance between forces of immunostimulation and immunosuppression by the tumor, which ultimately avoids its own destruction by modulating the host's immune response. It has been shown that T-helper cell activity in BT patients is suppressed. In vitro suppression of helper cells leads to failure of immunoglobulin secretion and decreased T-cell cytotoxicity. Abnormal IL-2 production, as well as impaired IL-2 receptor expression, may also play a part in BTs. TILs from human gliomas lose their ability to proliferate in culture after a few weeks, when IL-2R expression declines (16). Normally, MHC-I Ags are not present on the surface of astrocytes and are very poorly expressed on the surface of glioma cells (17–19). Several lymphocyte functions are blocked by soluble factors secreted by gliomas (transforming growth factor  $\beta$  [TGF- $\beta$ ], IL-10, and insulin-like growth factor-1). Cytokines, such as TGF- $\beta_2$  downregulate expression of class II Ags (20), inhibit the activity of lymphokine-activated killer (LAK) and NK cells, and suppress T-cell proliferation (21). GBMs have been shown to produce prostaglandin E<sub>2</sub>, which is a negative regulator for the generation of LAK cell activity. Prostaglandin E<sub>2</sub> suppresses T-cell proliferation induced by mitogenic anti-CD3 Abs, and downregulates the class II Ag human leukocyte antigen-DR (HLA-DR). Thus, the immunosuppressive elements produced by the tumor overwhelm the immunosurveillance of the host in the course of tumor progression.

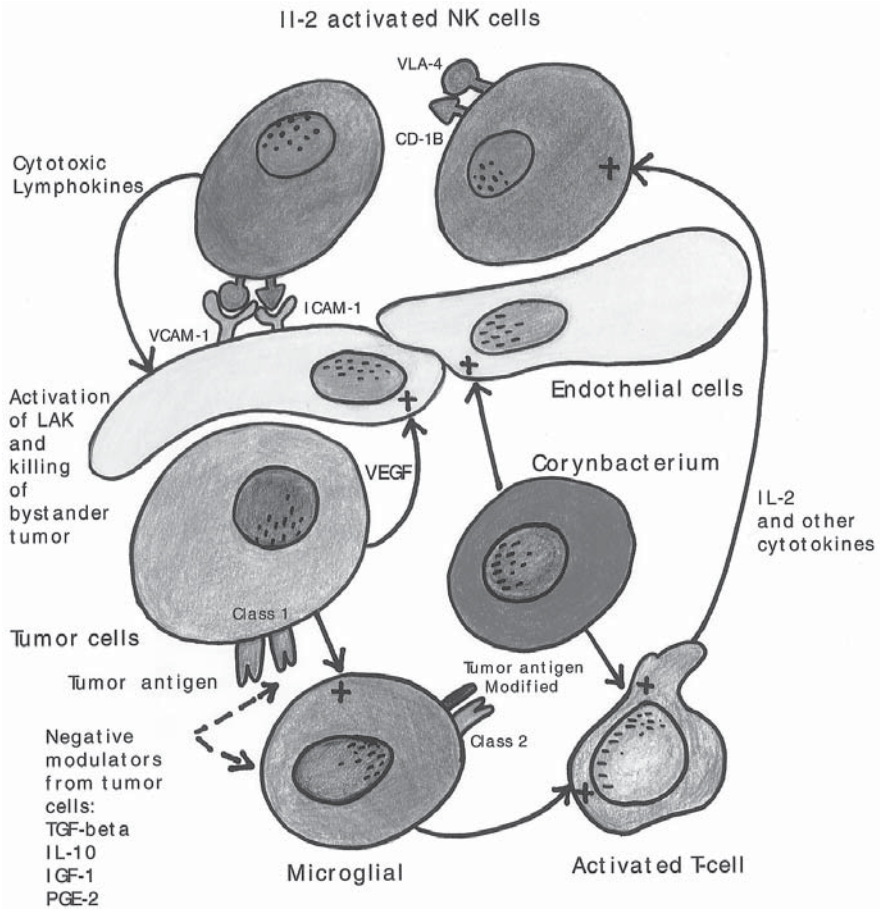
### 4. CAN IMMUNOSURVEILLANCE BE IMPROVED BY USING NONSPECIFIC IMMUNOSTIMULANTS?

In the past two decades, attempts to artificially modify the immune environment and responses have been tried in the treatment of neoplastic disease. Over a century ago, Coley treated malignant tumors with repeated inoculations of

erysipelas, in an attempt to activate the immune system in a nonspecific fashion (22). In the late 1970s, Conley reported that mice with chronic *Toxoplasma* infection showed delay in growth of implanted malignant tumors, both in the brain and peripherally. Histologically, tumors in the brains of infected mice had a marked mononuclear cell inflammatory reaction around the implanted ependymoblastoma (23). Conley postulated that nonspecific immunodulation of the host by the *Toxoplasma* (an organism known to infect all cells in the body, including those in the brain) produced a population of activated macrophages that were cytostatic to tumor cells. However, another immunostimulant, *Corynebacterium parvum*, had no inhibitory effect on BTs, but was highly effective against tumor growth in the periphery (24–26). *Toxoplasma* is known to cross the BBB, but *C. parvum* does not. Thus, the usefulness of an immunostimulant appears to be governed by its ability to stimulate immunologically competent cells within the parenchyma of the organ(s) bearing the tumor(s) (27). Further investigation has demonstrated that inflammatory cells extracted from *Toxoplasma*-infected brain showed a predominance of cytotoxic T cells (Lyt 2+) over helper T cells (L3T4+) (28). Hauser et al. (29) was able to demonstrate that both acute and chronic *Toxoplasma* infections in mice were associated with an increase in NK cell activity. It is probable that there is more than one population of mononuclear cells that function as effector cells in this nonspecific antitumor response.

## 5. MECHANISMS OF NONSPECIFIC IMMUNOMODULATION

Using a metastatic BT model in mice, Kennedy et al. (30) was able to retard the growth of metastatic KHT mouse sarcomas in the brain by priming the animals with direct injections of *C. parvum* intracranially. They observed a moderate to intense inflammatory response in the inoculated brains, resulting in a significant reduction in cerebral metastatic disease. Intuitively, this nonspecific killing of tumor cells was probably the result of a variety of factors. *C. parvum* has been shown to promote IFN production and to increase the local activity of cytokines (31), together with activation of tumor-specific T-cells (32) and NK cells. A number of cytokines, such as IL-2, IL-6, and IL-3, have also been shown to be upregulated in this model. IL-6, for example, is thought to eliminate glioma by neutralizing the glioma-induced suppression of T-cell proliferation and IFN- $\gamma$  production (33). Furthermore, intracerebral injection of *C. parvum* induces a local inflammatory cellular infiltrate and upregulates intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) on vascular endothelium throughout the entire central nervous system. Expression of the antimurine-endothelium monoclonal antibody (MECA)-32 Ag in the vicinity of the injection site suggests an altered functional status of the vascular endothelium, and, in turn, the BBB. The expression of VCAM-1 is indicative of



**Fig. 1.** Immunostimulation and modulation by *Corynebacterium*. A schematic representation of the activation of NK cells and upregulation of VCAM and ICAM by the bacterium.

having active recruitment of lymphocytes in response to *C. parvum* induction (34). It is plausible that, by modulating the expression of the VCAM-1 and ICAM-1 pathways, *Corynebacterium* promotes the generation of LAK cells (Fig. 1). The latter are non-Ag-specific killer cells, and can initiate killing of the “by-stander” tumor cells in a non-MHC-restricted manner. In fact, ICAM-1 has been shown to facilitate the binding of TILs to GBM via the LFA-1.

Another nonspecific immunostimulant, 2,3,5,6-tetrahydr-5-phenylimidazo-2,1 $\beta$ -thiazole hydrochloride (Levamisole), has been administered to patients with anaplastic astrocytoma, following resection, radiotherapy, and 1,3-bis-(2-chloroethyl)-1-nitrosourea. Patients treated with Levamisole alone did not show a significant improvement in survival. However, when combined with

active immunization with *Bacillus Calmette-Guerin* (BCG), Levamisole was found to be therapeutically effective against an Avian sarcoma virus (ASV)-induced glial tumor in rats (35). It has been postulated that Levamisole increases NK cells and activates T-cells, so as to potentate the effect of BCG. In another organ system, intravesical treatment with BCG reduced bladder tumor growth. This antitumor effect was attributed primarily to the recruitment of NK cells and the augmentation of their antitumor activity (36). Purified BCG cell wall has also been shown to be effective in preventing the growth of gliomas, when injected simultaneously with tumor cells into the brain. In this case, BCG acts as a nonspecific stimulant similar to *C. parvum* (37). Other nonspecific immunomodulators, such as OK-432 (lyophilized powder of *Streptococcus pyogenes*) and PS-K (protein-bound polysaccharide kureha form *Basidiomycetes*) enhance cytokine production. OK-432 has a cytotoxic effect on tumors by activating mononuclear cells through the Fas signaling pathway (38).

Recent advances in gene therapy suggest the feasibility of Ag modulation. Lampson (39) demonstrated that a modified rat glioma (9L/*LacZ*), expressing the *LacZ* reporter gene in a stable fashion, could provoke an inflammatory response. When *s-Myc* was introduced into 9L or C6 glioma cells, it enhanced the presentation of tumor Ag from these cell lines and upregulated the activity of the host immune response, resulting in the inhibition of tumor growth (40).

## 6. SHOULD THIS ASPECT OF TREATMENT CONTINUE TO BE EXPLORED?

Although using chronic *Toxoplasma* infection or direct injections of *C. parvum* into the brain is an impractical therapeutic measure against BTs in humans, advances in the fields of molecular biology and immunology have revitalized an interest, in tumor immunotherapy. Rather than nonspecific stimulation, using targeted, specific Ag stimuli holds more promise for increased efficacy. The use of bone marrow-derived dendritic cells, which can be primed against a specific tumor using endogenous or reconstituted Ags in vitro (41,42) may increase immunologic defenses on the part of the host. Other promising areas of research include vaccination with autologous tumor-derived heat-shock protein-peptide complexes. Heat shock proteins chaperone the Ag repertoire of the cells from which they are derived, bypassing the need to identify specific Ag epitopes (43). Also, IL-2/IFN- $\gamma$ -secreting allogeneic fibroblasts may be effective in eliciting NK/LAK cell-mediated antitumor responses (44).

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## Biological Principles of Brain Tumor Immunotherapy

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*Amy B. Heimberger, MD,  
Darell D. Bigner, MD, PhD,  
and John H. Sampson, MD, PhD*

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### 1. INTRODUCTION

The objective of this chapter is to discuss the biological principles that currently guide the design of immunotherapies (ITs) directed against central nervous system (CNS) tumors. Direct translation of systemic ITs currently in use to CNS tumors is limited by the heterogeneity of gliomas, immunosuppression mediated by cytokines elaborated by gliomas, and the possible induction, in the case of nonspecific ITs, of potentially lethal autoimmune reactions. In order to design rational ITs, a clear knowledge of immunological responses within the CNS is required. The basic tenet of “immunological privilege” is placed within the more proper context of immunological suppression. Emerging concepts, such as antigen (Ag) presentation within the CNS and the presence of CNS comple-

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ment, are presented as well, because these areas represent new potential areas for therapeutic intervention. Discussion of application of this information, including manipulation of cytokines, adoptive IT, vaccination, and tumor-specific Ag approaches, is reserved for other chapters.

## 2. OVERVIEW OF THE IMMUNE SYSTEM

Immune responses (IRs) are initiated by uptake within antigen-presenting cells (APC) of foreign protein Ags, which are subsequently degraded into small peptides. These peptides become associated with class I or class II major histocompatibility complex (MHC-I or -II) molecules, which are expressed on the surface of the APC (Fig. 1). T-cells recognize Ags within the context of these MHC–Ag complexes via cell surface receptors. More specifically, CD4<sup>+</sup> (helper) T-cells recognize peptide–class II complexes; the CD8<sup>+</sup> (cytotoxic) T-cells recognize peptide–class I complexes. The recognition of the peptide–MHC complex on APC results in activation and clonal expansion of the naïve T-cell. Full activation of a T-cell requires, in addition to receptor engagement, co-stimulation by additional receptor–ligand interactions between the APC and the T-cell. Absence of co-stimulation, as when a naïve T-cell encounters Ag presentation by a tumor cell, can lead to a state of unresponsiveness or anergy. Upon appropriate activation, the T-cell mediates a wide range of immunological functions. For example, helper T-cells increase the capacity of B-cells to develop into antibody (Ab)-producing cells, increase the microbicidal capacity of macrophages, and mobilize inflammatory responses; cytotoxic T-cells directly lyse target cells.

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**Fig. 1.** (*see facing page*) Activation of the immune system. Exogenous immunogens are captured by the APC and are processed within cytoplasmic vesicles. Processing of the Ag involves denaturation and partial proteolytic digestion into short peptides. The resulting peptides associate with MHC-II and are presented on the surface of the APC ①. A CD4 Th-lymphocyte, expressing a TCR that recognizes a distinctive peptide–MHC complex presented by the APC, will then become activated ②. The activated Th-lymphocyte then promotes the growth, differentiation, and function of the B-cell either by direct contact (CD40) or by the secretion of cytokines (IL-2, IL-4, and IL-6). Activated B-cells then differentiate into plasma cells, which secrete Abs specific for the immunogen. Abs can neutralize toxins, prevent the attachment of virus to the cell, function as opsonins to promote phagocytosis, activate complement, and mediate Ab-dependent cell-mediated cytotoxicity ③. Additionally, the activated lymphocyte will release cytokines or other products that will attract inflammatory cells. Discharge of bioactive substances into the local tissue can lead to increased local vascular perfusion, capillary permeability, accumulation of extravascular fluid, and pain √. MHC-I is expressed on virtually all cell types and is recognized by CD8 cytotoxic T-lymphocytes (CTLs). The CTL is activated by the presence of foreign Ags, such as a virus, within the context of MHC-I on the surface of a cell, and by IL-2 secreted by the activated helper T-lymphocyte. Upon receiving both signals, the CTL destroys the target cell by release of toxins or by inducing apoptosis. Adapted with permission from ref. 189.

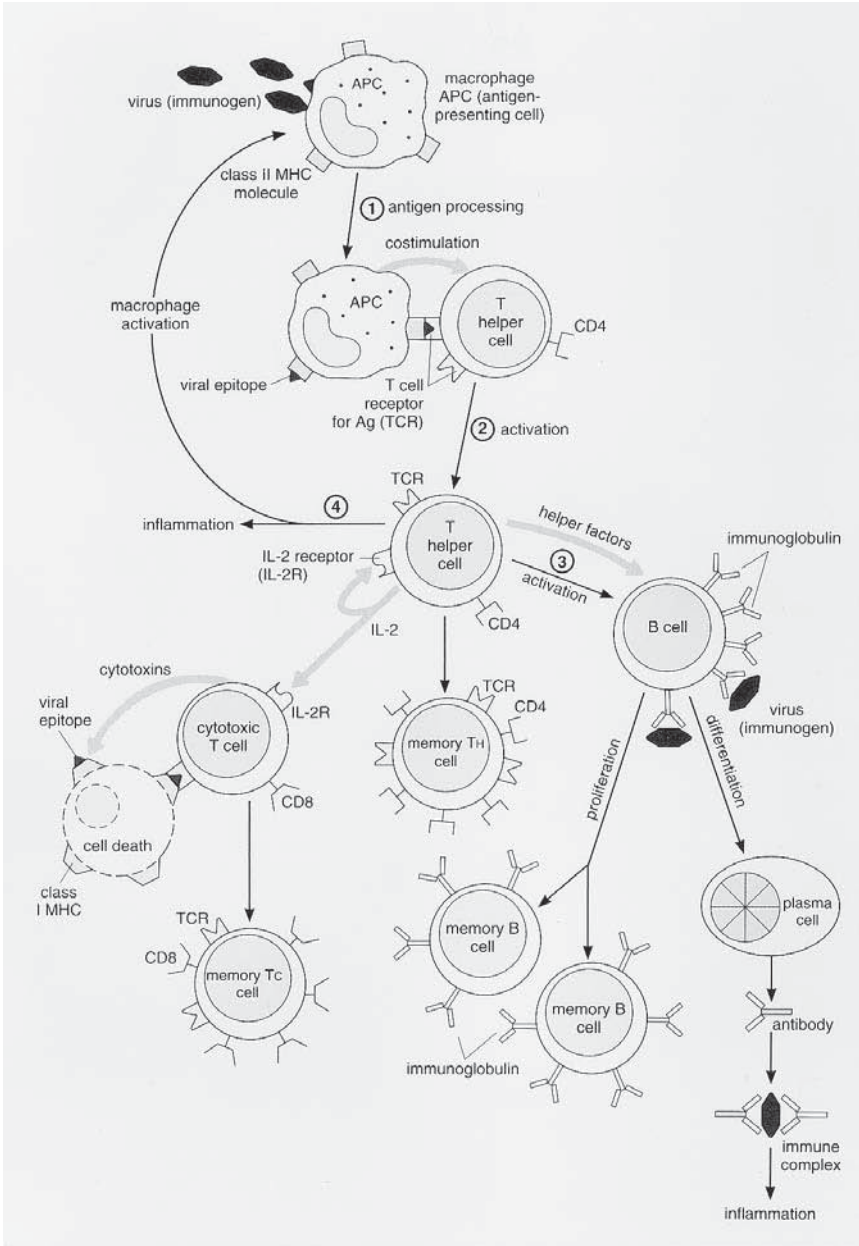


Fig. 1

The specific helper T-cell population ( $Th_1$  or  $Th_2$ ), which is generated from naïve T-cells ( $Th_0$ ), is a property of the APC and local cytokine production (1).  $Th_1$  cells produce interleukin (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ), and enhance the microbicidal activity of monocytes and macrophages.  $Th_2$  cells produce IL-4, IL-5, and IL-10, and assist in Ab production via class-switching for humoral immunity (2).

Crosslinkage of cell surface immunoglobulin (Ig), by an encounter with an Ag (crosslinkage-dependent B-cell activation) or by interaction with a CD4+ helper T-cell (cognate help), can activate B-cells. The activation of the B-cell by the helper cells is a property of cell surface molecules expressed by the T-cell (gp39–CD40 ligand interaction) and the production of cytokines. Upon activation, the B-cell differentiates into either an Ab-secreting cell or a memory cell. Upon rechallenge, the memory cell produces Abs in greater magnitude, more promptly, and of higher affinity, compared to the initial Ab response. Affinity maturation is a property of somatic hypermutation in the variable region of the Ab, which results in greater affinity and subsequent preferential selection of specific B-cells. Furthermore, the B-cell is capable of switching the heavy-chain constant region, thus allowing the production of Abs capable of mediating distinct biological functions, while retaining Ag-specificity. These Abs mediate targeting for effector cells and complement binding.

### 3. HETEROGENEITY OF TUMORS

Like other solid tumors, the heterogeneity of malignant gliomas has been confirmed at the morphologic, biologic, genetic, and antigenic levels (3–6). The sum of the evidence strongly suggests that such heterogeneity is a consequence of neoplastic glial transformation. This diversification renders tumor cells differentially susceptible to various therapeutic modalities and offers one biologic explanation for therapeutic resistance. The implications of this inherent heterogeneity are profound and must be recognized in order to promote the design of rational therapeutic approaches, such as active specific IT, which may require an individualized approach. Using Abs of various specificities and cytotoxicity assays, such complex Ag in disparities have been clearly identified in animal tumor models (7–9) and human glial tumors (10–14). Even among clones derived from a single established cell line, reactivity with a panel of monoclonal antibodies (MAbs) of varied specificity demonstrated an individualized pattern of antigenicity (13,14).

Nowell (15,16) first proposed that tumors develop through a predictable series of genetic mutations with selective clonal expansion. Well-documented genetic alterations occurring within gliomas include *p53* mutations, epidermal growth factor receptor (*EGFR*) amplification, inactivation of the *CDKN2A* gene (9p21), overexpression of platelet-derived growth factor, *p16* deletion, and loss of het-



erozygosity at 13q, 19q, 17p, and 10 (17,18). These alterations may provide specific targets that can be identified immunologically. Since progression within gliomas is established at the genetic level (19), a correlation between histologic progression and specific genetic abnormalities (20–22), which are clonally conserved, is being established.

Recently, numerous Ags associated with gliomas have been identified. Only a few, however, have been demonstrated as having significant diagnostic or therapeutic utility. These include the extracellular matrix-associated Ags (GP240 and tenascin), the membrane-associated ganglioside molecules, and the overexpressed deletion variant of the epidermal growth factor receptor (*EGFRvIII*) (23). MAbs that are specific to the *EGFRvIII*, and do not recognize the wild-type *EGFR*, significantly enhance mean longevity via intratumoral injections in animals and will soon be in clinical trials.

#### 4. IMMUNOLOGICAL PRIVILEGE OF CNS

Classically, the brain has been characterized as being “immunologically privileged,” based on the protective nature of the brain’s environment to allografts and xenografts. Most early studies demonstrated, by attempting to transplant MHC-mismatched tissues into various organs, that the brain was a more permissive host (24). Data from this paradigm indicated that sensitization to Ags present within the brain was relatively diminished. Medawar’s Nobel-Prize-winning work (25) demonstrated that, in animals that had previously been sensitized to foreign tissue, subsequent rechallenge demonstrated that the IRs were often delayed or incomplete within the CNS. Additionally, a number of vaccination strategies in experimental animals were effective against tumors outside the CNS, but completely failed to have impact on tumors grown within the CNS (26,27). Sensitization of the immune system can be accomplished against intracranial tumors, if multiple, larger Ag doses are given (27,28). In a clinical study (29), patients successfully treated with biomodulators had tumor relapses within the brain, despite remissions extracranially. Taken together, the data indicate that the relationship between the brain and the immune system is not the same as that between the immune system and other organs.

Possible explanations for the immunological privilege of the brain include the absence of conventional lymphatics, the presence of the blood–brain barrier (BBB), and the presumed paucity of APC within the neural parenchyma. The concept of the BBB is based on studies demonstrating that intravital dyes and neuroactive compounds, which pass from blood into most tissues, fail to penetrate into the brain. By electron microscopy, tight junctions that blocked the passage of tracers across the capillary wall were localized to the cerebral capillary endothelium. Astrocytic endings surround the blood capillaries of the brain, form part of the physical basis of the restrictive BBB, and may play a role in the

induction of tight junctions between the endothelial cells (30). The BBB excludes large, water-soluble molecules in the bloodstream from the brain. However, this barrier can be broken down during inflammatory processes in the brain. In vitro studies indicate that cells (such as astrocytes) close to the CNS vessels, upon activation, elaborate cytokines (such as tumor necrosis factor [TNF]) that upregulate adhesion molecules (such as intercellular adhesion molecule [ICAM]), for immune cell binding. Furthermore, during inflammation, there is loosening of the tight endothelial junctions, which allows subsequent passage of lymphocytes, monocytes, and macrophages from the vasculature into the CNS.

The absence of lymphatic drainage within the brain was thought to block the afferent limb of the IR, thus also partially explaining immunological privilege. However, the trafficking of Ag particles from the brain and cerebrospinal fluid (CSF) to the draining cervical nodes is much larger than previously appreciated. Studies indicate that the interstitial fluid (ISF) is cleared to the CSF by specialized extracellular channels, Virchow-Robin spaces, which surround blood vessels as they penetrate the brain. Current theory suggests that the secretion of the ISF by the cerebral capillary endothelium through coupled transport of solutes and water generate a driving force for bulk flow from the brain to the CSF, with subsequent drainage of the ISF from the tissue (31). The ISF and CSF then drain either into dural sinuses or along the subarachnoid space. Substances draining from the cranial subarachnoid space via the olfactory pathway can be cleared to the nasal mucosa and then into the deep cervical lymph nodes. Substances draining from the dural sinuses become blood-borne and subsequently circulate to the spleen. As observed with radiolabeled albumin injected into the brain tissue, the protein begins to appear in the deep cervical lymph in about 2 h. Maximal concentrations were achieved in 15–20 h, and drainage persisted for several days (32). High-mol-wt proteins (33), lymphocytes, and macrophages have also been injected into the brain and ventricles and subsequently recovered from the cervical lymph trunks, which indicates that there is lymphatic drainage from the brain with a prolonged period of Ag exposure in draining lymph nodes (31).

Cserr et al. (34) developed a rat intracranial cannula model in which there was normal BBB permeability. Microinfusion of albumin into the CNS yielded a prolonged, Ag-specific serum Ab response, primarily IgG<sub>1</sub> and IgG<sub>2</sub>, with Ab-secreting cells identified in the cervical lymph nodes and spleen. The magnitude of the serum Ab response was higher when compared with systemic administration, and an increased ratio of IgG<sub>1</sub> to IgG<sub>2</sub> was found. Furthermore, delayed-type hypersensitivity was suppressed (35), which presumably contributes to the ability of allografts to survive for prolonged periods within the brain. Thus, contrary to the classical interpretation of the brain as not being in communication with secondary lymphoid organs, an immune response to CNS-administered Ag is present and regulated.

## 5. IMMUNOSUPPRESSION MEDIATED BY MALIGNANT GLIOMAS

Although there is clinical evidence for cell-mediated anti-glioma activity (36,37), immunosuppression in patients with primary intracranial tumors has been well documented. Glioma patients have been shown to have low peripheral lymphocyte counts, reduced delayed-type hypersensitivity reactions to recall Ag, impaired mitogen-induced blastogenic responses by peripheral mononuclear cells, and increased CD8<sup>+</sup> suppressor T-cells (38). These findings indicated a degree of anergy that was proportional to the degree of anaplasia of the neoplasm (39). The lymphocyte deficit involves the T-helper (CD4<sup>+</sup>) subsets with decreased T-cell activity in vitro (40–42). Furthermore, there is diminished induction of Ig synthesis by B-cells in vitro from the peripheral blood of patients with intracranial tumor, probably related to diminished T-helper activity (43). However, humoral isotypes in vivo, within the serum of patients with glioblastoma multiforme (GBM) or malignant glioma, are usually normal. Initial characterization of the impairment in T-cell activation revealed that it was not the result of an inability of the cells to bind mitogen (44), but resulted from a defect in lymphocyte activation, with IL-2 production significantly diminished (44,45). However, the addition of recombinant IL-2 failed to improve the proliferative abilities of lectin-stimulated T cells (46). Further studies of lymphocytes obtained from patients revealed that lectin-stimulated T-cells do not express the high-affinity IL-2 receptor, as evidenced by ligand-binding studies with <sup>125</sup>I-labeled IL-2 (47). Recent data indicates that diminished responsiveness of peripheral T-cells is associated with impaired early transmembrane signaling through the T-cell receptor (TCR/CD3) complex (48).

Numerous reports of decreased or defective systemic cell-mediated immunity in brain tumor patients are counterbalanced by the local IR within the CNS tumor (49). Mononuclear cell infiltrates are common within the parenchyma of human gliomas (50), with several studies (50–52) attempting to correlate the intensity of infiltration with survival. Although these studies reported prolonged survival correlating with the presence of lymphocytic infiltration, this has not been consistently seen (53). Current concepts in malignant melanoma stress that it is the pattern of the lymphocytic tumor infiltrate that is an indicator of improved prognosis (54,55), but this has not yet been histologically verified in gliomas.

Human malignant glioma cell culture supernatants have been shown to suppress IRs in vitro (41,56,57), proliferation of lymphocytes (41), IL-2 synthesis (40), and IL-2R expression (58). Although IL-10 (59,60) and prostaglandin E<sub>2</sub> (61,62) have been implicated, various isoforms of transforming growth factor- $\beta$  (TGF- $\beta$ ) have been most extensively studied (40,56,57,63,64). The inhibitory factor previously termed “glioblastoma-derived T-cell suppressor factor” has in fact been shown to be a member of the TGF- $\beta$  family (63). TGF- $\beta$ <sub>1</sub> and TGF- $\beta$ <sub>3</sub>

mRNA have been reported to be present in glioma cells, but the major isoform secreted *in vitro* and *in vivo* is TGF- $\beta_2$ . TGF- $\beta_2$  has been demonstrated in all grades of gliomas, without a demonstrable correlation to the degree of malignancy (65). Astrocytes produce a latent form that must be proteolytically cleaved for biological activity. TGF- $\beta$  has many systemic immunosuppressive properties, including the reduced production of IL-1, IL-2, and IFN- $\gamma$ ; downregulation of MHC II; and reduction of cytotoxicity by natural killer cells. Within glioma patients, TGF- $\beta$  has been demonstrated to have a suppressive effect on glioma-infiltrating lymphocyte proliferation and cytotoxic activity (66), and probably mediates the diminished formation of high-affinity IL-2R on the surface of CD4<sup>+</sup> T-cells (67).

The potential of immunosuppressive factors, such as TGF- $\beta$ , to abolish a cell-mediated antitumor IR has been demonstrated experimentally. Torre-Amione et al. (68) transfected a highly immunogenic and easily rejected subcutaneous fibrosarcoma cell line with TGF- $\beta$  and demonstrated that, after transfection, the fibrosarcoma cell line was able to completely escape immune rejection. The tumor line retained expression of MHC-I and the tumor-specific Ag, but failed to stimulate a primary cytotoxic lymphocyte response *in vitro* or *in vivo*. In the reciprocal experiment, rodents bearing intracranial 9L were subcutaneously immunized with 9L cells and genetically modified with an antisense plasmid vector, to inhibit TGF- $\beta$  expression, which resulted in significant increased median survival (69). In contrast, Ashley et al. (70), utilizing an immunocompetent syngeneic murine model, found that TGF- $\beta$  inhibited induction of antitumor cytotoxicity when the tumor cells were given subcutaneously, but not when they were given intracranially. They concluded that the net effect *in vivo* of TGF- $\beta$  production within high-grade glioma was tumor inhibition (70).

Various isoforms of TGF- $\beta$  (71) are secreted by gliomas (67). A high-mol-wt form of biologically active TGF- $\beta$ , derived from malignant gliomas, was proposed as possibly having a role in regulating receptor and ligand expression on gliomas by providing an autocrine and/or paracrine mechanism for stimulating continuous glioma cell proliferation (72). The role of TGF- $\beta$  therefore appears to be more complex than initially presumed. TGF- $\beta$  not only acts as an immunological modulator, but also as a mediator of tumor invasion and adhesion (73) by interacting with a variety of tumor suppressor genes via secondary messengers (74).

## 6. MHC EXPRESSION WITHIN CNS

The expression of MHC and Ag presentation within the CNS has been controversial. Much of the data has been challenged, but these discrepancies can be resolved, if technical and biological considerations are addressed. Controversy because of technical considerations is often the result of a failure to differentiate

surface vs internal expression; failure to control for cross-reactivity by employing MAbs with specificity to different determinants on the molecule of interest; or failure to differentiate data from genetic studies, which confirms the capacity to synthesize a protein from the actual presence of a functional protein product. Similarly, biological considerations require that species and tissue of origin be clearly defined.

Although both MHC-I and -II molecules can be detected within the CNS of most species, their distribution is irregular, and their expression is under strict regulatory control and is limited to specific cell types. MHC-I expression *in vivo* within the normal CNS is concentrated on the endothelial cells and parenchymal cells, with no definitive class I staining associated with normal neurons (75–77). In rodents, detectable protein is concentrated at the luminal surface of the blood vessel (78,79) and may reflect proteins from the serum that have been absorbed into the endothelium (79). Induced or occasional class I staining has been identified on microglia (Table 1; 80,81), ependymal cells (81,82), and stromal cells.

MHC-II molecule expression in the normal CNS is limited to subsets of microglial cells, especially in the white matter (81,83,84) and CNS dendritic cells (DCs) (85). Although there is general agreement that neurons and oligodendrocytes do not express MHC-II molecules under normal conditions, disagreement remains as to whether endothelial cells and astrocytes might express class MHC-II, especially under pathologic conditions such as multiple sclerosis and experimental allergic encephalomyelitis (EAE) (86–90). Immunohistochemical staining with Abs that recognize MHC-II molecules also becomes evident along the ependyma after stereotactic injections of IFN- $\gamma$  (78).

In contrast to analyses done on tissue derived from *in vivo* sources, all major CNS cell types, except neurons, have demonstrated a capacity to express MHC-I *in vitro* (91,92). Both human and murine astrocytes and oligodendrocytes have shown class I expression *in vitro*, which can be upregulated by IFN- $\gamma$  (93–97). In one report (92), class I was upregulated on neurons *in vitro* by IFN- $\gamma$ . However, this has come under question, and current consensus is that primary CNS neurons will not express class I molecules, even after exposure to IFN- $\gamma$  (95).

Astrocytes and oligodendrocytes have been shown to possess the capacity for class II expression *in vitro* under various conditions, as well, although this expression still remains below that usually found on microglia *in situ* (93,94, 96–100). Evidence has been accumulating to suggest that class II expression that may have functional significance can occasionally be observed on astrocytes *in vitro*. For example, pure astrocytes cultured from newborn rats are capable of presenting protein Ag to Ag-specific helper T-cell lines derived from rats previously sensitized *in vivo* (101,102). Within 24 h of astrocyte/lymphocyte co-culture in the presence of myelin basic protein (MBP), these

**Table 1**  
**Immunological Surface Markers on CNS Cells**

<i>Determinant</i>	<i>DC</i>	<i>Monocyte</i>	<i>Granulocyte</i>	<i>B- and NK cells</i>	<i>T-cell</i>	<i>Microglia</i>	<i>Astrocyte</i>
MHC-I	+	+	+	+	+	+	+
MHC-II	+	±	-	+	-	Inducible	Inducible
Leukocyte antigen							
CD45	+	+	+	+	+	+	
Adhesion							
CD11a	+	+	+	+	+	Inducible	
CD54	+	+	Trace	+	-	+	Inducible
CD58	+	+	+	+	-		+
T-cell markers							
CD1	-	-	Trace	Trace			+
CD2	±	-	-	-	+		
CD4	-	-	-	-	+		
B-cell markers							
CD40	+	Trace	-	+	-	Inducible	-
NK cell markers							
CD57	-	-	-	+	-		
Co-stimulatory							
CD80	+	+		Inducible		-	Inducible
CD86	+	+		+	+	Inducible	-
Myeloid							
CD13	-	+			-	+	
CD14	-	+	Trace	-	-	Inducible	

The levels of MAb staining on various cells are indicated as follows: -, no detectable staining; +, detectable staining; ±, variable results reported in the literature; Inducible, present upon activation (176-186).

T-cells were seen in aggregates around the astrocytes and subsequently formed blast cells that proliferated. The reaction, which was Ag- and MHC-restricted, resulted in upregulation of MHC-II molecules on the astrocytes. In another study, however, freshly isolated astrocytes exposed to IFN- $\gamma$ , which upregulates MHC-I and -II expression, were not able to activate naïve alloreactive CD4<sup>+</sup> T-cells, but were able to stimulate a primed alloreactive CD4<sup>+</sup> T-cell line. Similarly, unprimed CD8<sup>+</sup> cells proliferated slightly in the presence of stimulator astrocytes, but significant stimulation was IL-2-dependent. This implies that activation of T-cell subsets by astrocytes within the CNS can only follow priming by another type of APC (103).

Expression of MHC-I and -II molecules on glial tumor cells has been less well defined. Established glioma cell lines are clearly capable of expressing either MHC-I or -II molecules in vitro (104,105). Expression of MHC can usu-

ally be enhanced by IFN- $\gamma$  *in vitro*, but evidence suggests that this cytokine may not have the same effects on tumor cells *in vivo* (105). Although MHC-I or -II molecule expression is not a prominent feature of glial tumors, expression of both classes of molecules on a small subset of malignant astrocytes has been demonstrated fairly convincingly (76). In development of ITs mediated by cytotoxic T-cells, which are less discriminatory against targets expressing high MHC-peptide densities, quantification of MHC expression in neoplastic CNS tissue may be a consideration, because minimal levels of MHC-peptide complex are necessary for lysis (106,107).

## 7. CANDIDATES FOR APC WITHIN CNS

A variety of cells, including microglia, perivascular macrophages, meningeal macrophages, intraventricular macrophages, DCs, epiplexus cells, and astrocytes, have been implicated as the major APC in the CNS (108).

Microglia are capable of presenting Ag to helper T-cells *in vivo* (109) and have been shown to have some phenotypic and functional characteristics of both macrophages and DCs (110,111; Table 1). Although microglia appear quiescent regarding endocytic and secretory function, they express: MHC-II Ags *in situ* constitutively (111,112); T-cell co-stimulatory molecules, such as lymphocyte function-associated Ag-1 and -3, ICAM-1 (113), and B7 (114); and complement receptor 3 Ags (85). Astrocytes also express ICAM-1 and lymphocyte function-associated Ag-3, although to a lesser degree than microglia (113). Functionally, microglia can cluster CD4<sup>+</sup> T-cells (111) and induce T-cell responses in a mixed lymphocyte-type reaction *in vitro* (111) and are capable of phagocytosis (115), cellular cytotoxicity (116), and chemotaxis (117).

Microglia differ from DCs in immunophenotypic characteristics, relative phagocytic capacity, and turnover rate. McMenamin and Forrester (85) suggested that, since DCs have a high turnover rate, if microglia were equivalent, then they would have a similar degree of turnover within the CNS. Based on radiation chimera models, however, turnover appeared low. Although microglia are capable of division, they are replenished from the monocyte pool. Thus, microglia do not have a life-cycle akin to DCs (85). Because there has been confusion in the literature regarding strict definitions of microglia and DCs, some data describing microglia may in fact be more indicative of DCs.

Initially, DCs were thought to be absent from the CNS. This conclusion was based on immunohistochemical investigations (108) of MHC-II expression in conventional tissue preparations from a variety of species, which reported expression on only rare perivascular cells. Recent observations (118) based on immunofluorescence and morphological appearance by electron microscopy, however, indicate that the choroid plexus does contain an extensive population of MHC-II<sup>+</sup> DCs. Additionally, cells consistent with the DC lineage have been



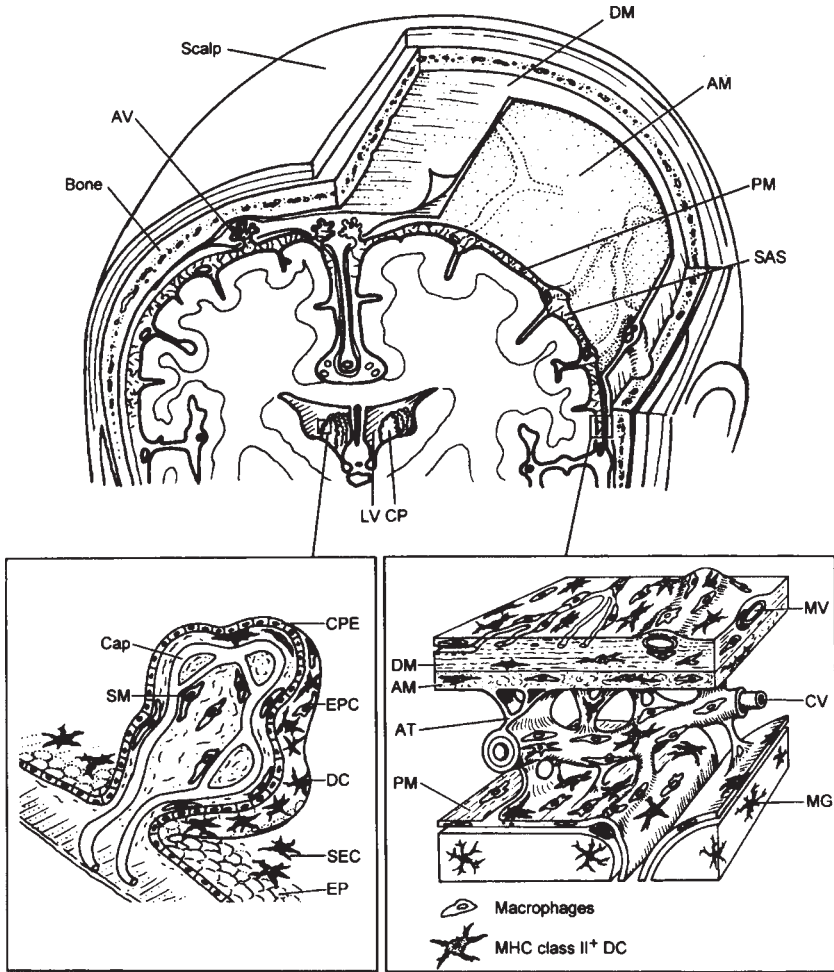
identified in whole mounts of rat dura (85; Figs. 2 and 3). Responses elicited by placement of inflammatory stimuli into the neural parenchyma are minimal compared with responses to such stimulation in the ventricles or subarachnoid space (119), and McMenamin has proposed that this may result from the paucity of DCs within the parenchyma. The initial stages of autoimmune disorders often involve paraventricular, leptomeningeal, and perivascular sites and may be a consequence of the extensive DCs present at these sites.

Phenotypic and functional analysis of peripheral DCs from patients with a variety of neoplasms has shown them to be similar to those produced from healthy donors (120–122). The authors have found that DCs isolated from the peripheral blood of patients with all grades of gliomas are phenotypically similar to healthy donors. The functional analysis of DCs from glioma patients is currently underway, and preliminary evidence supports the notion that systemic DCs are not functionally altered, as is the case with the lymphocytes in glioma patients. Furthermore, in contrast to the suppressive role of TGF- $\beta$  on lymphocytes, TGF- $\beta_1$  seems to exert a maturation role for DCs, according to published reports (123). DCs may become functionally impaired, however, upon infiltration into tumor. For example, DCs isolated from rat colon carcinoma have been demonstrated to be poor stimulators of primary allogeneic T-cell proliferation, and they failed to express B7, an essential co-stimulatory signal for T cells (124).

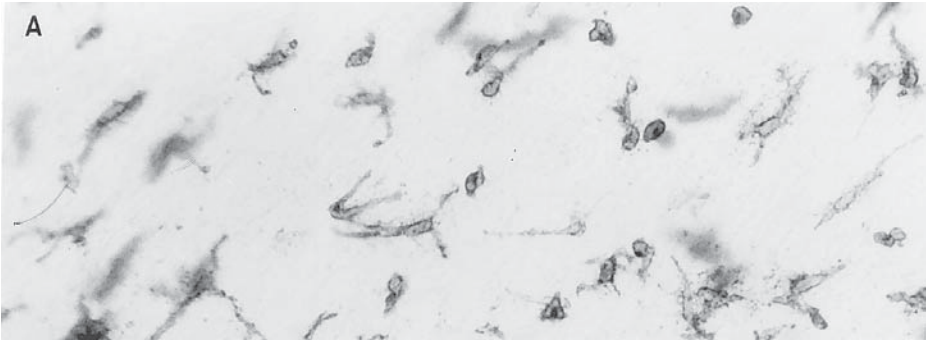
Other candidates for APCs within the CNS are the macrophages, which are recruited from the systemic circulation into the CNS to phagocytize degenerating cells during development. These cells remain as the resident macrophage population in the meninges, ependymal region, and choroid plexus (125). The role of CNS macrophages, including tissue repair and the elaboration of cytokines, is presumably similar to that of the systemic macrophage. Identification of a single cell type as the definitive APC within the CNS remains unresolved, and the defining quality may be a property of the Ag itself, the location of the Ag with the CNS, and/or local cytokine production.

## 8. IMMUNOLOGICAL RESPONSES WITHIN CNS

Lymphocytes in the CNS of healthy humans or rodents are a rare finding (126,127). However, during neuroinflammatory responses, such as multiple sclerosis and EAE, lymphocytes are abundant within the CNS (128). To elucidate the parameters that dictate lymphocyte infiltration into the CNS (129), healthy rats were injected with either concanavalin A (Con A)-activated CD4<sup>+</sup> T-cell clones recognizing an MBP epitope or a nonspecific epitope, bulk splenocytes, or Con A-activated bulk splenocytes (Fig. 4). Large numbers of all of these lymphocyte preparations, except the inactivated splenocytes, could be detected in the CNS within 3 h, with concentrations peaking around 12 h. The concentra-



**Fig. 2.** The skull, choroid plexus, and meninges. **(Upper)** Schematic three-dimensional drawing (in the coronal plane) of the skull, cranial cavity, meninges, brain, and ventricles containing the choroid plexus. **(Bottom left)** The structure of the choroid plexus and distribution of macrophages and DCs. **(Bottom right)** The distribution of macrophages and DCs in the meninges. AV, arachnoid villi; AM, arachnoid membrane; AT, arachnoid trabeculae; Cap, capillaries; CPE, choroid plexus epithelium; CV, cerebral vessel; DC, dendritic cell; EP, ependyma; EPC, epiplexus cell (type of macrophage); DM, dura mater; CP, choroid plexus; LV, lateral ventricle; MG, microglia; MV, meningeal vessel; PM, pia mater; SM, stromal macrophages; SEC, supraependymal cells; SAS, subarachnoid space. Adapted with permission from ref. 85.

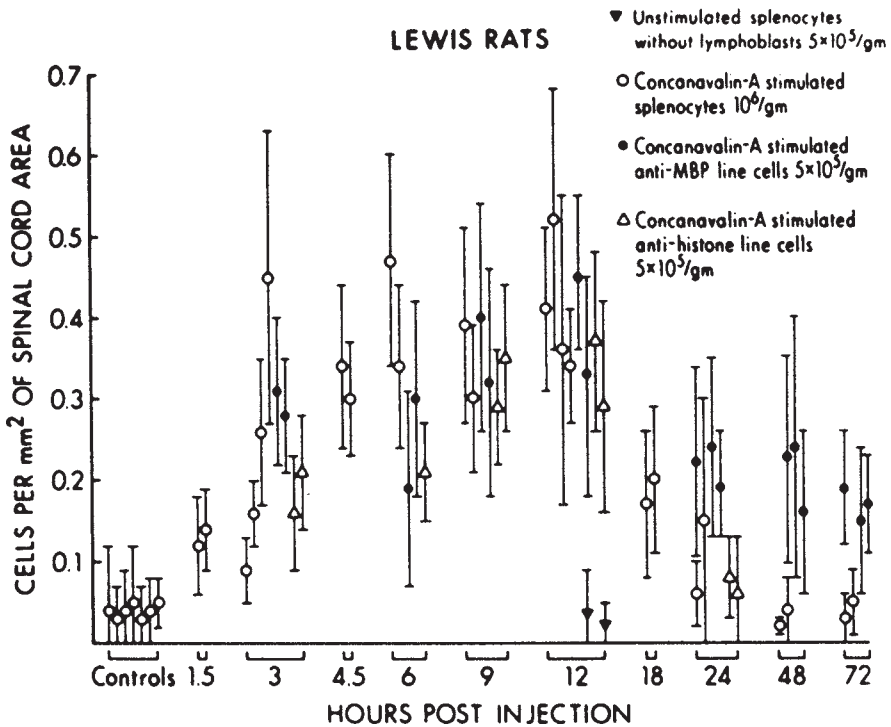


**Fig. 3.** Rat dural whole mount stained with Ox6 displaying dendritic MHC-II cells ( $\times 600$ ). Adapted with permission from ref. 85.

tion of nonspecifically activated lymphocytes diminished over the next 24–48 h, but lymphocytes with specific reactivity for Ags within the CNS remained at high concentrations for  $>72$  h. This indicates that lymphocytes require activation prior to entry into the CNS, but that Ag specificity is not necessary for entry into the CNS. The ability of activated lymphocytes to invade normal brain parenchyma was also demonstrated in another study (127) using a graft-vs-host disease model. In this study, graft-vs-host disease was initiated systemically, by infusing F1 hybrid rats with parental lymphocytes, which produced a scattered infiltration of lymphocytes into the CNS and coincident upregulation of MHC molecules on host CNS cells (127).

Further evidence that lymphocytes require activation prior to entry into the CNS was provided by a transgenic murine model in which  $\beta$ -galactosidase, under the control of a glial fibrillary acidic protein promoter, was expressed in astrocytes. Peripheral immunization with  $\beta$ -galactosidase in complete Freund's adjuvant (CFA) resulted in an Ag-dependent infiltration of the CNS by mononuclear cells, principally  $CD4^+$  T-lymphocytes, some  $CD8^+$  T-cells, and monocyte/macrophages, into the CNS parenchyma and perivascular regions (130). Together, these results indicate that T-cell activation is the fundamental signal allowing surveillance of the CNS.

Sampson et al. (131) provided definitive evidence that systemic T-cells could gain access into the CNS, and that these T-cells could have a therapeutic effect against poorly immunogenically established intracerebral tumors. Irradiated murine melanoma cells, which were transfected with cytokines and implanted systemically, could initiate an efficacious cell-mediated response against intracranial melanoma. Lymphocyte subset depletion studies indicated that, when  $CD8^+$  T-cells were depleted, there was an absence of  $CD4^+$  T-cells within the brain tumor as well. This was not the case in subcutaneous tumors, suggesting a



**Fig. 4.** The time-related alterations in the concentration of T-cells per unit area in the lower spinal cord of syngeneic rats. Each bar depicts the mean and standard deviations of the T-cell concentration in the spinal cord of one animal. The quantity and type of cells injected are indicated. Controls were uninjected rats, or rats receiving an injection of physiological saline via tail vein. In determining the concentration of cells in the spinal cord of a rat, approx 1 cm<sup>2</sup> total area of 5- $\mu$ m thick cryostat sections, taken at least 15  $\mu$ m apart, were analyzed. Adapted with permission from ref. 129.

potential difference between the CNS and other tissues regarding lymphocyte migration. This may have significant implications for autoimmune diseases of the brain. Overall, such studies lend enthusiasm to the possibility that T-cells activated during a systemic antitumor response are not strictly barred from the CNS.

Most studies examining humoral responses within the CNS (132–134) are based on pathological states. The presence of Igs within the CSF implies passage of activated, Ag-specific B-cells from their systemic sites of activation into the CNS. Based on the fact that activated T-cells have the capacity to enter the brain, the prevailing hypothesis is that activated B-cells migrate into the brain as blasts and differentiate into Ab-secreting cells. Support for this hypothesis is

provided by systemic Ag immunization, with subsequent identification of lymphocytes secreting Ag-specific Abs within the CNS (135). In pathological states, plasma cells secreting IgG, IgA, and IgM isotypes within the CNS have been identified with immunocytochemistry (136). Glioma patients have demonstrated production of antiglioma Abs in indicating that the Ag-recognition system is partially working (137). Characterization of these Abs indicates a skewing to the IgM isotype, which may be a property of Ag recognition, but may also be secondary to an immunosuppressive mechanism that prevents the elaboration of an IgG IR. For example, GBM cells produce IL-6, which is a potent B-cell stimulator. However, these same cells also elaborate TGF- $\beta_2$ , which suppresses secretion of IgG and IgM by B-cells.

Ab responses within the CNS were not thought to be complement-mediated, since complement concentrations are low in the CSF (34). Furthermore, inoculation of soluble Ag into the anterior chambers of the eyes of rodents, also a site of immunological privilege, failed to produce sufficient amounts of complement fixing (IgG<sub>2</sub>) Abs (138). Although the liver is the primary source of plasma complement, there has been recent evidence that cells such as macrophages, fibroblasts, endothelial cells, leukocytes, synovial lining cells, and astrocytes can produce complement components that are responsible for bacterial opsonization, phagocytosis, and lysis. Lévi-Strauss and Mallat (139) first provided evidence that astrocytes are capable of elaborating complement. They demonstrated that a rodent astrocyte line produced C3 and factor B *in vitro* (139). Since then, human astrocytes have also been shown to secrete all components of the classical, alternative, and terminal complement pathways, as well as regulatory proteins (140). Functional activity of these components has been confirmed by assembling a lytic complement pathway from the products generated in culture. In instances of low levels of expression, IFN- $\gamma$  or TNF- $\alpha$  can enhance synthesis (141). The same authors proposed that, because astrocytes can synthesize inflammatory cytokines, complement biosynthesis may be turned on in an autocrine fashion.

Astrocytes and astrocyte cell lines are resistant to complement lysis secondary to membrane-bound inhibitor (CD59) and decay-accelerating factor. Blocking CD59 with a specific Ab renders astrocytes susceptible to lysis by complement; blocking the inhibitors of the activation pathway (such as decay-accelerating factor) has little effect on lytic susceptibility (140). Thus, CD59 appears to be the crucial factor in protecting these cells from complement lysis. The contribution of other cells, such as microglia, to the complement pathway remains to be ascertained, but astrocytes, oligodendrocytes, and neurons all have the ability to spontaneously activate complement *in vitro*. Some components of complement, such as C3, C4, and C9, are expressed at the messenger RNA level

in neuroblastoma and astrocytoma cell lines (142), but whether gliomas can form functional complement components is not presently known.

## 9. EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

Induction of EAE, an autoimmune, inflammatory, demyelinating process probably mediated by CD4<sup>+</sup> T-cells, is a potential risk when vaccinating patients with tumor homogenates possessing potentially encephalitogenic CNS proteins. Induction of a postvaccination encephalomyelitis in humans was first reported in 1885, when patients were vaccinated against rabies with spinal cord tissue derived from rabbits that were infected with the rabies virus. Subsequent evaluation identified that the Ag component was derived from the spinal cord component and not from the virus itself. EAE was first produced experimentally in animals in the 1930s (143) by repeated inoculation of brain matter within monkeys. The primates subsequently developed a paralytic disease that correlated with a cellular infiltrate of the brain and spinal cord. EAE was difficult to induce consistently, and not until the discovery of strong adjuvants, such as CFA, could EAE be induced reproducibly (144).

EAE can be induced with adjuvant by immunization with MBP, myelin proteolipid protein (145,146), myelin oligodendrocyte glycoprotein (147), glial fibrillary acidic protein, and the astroglial calcium-binding protein, S-100 $\beta$  (148). Since there is the ability to induce autoimmune encephalitis, not only with MBP, but also with many of the CNS components, a preparation containing these components, as well as other undiscovered components, has the potential to induce EAE (Table 2). EAE can be readily induced in rats, guinea pigs, mice, sheep, and monkeys after a single injection of CFA and either autologous or heterologous CNS tissue homogenate (149).

ITs against CNS Ag will potentially need to be evaluated in susceptible strains for induction of EAE. For example, DCs pulsed with MBP can activate lymphocytes and subsequently transfer severe EAE in Lewis rats, which indicates that this therapy has the potential for induction of autoimmunity (150). Susceptibility is strain-dependent; both EAE-susceptible and -resistant rodent strains have been identified. Sensitive mouse strains include SJL (H-2<sup>s</sup>), A.SW/Sn (H-2<sup>s</sup>), PL (H-2<sup>u</sup>), and DBA1/J (H-2<sup>q</sup>). Partially resistant strains include C57BL/6 (H-2<sup>b</sup>) and A.TL (H-2<sup>s</sup>). Resistant strains include A/J (H-2<sup>a</sup>), BALB/c (H-2<sup>d</sup>), NZB (H-2<sup>d</sup>), AKR/J (H-2<sup>k</sup>), CBA/J (H-2<sup>k</sup>), and BRVR (H-2<sup>k</sup>) (151–153). Susceptibility to EAE has been correlated to the MHC background (151), but this has been disputed (154) and more recent evidence indicates additional factors may contribute. For example, in resistant strains of mice, except AKR, EAE can be induced by treatment of the mice with anti-IFN- $\gamma$  MAbs (155). Furthermore,

**Table 2**  
**Vaccination Protocols**

<i>Antigen</i>	<i>Adjuvant</i>	<i>Reference</i>
<b>Vaccination protocols that induce EAE in susceptible animals</b>		
Brain or spinal cord homogenate	Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) with Bacilli Calmette-Guerin cell wall or <i>Mycobacterium tuberculosis</i>	(144,187)
Glial fibrillary acidic protein	Not specified	(148)
Glioblastoma	CFA or IFA	(149)
Myelin basic protein	CFA	(145,188)
Myelin oligodendrocyte glycoprotein	CFA	(147)
Myelin proteolipid protein	IFA	(146)
S-100 $\beta$	Not specified	(148)
<b>Vaccination protocols that do not induce EAE in susceptible animals</b>		
Glioblastoma	None	(149)
Cultured cell lines	CFA or IFA	(149,164)

in rats, hyperinducibility of MHC-II on astrocytes correlated with strain-specific susceptibility to EAE (156). Finally, astrocytes may be elaborating chemo-attractant cytokines for leukocyte trafficking in EAE (157), which may be variable in various strains of rodents, but this has not yet been evaluated.

Primarily Ag-specific CD4<sup>+</sup> T-cells (of the Th<sub>1</sub> subtype) mediate EAE. Identification that lymphocytes were involved in the autoimmune effects was based on passive transfer experiments (158), which were confirmed with depletion studies (159). Passive transfer of CD4<sup>+</sup> T-cells, with induction of EAE in naïve histocompatible recipients (160), provided definitive evidence for the effector function of the CD4<sup>+</sup> T-cell.

Given the ranges of protocols that routinely use vaccination with CNS tissue for the production of lethal EAE in nonhuman primates (149) and the documented susceptibility of humans to autoimmune disorders within the CNS, the induction of such autoimmune responses is of concern for clinical trials using unfractionated CNS Ags. Based on previous IT trials of humans with brain tumors, in which there were two possible cases of EAE (161,162), induced autoimmune responses remain a consideration. The majority of patients involved in active immunization with human glioma tissue or cells failed to display evidence of EAE (163). Induction of EAE in humans may then be dependent on the adjuvant utilized or on the Ag preparation. For example, in a hyperimmunization protocol using human glioma cells as the Ag preparation and Bacillus Calmette-Guerin cell wall as the adjuvant, there was no evidence of EAE in nonhuman



primates (164). However, a single injection of glioma tissue with CFA induced fatal EAE in nonhuman primates. Adjuvant has been demonstrated as a necessary component for the induction of any significant detectable glioma-associated reactivity in animals (Table 2).

In the scenario of an IT with the potential to induce EAE, a variety of strategies could be utilized to block induction. Because a single T-cell recognizes only a single peptide fragment within the context of MHC (165), immunological responses typically recruit multiple T-cell clones that recognize a variety of peptide sequences within a protein. In the case of EAE, the MBP-specific, encephalitogenic CD4<sup>+</sup> T-cell response is characterized by an unusually strict epitope dominance (166). Recently, EAE has been successfully blocked in vivo by preventing T-cell recognition with synthetic peptides, and this represents a potential therapeutic modality (167). Other interventions include alteration of either systemic or local cytokine production. For example, encephalitogenic T-cells produce TNF, which acts on endothelial cells to cause expression of ICAMs and subsequent passage of the T-cells across the BBB into the parenchyma. Lesion formation could be blocked by MAbs against TNF (168,169). Alternatively, attempts to shift the IR from Th<sub>1</sub> to Th<sub>2</sub> via oral administration of MBP have been reported (170) to inhibit EAE, or the Th<sub>1</sub> phenotype can be downregulated utilizing cytokines such as TGF- $\beta$ . Finally, the rationale for selecting tumor-specific Ag approaches in IT is based on the fact that the targeting Ag preparation does not contain CNS Ags that could possibly induce an autoimmune response.

## 10. SUMMARY

The interaction between the brain and the immune system is unlike the interaction between systemic tissues and the immune system. The concept of “immunological privilege” is clearly not absolute, with the presence of T-cells, B-cells, natural killer cells (49), and APCs within the CNS suggesting some amount of immunological responsiveness. Despite immunological privilege and a degree of immunosuppression, elaborated in part by the glioma, systemic sensitization of the immune system against murine and rat intracerebral glioma (171–174) and melanoma (131,175) has been achieved.

Ultimately, ITs for gliomas appear promising, but the unique characteristics of CNS tumors will need to be considered for rational design of therapeutics. Novel approaches to antitumor IT will need to be independently and thoroughly evaluated against tumors within the CNS, despite successes with these techniques outside the CNS. Recent identification of tumor-associated Ags, such as the mutant *EGFRvIII*, may provide selective targeting to gliomas, with a theoretically decreased risk of induction of EAE.

Many of the previous attempts to treat glioma patients with ITs, such as lymphocyte transfer, vaccination with glioma cells, and the use of some cytokines,

have not met with significant success. Emerging concepts within the field of immunology, advances in molecular techniques, and a greater understanding of the interaction between the CNS and the immune system provide background for more rational and perhaps more efficacious treatments.

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# III

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## ADOPTIVE CELLULAR IMMUNOTHERAPY OF BRAIN TUMORS

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# 6

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## Systemic T-Cell Immunotherapy for Brain Tumors

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*Gregory E. Plautz, MD and Suyu Shu, PhD*

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### 1. INTRODUCTION

Malignant brain tumors (BT) are difficult to treat effectively using current surgery, radiotherapy, and chemotherapy approaches (1–3). The authors have explored the use of T-cell immunotherapy (IT) to treat BTs in an extensive series of preclinical studies and, more recently, in clinical trials. Theoretically, the existence of the blood–brain barrier (BBB) and the lack of a lymphatic drainage system in the brain would impede the ability to elicit an afferent immune response (IR). Indeed, early studies using transplantation of allogeneic tissues established the concept of “immune privilege” for the brain (4,5). However, there is ample clinical (6,7) and experimental (8) evidence that the brain is permissive for T-cell-mediated efferent IRs.

There are several theoretical advantages of using T-cell IT for the treatment of malignant BTs. First, the recognition of antigens (Ags) by T-cells is highly specific, potentially targeting the response to unique tumor Ags and limiting

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toxic effects on normal tissue. Second, activated T-cells have the capacity to traffic into BTs following systemic delivery. This finding (8) could potentially address limitations imposed on the delivery of therapeutic agents across the brain endothelium or on local diffusion following intracavitary delivery. Third, T-cells can be amplified, and persist as memory cells to potentially provide a continuous antitumor effect of extended duration, which is independent of the tumor cell cycle.

Despite these theoretical advantages, previous clinical applications of IT for primary BTs or brain metastases have been mostly disappointing (9–17). Observations from trials of cytokine therapy have suggested that intracranial metastases progress in cases in which there is a therapeutic response at other sites of disease (18). In addition, systemic interleukin-2 (IL-2) causes edema around intracranial tumors that is often a dose-limiting side effect (19,20). Because IL-2 has been used in virtually all clinical applications of cellular IT, the presence of brain metastases remains an exclusion criteria for most cancer IT protocols. Furthermore, patients with malignant gliomas often have diminished IRs, which may, in part, result from immunosuppressive substances released by gliomas (21–26). The combination of these factors has tempered enthusiasm for applications of IT for BTs.

The authors have developed an approach to cancer IT that uses systemic adoptive transfer of tumor-draining lymph node (LN) T-cells that have been activated *ex vivo*. To test whether this approach would be useful against tumors in immuno-privileged sites, an experimental intracranial tumor model system was utilized. As described below, adoptive transfer of tumor-reactive T-cells was able to cure intracranial tumors. The parameters that controlled treatment efficacy for BTs differed from those involved in treatment of tumors at other anatomic sites. For example, the addition of systemic IL-2 was found to be inhibitory to the therapeutic efficacy of transferred T-cells for the treatment of BTs; a similar regimen of IL-2 often augmented the treatment of pulmonary metastases and peritoneal ascites tumors. These experiments clearly demonstrate that the immune reaction to intracranial tumors needs to be considered as a unique phenomenon. These features stimulated changes in the design of the authors' clinical trials of adoptive IT for patients with malignant gliomas. The clinical responses observed in some patients confirms the rationale of this approach to BT immunotherapy.

## **2. ADOPTIVE IT USING T-CELLS REACTIVE AGAINST TUMOR AGs**

Advances in the understanding of T-cell recognition of Ags and the nature of tumor Ags indicate that autologous tumor cells are, at present, probably the best source of Ag, and that autologous T-cells are required for therapy. T-cells recognize Ags in the form of peptide fragments of proteins, which are processed

intracellularly and presented by the major histocompatibility complex (MHC) molecule (27,28). Experimental evidence from carcinogen-induced animal tumors suggests that the tumor-specific transplantation Ags that elicit the strongest T-cell responses are unique to each tumor, rather than broadly shared Ags (29–31). More recently, peptides derived from either tissue-restricted normal proteins or mutant proteins that cause malignant transformation have been defined. Experiments and clinical studies are ongoing to determine whether such peptides are effective for eliciting a protective IR (32–34). Nevertheless, until a broadly shared glioma-specific Ag is defined, autologous tumor cells will remain as the best source of tumor Ags. Because humans have numerous MHC alleles, an autologous source of T-cells is required to correctly recognize the tumor Ags. The heterogeneity of human cancer patients has made it difficult to dissect the antitumor IR using direct experimental approaches. Therefore, the fundamental principles of adoptive IT have been established using experimental tumors of defined immunogenicity that can be transplanted among genetically identical hosts and treated with T-cells at defined doses.

### **3. TUMOR-DRAINING LNs AS OPTIMAL SOURCE OF T-CELLS**

The observation in mouse tumor models that hosts bearing progressively growing, poorly immunogenic tumors had tumor-reactive T-cells was conceptually important (35). This indicates that, although the host was able to mount an IR to the growing tumor, the magnitude or characteristics of the IR were insufficient to control the growth of the progressive neoplasm. Moreover, animal models using tumor-bearing hosts, in which issues of tolerance and tumor-mediated immune modulation are present, are much more relevant to clinical situations than are experiments in naïve animals. Experiments to identify the site of tumor-reactive T-cells demonstrated that tumor-draining LNs were a much better source than the spleen. Furthermore, there was essentially no antitumor activity in distant LNs. This finding is consistent with the physiologic role of LNs to recruit naïve T-cells and facilitate initial Ag stimulation. The tumor-draining LNs undergo tremendous hypertrophy, increasing 10–20-fold in cell number from d 6 to 14 after tumor inoculation.

### **4. REQUIREMENT FOR EX VIVO ACTIVATION OF LN T-CELLS**

An important characteristic of tumor-draining LN T-cells is that they are immature, with weak effector function. When transferred directly to a secondary host, even with a small tumor burden, they usually do not mediate tumor regression. However, after ex vivo stimulation, they acquire potent antitumor reactivity. Adoptive transfer of these cells into a tumor-bearing host mediates tumor regression and the establishment of a long-term memory response (36,37). The antitumor response is highly specific, and independently derived tumors

**Table 1**  
**Efficacy of Anti-CD3-Activated**  
**LN T-Cells Against Pulmonary Metastases**

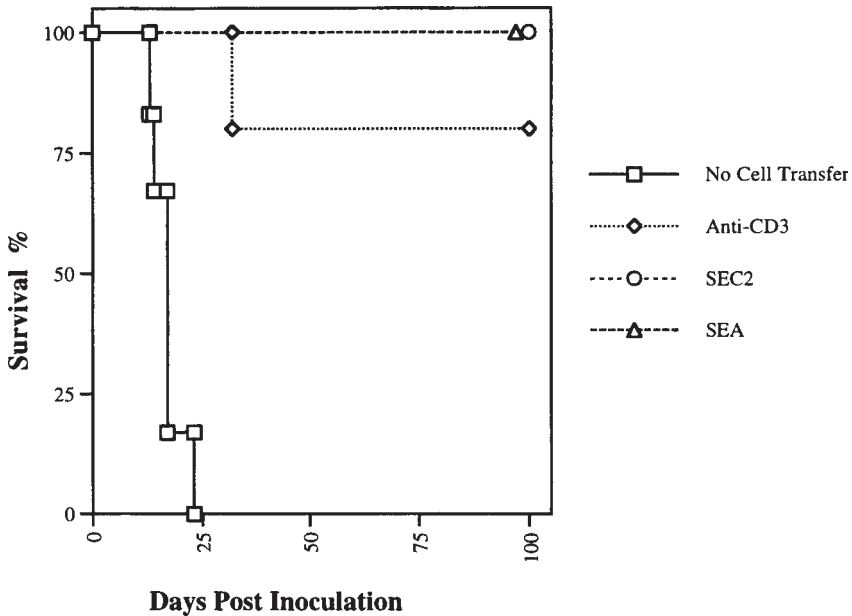
<i>No. cells</i>	<i>Mean no. metastases</i>
0	156
$6 \times 10^6$	0
$3 \times 10^6$	7
$1.5 \times 10^6$	65

Modified with permission from ref. 38.

of the same histologic type are not rejected. Moreover, adoptive transfer of LN T-cells from normal animals activated *ex vivo* by the same procedure is not therapeutic. This indicates that sensitization to specific tumor Ags *in vivo* is required, and that LN T-cells require a differentiation step to become fully functional.

Initially, tumor cells were used to provide *in vitro* stimulation (IVS). However, the clinical application of IVS was difficult, because a sufficient number of tumor cells was frequently not available. An important advance in the strategy to activate LN T-cells was the use of Ag-independent stimulation *ex vivo*. The requirement for Ag can be bypassed during *ex vivo* activation by stimulating the T-cells through the T-cell receptor (TCR-CD3) complex using monoclonal antibodies (MAb) against CD3 (Table 1; 38). Another effective approach to *ex vivo* stimulation is treatment with bacterial superantigens (Fig. 1; 39). Superantigens provide an potent mitogenic signal to T-cells expressing certain TCR V $\beta$  regions (40). The use of anti-CD3 MAb or bacterial superantigens has theoretical, as well as strong practical, advantages. These mitogens activate both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells; tumor cells often only stimulate CD8<sup>+</sup> T-cells. Even though the activation of T-cells with anti-CD3 MAb or bacterial superantigens is Ag-independent, the T-cells retain their specificity in adoptive IT (Fig. 2).

A general procedure for *in vitro* activation of tumor-draining LN T-cells has been culture with immobilized anti-CD3 or superantigens for 2 d, followed by culture in 24 IU/mL of recombinant IL-2. A high proportion of the T-cells express the high-affinity IL-2 receptor after the initial 2-d stimulation with anti-CD3 or bacterial superantigens. They subsequently undergo rapid proliferation in a low concentration of IL-2 (24 IU/mL), while retaining their antitumor function. Culture in IL-2 thus serves to amplify the number of T-cells that can be used for therapy. If the cells are cultured in higher concentrations of IL-2 (>240 IU/mL), they lose their antitumor function, even though they proliferate to a greater extent (38). In nearly all applications of cellular IT for cancer, systemic IL-2 has been administered to the recipient. Theoretically, IL-2 serves to promote the



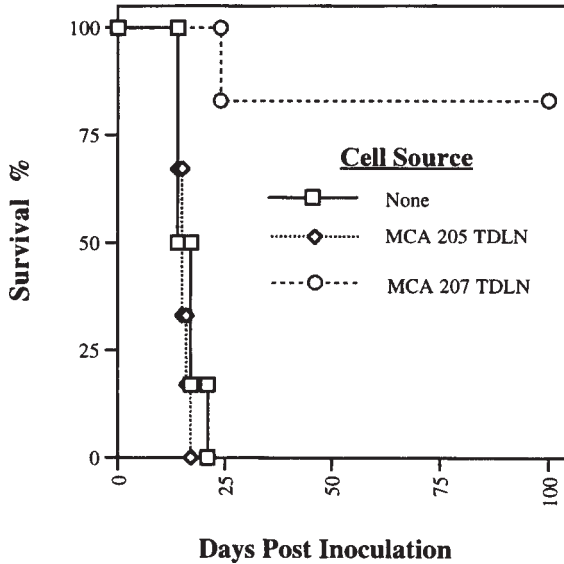
**Fig. 1.** MCA 205 tumors were inoculated intracranially, and mice were treated 3 d later with 5 Gy WBI, followed by systemic adoptive transfer of  $15 \times 10^6$  of the indicated tumor-draining LN cells and monitored for survival. Immobilized anti-CD3 MAb, 1 mcg/mL soluble *Staphylococcal* enterotoxin C2 (SEC2), or 20 ng/mL soluble SEA were added during the first 48 h of ex vivo activation.

survival and function of the transferred cells. However, the anti-CD3- or super-Ag-stimulated T-cells do not require systemic IL-2 after adoptive transfer to function, presumably because tumor-reactive CD4<sup>+</sup> cells are also activated and can provide this cytokine to the CD8<sup>+</sup> cells in vivo. As will be discussed in Section 5, IL-2 has profound effects on the outcome of adoptive IT of BTs.

It is apparent from these preclinical studies that an IR is generated against unique tumor Ags in the tumor-bearing host. Although this response initially fails to control tumor growth, the tumor-reactive T-cells can be artificially manipulated ex vivo to induce differentiation into mature effector cells and to amplify the number of tumor-reactive T-cells. However, there are strict requirements on the activation conditions to produce cells with the highest therapeutic activity, and deviation from the proper sequence fails to produce useful cells.

## 5. UNIQUE ASPECTS OF ADOPTIVE IT OF BTs

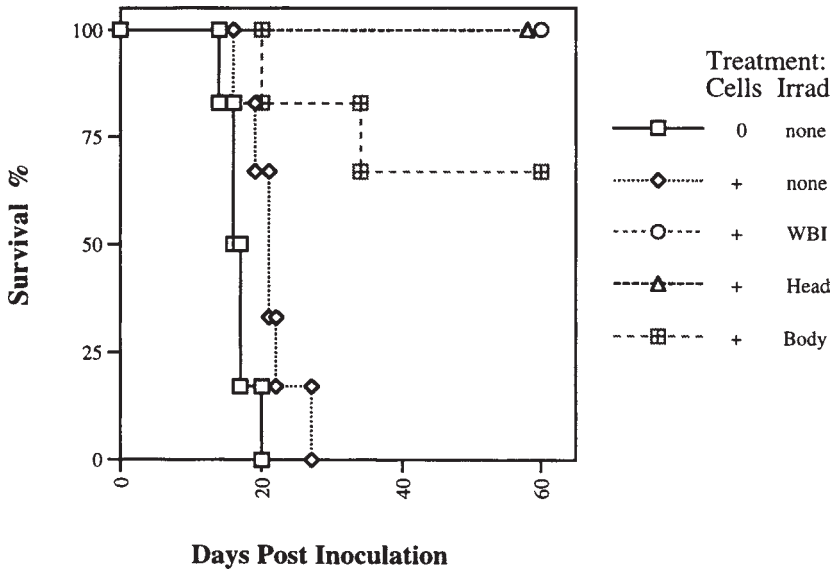
To define parameters that influence the outcome of adoptive IT, the authors used an experimental mouse model of BTs, which was induced by intracranial



**Fig. 2.** MCA 207 tumors were inoculated intracranially, and mice were treated 3 d later with 5 Gy WBI and activated LN cells derived from mice bearing either MCA 205 or MCA 207 tumors. LN cells were activated with staphylococcal enterotoxin C2, and  $15 \times 10^6$  of the indicated cells were transferred intravenously, and survival was monitored.

inoculation of tumor cells (41–43). For many of these studies, two weakly immunogenic fibrosarcomas (MCA 205 and MCA 207) were used. However, these tumors grow as nodules following intracranial inoculation. To provide a model of primary BTs, a murine glioma (GL 261) with an infiltrative pattern of growth in the brain was also used (44). These models demonstrated that the parameters governing the response to adoptive IT in the brain differed in many respects from treatment of tumors at other anatomic sites.

It was initially found that sublethal whole body irradiation (WBI) with 5 Gy of  $\gamma$ -irradiation promoted the therapeutic activity of the transferred T-cells. WBI has numerous effects that could potentially contribute to this response, including direct effects on tumor cells, effects on the tumor vasculature, and effects on the host immune system. To determine which of these effects were predominant, local irradiation of the head or irradiation of the body from the neck down was performed (Fig. 3). The results of these experiments suggested that the local effects of irradiation were predominant, because head irradiation was as effective as WBI, but body irradiation, with shielding of the head, was less effective (45). Direct effects on tumor cells were apparently most important, because preirradiation of the host, prior to tumor inoculation, did not augment the therapeutic effect. Moreover, studies on the migration of transferred T-cells demonstrated that irradiation did not promote trafficking of T-cells into the tumor. WBI



**Fig. 3.** Mice were inoculated intracranially with MCA 205 tumor, and were treated with 5 Gy of either WBI, irradiation of the head only, or irradiation of the body caudal to the neck. Mice also received  $15 \times 10^6$  *Staphylococcal* enterotoxin C2-activated cells intravenously, following irradiation.

alone had only a minimal impact on the length of survival of mice bearing intracranial tumors, and a dose of 5 Gy had a modest effect on *in vitro* proliferation of the tumor lines used for these experiments. Apparently, irradiation made the tumor cells more susceptible to IRs or improved Ag presentation within the tumor.

Regression of intracranial tumors is dependent on the transfer of both CD4<sup>+</sup> and CD8<sup>+</sup> cells (Table 2). In early studies of adoptive IT for pulmonary metastases, systemic IL-2 promoted the therapeutic activity of the transferred anti-CD3/IL-2-activated tumor-draining LN cells. Presumably, IL-2 promotes the survival and function of the adoptively transferred T-cells, and it is routinely used in clinical adoptive IT protocols. It was, therefore, surprising to find that systemic IL-2 abrogated the adoptive IT of intracranial tumors (Fig. 4; 43). This effect was apparent, even if the administration of IL-2 was delayed up to 3 d after T-cell transfer. As discussed in Section 8, this result had a significant impact on the design of the authors' clinical trials.

## 6. IDENTIFICATION OF THE SUBSET OF LN T-CELLS WITH THERAPEUTIC ACTIVITY

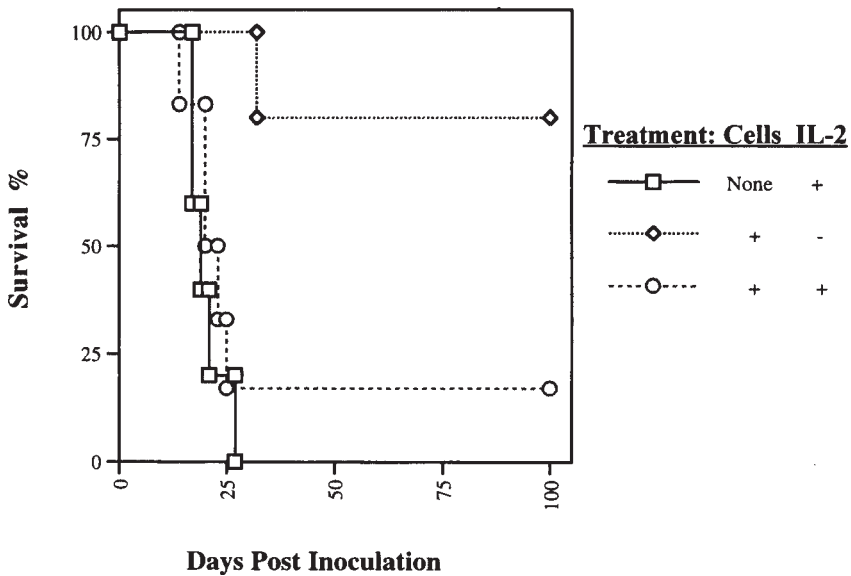
The frequency of T-cells that respond to typical Ags is very low. It is likely that most of the T-cells in the tumor-draining LNs do not have antitumor reac-



**Table 2**  
**Requirement for CD4 and CD8 T-Cells**  
**for Treatment of Intracranial Tumors**

<i>Treatment</i>	<i>Cells</i>	<i>Median survival (d)</i>
None	0	19
Rat IgG	$3 \times 10^7$	>60
Anti-CD4	$3 \times 10^7$	21
Anti-CD8	$3 \times 10^7$	21

Modified with permission from ref. 43.



**Fig. 4.** Mice were inoculated intracranially with MCA 205 tumors, and were treated 3 d later with 5 Gy WBI and intravenous transfer of  $15 \times 10^6$  activated tumor-draining LN T-cells.  $9 \times 10^4$  IU IL-2 was administered intraperitoneally twice a day, for a total of eight doses, starting immediately after cell transfer.

tivity. Consequently, the ability to isolate tumor-reactive cells and eliminate irrelevant cells has theoretical and practical appeal. To pursue this strategy, the authors noted that there was a small increase in the percentage of T-cells with downregulated CD62L (L-selectin) expression in tumor-draining LNs compared with normal LNs. L-selectin is expressed at high levels on the surface of naïve T-cells and facilitates their trafficking to LNs (46,47). The decrease in L-selectin

**Table 3**  
**Efficacy of L-Selectin<sup>low</sup> T-Cells**  
**for Treatment of Intracranial Tumors**

<i>Treatment</i>	<i>Cell no.</i>	<i>Median survival (d)</i>
No cells	0	21
Total cells	$15 \times 10^6$	38
L-selectin <sup>high</sup>	$15 \times 10^6$	21
L-selectin <sup>low</sup>	$2 \times 10^6$	>60

Modified with permission from ref. 50.

expression is thought to permit recently activated T-cells to circulate systemically (48,49). Since downregulation of CD62L molecules on T-cells often signifies recent T-cell activation, the authors tested the therapeutic activity of T-cell subsets that were segregated based on their level of CD62L expression. A minor subset (15–25%) of tumor-draining LN T-cells, with low expression of L-selectin, was separated from the remainder of the LN cells. The L-selectin<sup>low</sup> subset was found to contain all of the therapeutically active T-cells (Table 3; 50). In contrast, the remainder of the LN T-cells with high levels of L-selectin had no antitumor reactivity in vitro or in vivo. The isolated L-selectin<sup>low</sup> cells proliferate more rapidly than the unseparated LN cells. More importantly, there was evidence of partial suppression of the therapeutic cells by the L-selectin<sup>high</sup> T-cells in the unseparated LN cultures, compared with the isolated L-selectin<sup>low</sup> cultures. In addition, the L-selectin<sup>low</sup> cells infiltrated tumors following adoptive transfer to mice with intracranial tumors, while the L-selectin<sup>high</sup> cells did not (44).

## 7. ANTITUMOR FUNCTION OF ADOPTIVELY TRANSFERRED T-CELLS

The specificity of adoptive IT indicates that the T-cells need to encounter Ag in vivo through their TCRs in order to mediate an antitumor response. Experimental evidence also suggests that direct contact of T-cells with either tumor or Ag-presenting cells within the tumor are needed. First, the L-selectin<sup>low</sup> cells with antitumor function infiltrate tumors; the ineffective L-selectin<sup>high</sup> cells do not. Second, the authors have demonstrated that treatment of T-cells with pertussis toxin, which prevents systemically transferred T-cells from leaving the vasculature and infiltrating, abrogates tumor regression. The pertussis toxin-treated T-cells were viable and retained their antitumor function when placed in physical contact with tumor cells, confirming the necessity of trafficking of systemically transferred T-cells.

Once at the tumor site, the T-cells can potentially interact directly with tumor cells or indirectly through Ag-presenting cells. Although the tumor the authors have used for many of the adoptive IT experiments does not express MHC-II molecules, CD4<sup>+</sup> T-cells are required for tumor regression. The authors have demonstrated that transferred CD4<sup>+</sup> cells infiltrate tumors. Therefore, tumor Ag presentation by MHC-II-positive cells within the tumor must be required for the regression of intracranial tumors. An intriguing recent finding is that CD4<sup>+</sup> cells prepared from the L-selectin<sup>low</sup> subset, are, by themselves, sufficient to mediate regression of intracranial tumor (51). This is in contrast to the requirement for both CD4<sup>+</sup> and CD8<sup>+</sup> cells when the total population of tumor-draining LN cells are activated (43). This indicates that, during the required ex vivo activation, there are multiple pathways for the development of effector function; and the effector functions that emerge from ex vivo activation are influenced by the components of the cell mixture. It also suggests additional strategies for regulating the antitumor response by manipulating the conditions of ex vivo activation.

## 8. CLINICAL TRIAL OF ADOPTIVE IT FOR MALIGNANT GLIOMAS

The authors have extrapolated laboratory findings from preclinical studies of adoptive IT of intracranial tumors to develop a strategy for a clinical trial in patients with malignant gliomas (52). For this clinical trial, patients were vaccinated with irradiated autologous tumor cells admixed with granulocyte-macrophage colony-stimulating factor (GM-CSF). \* Vaccine-draining LNs were removed 7–10 d later, and T cells were stimulated with the bacterial superantigen *Staphylococcal* enterotoxin A (SEA). Although SEA is a defined superantigen with the ability to activate selected TCR V $\beta$ -bearing T cells, the authors' experience clearly indicated its mitogenic activity on most, if not all, human T-cells. Activated T-cells were then cultured in serum-free media containing a low concentration of IL-2 (60 IU/mL). In some instances, additional stimulation with anti-CD3 MAb on d 6–8 of culture was performed to induce additional proliferation. This initial clinical trial in patients with recurrent high-grade malignant gliomas established the feasibility of this approach and demonstrated that it was associated with minimal toxicity. Moreover, 3/10 patients had radiographic regression of recurrent malignant gliomas following T-cell transfer, suggesting that this approach had therapeutic effects for some patients.

Autologous tumor cells were used for the tumor vaccine. For greater than 80% of the patients, short-term cultures of autologous tumor cells were success-

\* Editors note: Although there were no reports of experimental allergic encephalomyelitis (EAE) in these BT patients, the potential for EAE has been previously reported with the use of autologous glioma cells and adjuvants (63–65).

fully established. The cultured tumor cells were used for the vaccine, except in instances when a single-cell suspension prepared from the fresh tumor was the only material available. One particular advantage of using the cultured cells was that the vaccine was free of the necrotic debris and accessory cells still present in the initial single-cell suspension prepared from the tumors. As mentioned above, autologous tumor cells were used because a shared tumor Ag has not yet been defined for malignant BTs, and experimental evidence suggests that unique tumor Ags elicit the most potent IRs. GM-CSF was used as an adjuvant because of its documented ability to augment IRs to vaccines and tumors (53–57). The vaccine site was chosen on the upper anterior thigh because of the proximity to inguinal LNs, which can be easily removed using a minor surgical procedure.

All of the patients in the initial clinical trial had recurrent malignant gliomas, and 9/10 of these patients had recurrent glioblastoma multiforme (GBM). In addition, all patients had residual tumor present at the time of treatment. In most cases, masses greater than 2 cm were present. These patients showed variable levels of immunosuppression. Although the patients were not on corticosteroids, two of the patients required two rounds of vaccination to generate LN hypertrophy. Moreover, the yield of LN cells was markedly lower for several of these patients than that from patients with other types of malignancies, (such as malignant melanoma and renal cell carcinoma), treated on similar adoptive IT protocols (58). This suggests that prior corticosteroid use and/or immunosuppressive factors produced by BTs may have blunted the response to the vaccine (59–61).

The sequential activation of LN cells with SEA, anti-CD3, and culture in low-dose IL-2 produced rapid proliferation of T-cells ranging up to 350-fold. The proliferation of T-cells after the initial stimulation with SEA was much more consistent and typically yielded a 10–20-fold increase in cell number. In contrast, the second round of activation with anti-CD3 was much more variable, and, in some cases, did not result in net proliferation. Slight modifications to the culture conditions have recently permitted greater than 50-fold expansion routinely after SEA activation. These culture conditions were for a 9-d period, in low concentrations of IL-2. In many cases, enough LN cells were obtained to generate greater than  $10^{10}$  T-cells after SEA activation. Many patients have now been treated with such short-term cultured T-cells. Not only did the single round of activation with SEA produce a consistent pattern of T-cell growth, but the antitumor efficacy was also preserved. Moreover, there was less opportunity for *ex vivo* clonal selection that is not driven by specific tumor Ags.

The objectives of the initial phase I study of adoptive IT for malignant gliomas were to determine the feasibility and toxicity of this treatment. The authors found that it was feasible to conduct the study even in patients with recurrent malignant gliomas. However, because of the level of immunosuppression and the rapid natural progression of this disease, patients with recurrent tumors were

not optimal candidates for this therapy. The toxicity of the treatment was minimal. Most patients experienced flu-like symptoms consisting of fever, myalgia, and nausea, which typically lasted less than 24 h. Three of the patients developed increased edema at the tumor site 3–4 wk after the T-cell transfer. However, all of these patients were experiencing rapid progression of their disease, and the edema was thought to be secondary to tumor growth. Notably, there were no abnormalities in normal brain tissue that were apparent on serial MRI scans, although no biopsies or autopsies were obtained to specifically look for evidence of EAE. In addition, the patients did not experience any neurologic symptoms besides those related to tumor progression. As demonstrated in Section 5, the preclinical studies, showing that systemic IL-2 administration abrogated the efficacy of adoptive transfer for intracranial tumors, led to its omission from this clinical trial. Consequently, this treatment could be delivered as an outpatient therapy, and the toxicity of the treatment protocol was minimal compared with similar trials of adoptive IT employing IL-2.

The rapid progression of disease in many of the patients with recurrent GBM prevented addressing the question of delayed toxicity. One potential concern is that the autologous vaccination or IR against the tumor could elicit an autoimmune response against normal brain tissue. The lack of apparent toxicity in the authors' recurrent glioma patients stimulated additional studies in patients with newly diagnosed malignant BTs, including several patients with grade 2 and grade 3 astrocytomas and mixed gliomas. None of these patients have developed any signs of neurologic toxicity, with follow-up approaching 1 yr after cell transfer for four of these patients.

Three of the 10 patients with recurrent malignant gliomas had radiographic regression of residual tumor, which lasted 6 mo in two cases and 13 mo in the third patient (Table 4). Because spontaneous regression of recurrent malignant gliomas is rare and the median survival is approx 5 mo, these three patients had evidence of a therapeutic effect compared to historical controls (62). Based on this evidence of a treatment effect in some patients with malignant BTs, a phase II clinical trial of adoptive IT for newly diagnosed GBM has been initiated.

## 9. CONCLUSIONS

The immune system can generate a T-cell response to unique tumor Ags, even in the tumor-bearing host. Vaccination with autologous tumor cells and GM-CSF as an adjuvant stimulates a response in draining LNs. Ex vivo activation of LN T-cells induces differentiation to effector function and rapid proliferation. Adoptive transfer of these T-cells to recipients with BTs induces tumor regression that is immunologically specific. Several features of adoptive IT for intracranial tumors differ markedly from the treatment of tumors at other anatomic sites, such as the lung and skin. These findings have contributed to a

**Table 4**  
**Clinical Outcome of Patients with Recurrent Malignant Gliomas Treated with Adoptive IT**

<i>Pt no.</i>	<i>Cell dose</i>	<i>Response (duration)</i>
1	$1.5 \times 10^{11}$	PD
2	$1.7 \times 10^{10}$	PD
3	$3.3 \times 10^9$	PR (6 mo)
4	$2.7 \times 10^9$	PD
5	$1.0 \times 10^{10}$	PD
6	$5.7 \times 10^9$	PD
7	$6.0 \times 10^{10}$	PR (13 mo)
8	$9.5 \times 10^8$	PD
9	$5.6 \times 10^{10}$	PR (7 mo)
10	$1.7 \times 10^{10}$	PD

PD, progressive disease; PR, partial response.  
 Modified with permission from ref. 52.

strategy for clinical trials of adoptive IT in patients with malignant BTs. This therapy is feasible and is associated with minimal toxicity. Moreover, the omission of systemic IL-2 from this clinical trial has permitted the entire therapy to be delivered in the outpatient setting. Initial clinical experience was obtained in patients with recurrent tumors; treatment of newly diagnosed patients following surgery and radiation therapy may permit therapeutic responses to be more readily observed. Ongoing studies to determine the response rate and duration of response will help ascertain whether adoptive IT could provide additional therapeutic benefits when combined with the standard therapies for GBM.

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## Cytotoxic T-Lymphocytes Reactive to Patient Major Histocompatibility Complex Proteins for Therapy of Brain Tumors

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*Carol A. Kruse, PhD and David Rubinstein, MD*

### CONTENTS

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### 1. CELLULAR THERAPY OF GLIOMAS

In patients with primary malignant brain tumors (BT), tumor growth has defied conventional therapies comprised of surgery, radiation, and chemotherapy (1–3). The growth and expansion of tumor cells throughout normal brain neuropil often cannot be contained without destroying normal brain tissue. Immunotherapy (IT) strategies became of interest to neuro-oncology researchers, because regeneration of normal brain is considered limited, at best, and because the potential for selectivity in tumor over normal cell destruction is seemingly inherent in the immune system.

One experimental cancer IT strategy employed early on for BT patients entailed adoptive IT, a classic form of passive immune therapy involving transfer of isolated lymphocytes back into the tumor host. Regional adoptive IT was considered a viable option, because researchers felt that immune cell infiltrates into the brain, following peripheral administration, might be limited because of the blood–brain barrier.

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The cellular therapy of gliomas began with nonactivated lymphocytes. As the IT field progressed, nonspecifically activated lymphocytes were used. Finally, the ability to treat gliomas with specifically activated lymphocytes was developed. Further refinement led to development of alloreactive cytotoxic T-lymphocytes (CTLs) and their clinical use in a limited number of glioma patients.

### ***1.1. Cellular Therapy of Gliomas with Nonactivated Lymphocytes***

The first attempts at adoptive cellular therapy involved placement of autologous, but nonactivated, immune cells directly into the central nervous system (CNS), either intratumorally (4–7) or intrathecally (8–10). Local adoptive therapy was later combined with the cytokine, interferon, with the hope of engendering a synergistic effect (11,12). Although well-tolerated, no clinical benefit was apparent, and this decreased enthusiasm for cellular therapy in this subset of cancer patients.

### ***1.2. Cellular Therapy of Gliomas with Nonspecifically Activated Lymphocytes***

When the recombinant form of T-cell growth factor, interleukin-2 (IL-2), became available in suitable quantities (13), and especially when a subset of cells known as lymphokine-activated killer (LAK) cells were identified (14), a resurgence in cellular therapy for BTs ensued. LAK cells are lymphocytes nonspecifically activated in short-term culture with IL-2. They are heterogeneous preparations identified to contain large numbers of natural killer (NK) cells (15) and lyse tumor in a non-major histocompatibility complex (MHC)-restricted fashion. The intratumoral placement of autologous LAK cells and other effector preparations nonspecifically activated with lectin were clinically tested in a plethora of trials conducted with small numbers of BT patients at multiple institutions (16). Reasonable clinical results were obtained when LAK cells and IL-2 were given multiple times over an extended period (17). In a trial of 19 patients, (15 glioblastoma multiforme [GBM] and 4 anaplastic astrocytoma [AA]), two complete (1 AA, 1 GBM) and two partial (2 GBM) responses were obtained. The median survival for the GBM patients was 53 wk after reoperation compared to 25.5 wk for 18 GBM patients that were reoperated and treated with chemotherapy. Eight of the 15 GBM patients treated with IT survived more than 1 yr (53%) while only 1/18 treated with chemotherapy was alive at 1 yr (<6%). Although there seemed to be a significant increase in GBM survival with IT, because 6/15 GBM patients had additional surgery or biopsy and chemotherapy after IT, the contribution of subsequent treatment to the increased survival cannot be determined (17).

Improvements to cellular therapy are now being investigated by coupling effector cells with targeting agents, such as bispecific antibodies (Ab) (18–20). For instance, 8/10 patients treated with local cellular therapy using LAK cells coated with bispecific Abs had a remarkable clinical effect (18). Using an anti-

CD3 monoclonal Ab chemically conjugated to antiglioma monoclonal Ab, 4/10 patients with malignant gliomas showed regression of tumor, and, in another four patients, computed tomography (CT) and histology suggested eradication of the glioma cells left behind after surgery. No recurrence was detected in 10–18 mo of follow-up (18).

### ***1.3. Tumor-Specific Sensitization of CTLs***

In theory, tumor-sensitized cytotoxic CTLs (of CD3<sup>+</sup>/CD8<sup>+</sup> phenotype) are the ultimate effector cells to use for providing specificity. For BT therapy, generation of tumor-specific CTLs requires the recognition of antigen (Ag) expressed by tumor cells, but not by normal neurons or glial cells. They should be capable of trafficking to and directing specific destruction of tumor cells (21).

#### **1.3.1. *IN SITU* SENSITIZATION**

The failure of the host immune system to mount an effective antitumor response in the brain is a result of several factors: the “immuno-privileged” nature of the brain (22), the secretion of immunosuppressive factors by gliomas (23,24), and the inability of microglia to stimulate a primary immune response (25). In addition, following specific activation, tumor-reactive lymphocytes must home to the tumor site and retain their activated state in numbers sufficient to overcome tumor growth.

Although glioma-associated Ags have been identified (26), to date there has been no identification of specific peptides generated from these or other molecules that are recognized by glioma-specific CTL in the context of MHC. Tumor-specific CTLs have been isolated from tumor-infiltrating lymphocytes (TIL) or tumor-draining lymphocytes (TDL). TILs are obtained from the tumor following surgical resection (27–30). They are then stimulated and expanded *ex vivo* prior to reintroduction into the host. Although the ability of TILs to recognize and lyse gliomas has been demonstrated *in vitro*, little efficacy has been demonstrated *in vivo* (31). In contrast, several groups have shown partial responses or cures *in vivo* with TDLs in preclinical rodent studies. TDLs are isolated following immunization with irradiated glioma cells. Isolated lymphocytes are then restimulated *in vitro* prior to adoptive transfer into the host. Several groups have subsequently demonstrated the efficacy of this protocol in animal studies (32–43).

#### **1.3.2. *EX VIVO* SENSITIZATION**

One limitation, for *ex vivo* sensitization of autologous precursor CTLs for glioma therapy, is tumor tissue. Tumor is not always resectable, dependent on its intracranial location. If it is, it often is partially necrotic because it outgrows

its blood supply or because of antitumor treatment(s) the patient has received. Either way, necrosis is a quality that will diminish the CTL response. If autologous tumor is obligatory for generating the biologic, many patients will not be eligible for such a therapy. Additionally, if tumor-specific Ags are minor Ags, the number of responding cells in a mononuclear cell pool will be low (44), regardless of whether sensitization proceeds *ex vivo* or *in situ*. If autologous responder cells are employed, the decreased number of CD4<sup>+</sup> helper cells in BT patients (45,46) could likely be overcome with an exogenous supply of IL-2 if sensitization proceeds *ex vivo*. However, *in situ* sensitization could be inhibited if the suppressor T-cell compartment is predominant.

Use of allogeneic tumors for sensitization would be practical, especially if common tumor Ags existed among gliomas. Recently, investigators have identified several glioma-associated Ags (26), some of which are shared with melanoma (47). This is not surprising because both gliomas and melanomas are neuroectodermal in origin.

#### ***1.4. Cellular Therapy with Specifically Activated Lymphocytes***

Kitahara et al. (48) were one of the first groups to investigate the use of tumor-sensitized CTLs for therapy. They co-cultured patients' lymphocytes with inactivated allogeneic tumor cells. Five patients were treated, and two responded to the treatment, which consisted of a series of intratumoral implants of autologous CTL. Presently, phase I clinical trials have been initiated with CTLs generated from tumor-draining lymph nodes after host immunization with tumor cells (36,49,50).

## **2. PRECLINICAL STUDIES WITH ALLOREACTIVE CTLs**

### ***2.1. In Vitro and In Vivo Studies Using Alloreactive CTLs for Treatment of Rat Gliomas***

Histocompatibility Ags are expressed at a high relative Ag density on lymphocytes and are also expressed on BTs (29,51,52). In contrast, histocompatibility Ags are not expressed on the majority of normal human brain cells, including neurons, astrocytes, and oligodendrocytes (22,53,54). Thus, tumors bearing host histocompatibility Ags should be selectively targeted for kill by allogeneic CTLs sensitized to MHC. Once alloreactive CTLs are administered into the brain, an immunologically semi-privileged site, they should have the ability to migrate through brain parenchyma to reach individually penetrating tumor cells and lyse them before they are targeted by the host immune system for destruction.

In preclinical work, alloreactive CTLs were shown to have features that would give them added advantages for cellular therapy over nonspecifically activated

lymphocytes. The authors demonstrated that alloreactive rat CTLs lyse rat glioma cells more efficiently than tumor-sensitized syngeneic CTLs *in vitro* (52). Furthermore, the authors demonstrated that alloreactive CTLs were effective *in vivo* in modified Winn assays (55) and in assays in which rats had established gliomas (56,57). Alloreactive CTLs also display movement through brain parenchyma, which would give them the opportunity to make contact with disseminating tumor cells (58).

## ***2.2. In Vitro Studies Using Human Alloreactive CTLs to Lyse Human Gliomas***

Studies were expanded to investigate methods for optimally generating human alloreactive CTLs with the desired functionality against human BT targets (59). Mononuclear cells derived from patient blood were expanded many-fold in hollow fiber bioreactors after OKT3 (Muromonab-CD3) and IL-2 stimulation. Mononuclear cells from healthy, human leukocyte antigen (HLA)-mismatched donors were isolated from leucopaks, a byproduct of apheresis procedures. The responding HLA-mismatched allogeneic cells were co-incubated with  $\gamma$ -irradiated sensitizing lymphocytes from the patient. Alloreactive CTLs developed over 2–3 wk in hollow fiber bioreactors perfused with medium containing a low concentration of IL-2. Their expansion usually resulted in largely a CD3<sup>+</sup>/CD8<sup>+</sup>/CD11a<sup>+</sup> lymphocyte preparation, although on occasion, CD3<sup>+</sup>/CD4<sup>+</sup> predominance occurred. In studies in which these cells were tested for their ability to lyse relevant tumor or concanavalin A blasts made from sensitizing and responder lymphocytes, the alloreactive CTLs were shown to lyse patient tumors or targets displaying HLA Ags matching those of the patient. Therapeutically usable numbers of human alloreactive CTLs could be generated in the hollow fiber bioreactors.

## **3. CLINICAL EXPERIENCE WITH ALLOREACTIVE CTLs**

### ***3.1. Intralesional Placements into Brain Parenchyma***

Based on the authors' *in vivo* and *in vitro* preclinical findings, approval was obtained to conduct a phase I clinical trial to test multiple intracavitary placements of alloreactive CTL and IL-2 for recurrent malignant glioma (BB-IND-5423). The study employed inactivated stimulator lymphocytes from BT patients and responder lymphocytes from healthy unrelated donors. Initially, the alloreactive CTLs were implanted into the resected tumor bed at surgical debulking. Subsequent infusions of CTLs were given through a subgaleal Rickham reservoir/catheter system installed at surgery. This chapter, as well as a prior report (60), describes the toxicity and long-term follow-up of patients who received this immune treatment.



### **3.1.1. IMMUNE TREATMENT SCHEMA AND CYCLES**

Up to five treatment cycles were possible. Each cycle involved 2–3 intracranial infusions of alloreactive CTLs within a 2-wk period. A different immunologically distinct donor was used at each treatment cycle. If the patients' tumors were stable or responding to therapy, as judged by magnetic resonance imaging (MRI) scans and neurological examinations, they could continue to the next treatment cycle. Treatment began at the time the patients received their first intracavitary implantation of alloreactive CTLs and IL-2, which usually coincided with the day of surgical debulking for tumor.

The overall schema called for expansion of patient lymphocytes the first week, then sensitizing and expanding alloreactive CTL cultures the second and third weeks. Patients underwent cranial surgery, and alloreactive CTLs were implanted by a blunt brain cannula directly into the resected tumor bed. The alloreactive CTLs were suspended in a 2–8 mL volume of Hank's balanced salt solution containing IL-2 (60,000 IU). Subsequent alloreactive CTL/IL-2 infusions were placed into the resected tumor bed through a reservoir–catheter system that was installed at the time of surgery. The patients were observed for 24 h postoperatively after CTL infusions.

### **3.1.2. MONITORING OF PATIENTS**

Patients were monitored for CNS and systemic toxicity by neurological exams and MRI scans. Each patient was examined 2 wk following hospital discharge, then every other month at the General Clinical Research Center floor of the University of Colorado Hospital. Systemic and CNS toxicity was determined by the National Cancer Institute (NCI) Common Toxicity criteria and was monitored for a minimum of 24 h subsequent to all CTL infusions. Toxicity for graft-vs-host reaction also was monitored at the beginning of each treatment cycle.

### **3.1.3. ISOLATION AND EXPANSION OF PATIENT PERIPHERAL BLOOD LYMPHOCYTES USED FOR SENSITIZATION**

Anticoagulated whole blood was diluted in buffered saline, layered over a density gradient medium, and centrifuged to isolate the mononuclear cells. To expand sensitizing cells, patients' mononuclear cells were suspended at  $10^7$  cells/mL in clinical-grade AIM-V synthetic medium containing gentamicin, 5% autologous serum, OKT3 (50 ng/ $10^7$  cells), and IL-2 (240 IU/mL). The cells were injected into the extracapillary space (ECS) of a preconditioned hollow fiber bioreactor, which acts as a continuous growth system. This was perfused at a rate suitable to allow the perfusion volume to be doubled every 1–3 d. By adding fresh AIM-V medium containing only IL-2 each time, the cells were gradually weaned from serum-containing medium. Lactic acid concentration was measured daily, and the rate of lactate production was plotted. Fresh medium was added every 4–5 d, or when the lactic acid concentration was extrapolated

to be above 0.5 g/L when measured 24 h later. Lactic acid production paralleled the expansion rate of the cells (59,60).

Cells were harvested from the ECS of hollow fiber units. Repeated harvests from a single artificial capillary culture (every 4–5 d) provided all the stimulator cells needed for the alloreactive CTL generation for a patient receiving five treatment cycles. Patient cells were cryopreserved in autologous serum containing 15% dimethyl sulfoxide (1 : 1 v/v). Cells were stored in liquid nitrogen until needed for sensitization. These cells maintained good viability (78–95%), when tested as late as 9 mo postcryopreservation, a time-point required for a possible 10-mo treatment period.

### 3.1.4. PROPAGATION OF ALLOREACTIVE CTLs AND QUALITY CONTROL ISSUES

Donor leucopaks (testing negative for HIV I/II, HTLV I/II, hepatitis B surface Ag, hepatitis B core Ab, hepatitis C Ab, and syphilis) were used as sources of responder cells. Donors were allogeneic to the patient and differed in at least three HLA-AB locus Ags (or two, if donors were homozygous at one allele). In 10/17 donors, a complete disparity at class I HLA-AB alleles was achieved in relation to the patient; 7/17 donors had one HLA-AB Ag in common (Table 1). Ags in common between patient and donor are indicated in Table 1 (shaded blocks).

The leucopaks generally contained  $1-5 \times 10^8$  mononuclear cells. The cells were isolated by density gradient centrifugation and washed several times with a balanced salt solution (59,60).

The patient's stimulator lymphocytes were washed, then irradiated (2500 Rads), before combining with allogeneic responder lymphocytes (i.e., one-way mixed lymphocyte culture). The lymphocytes were combined at responder-to-stimulator ratios of 5 : 1–1 : 1. They were placed into hollow fiber bioreactors and cultivated at 37°C with 5% CO<sub>2</sub> for 6–10 d with AIM-V medium containing 5% autologous donor serum and 60 IU IL-2/mL. As before, feeding was determined from daily plotted measurements of lactic acid concentration. CTL cultures in the ECS were left undisturbed for 5–7 d, then gentle mixing of the cells within the ECS occurred every 2–3 d. If low lactate rates were observed, cells were restimulated with OKT3 and cultured for an additional 1–2 wk.

To assure sterility of the harvested cells, aliquots of the alloreactive CTLs from each bioreactor were sent to the hospital clinical laboratories for bacteriological and fungal cultures 2–3 d before administration. On the day of administration, aliquots of the CTL infusates were again sent for culture, for limulus amoebocyte lysate assays for pyrogen, and for mycoplasma staining.

### 3.1.5. RESULTS

**3.1.5.1. Patient Profiles.** Six recurrent glioma patients (ages 26–46 yr) were treated. Accrual began in January 1995. Five/six patients had completed treatment by March 1996. A sixth patient began treatment in January 1998. Follow-

Table 1  
HLA Types of Patients and Donors

Patient	BTP1		BTP2		BTP3					BTP4		BTP5					BTP6				
	1	2	1	2	1	2	3	4	5	1	2	1	2	3	4	5	1				
HLA type	SELF	Donor 1	Donor 2	SELF	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	SELF	Donor 1	Donor 2	SELF	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	SELF	Donor 1	
A1																					
A2	X		X	X							X		X				X	X		X	
A3				X	X				X		X				X					X	
A11		X							X												
A24						X															
A26		X							X							X	X				
A28																			X		X
A29																X				X	
A31										X										X	
A32										X										X	
A33			X		X																
B7		X	X	X					X		X			XX					X		
B8						XX							X								
B13							X	X		X											
B14										X											
B17												X							X		
B18						X															
B27																X	X				
B38															X						
B41																X					
B44	X			X				X					X							X	
B49										X											
B50																					X
B51									X												
B58			X		X										X						
B60	X	X																		X	
B62								X													
B63									X						XX						

**Table 2**  
**Immune Therapy/Status**

<i>Patient no./ tumor</i>	<i>Cycle no./ infusions</i>	<i>Time to tumor progression/status</i>
BTP1/GBM	1/2 2/1	TTP 3 mo, recurrence at distant site, died 4 mo
BTP2/GBM	1/2 2/2	TTP 3 mo, local tumor recurrence, died 4 mo
BTP3/AODG	1/3 2/2 3/2 4/2 5/2	TTP 32 mo, died 40 mo
BTP4/AODG	1/3 2/1	Withdrew from study, alive with stable disease at 62 mo from the start of IT
BTP5/AA	1/2 2/2 3/2 4/5 5/3	Completed protocol, alive with stable disease at 62 mo from the start of IT
BTP6/GBM	1/2	TTP <1 mo, died 1 mo

Times to tumor progression (TTP) and death are given from the start of IT; GBM, glioblastoma multiforme; AODG, anaplastic oligodendroglioma, AA, anaplastic astrocytoma.

up on all patients was to January 2000. With toxicity as the primary concern, patients with a variety of histologic types were allowed to enroll. The pathologic diagnoses included three GBMs, two anaplastic oligodendrogliomas (AODGs), and one AA. All had failed treatment consisting of one or more surgeries and radiation (5000 cGy), at the minimum. All but two had failed chemotherapy, and two had also received radiosurgery treatment (60).

**3.1.5.2. IT Cycles/Status.** Patient data (number of cycles and infusions of alloreactive CTL received per cycle) are summarized in column 2 of Table 2; the current status of the six patients is summarized in column 3. Of the five treatment cycles that were possible, only two patients (BTP3 and BTP5) completed the entire series. All three glioblastoma patients (BTP1, BTP2, and BTP6) were deceased by the time of this writing. BTP1 was removed from the protocol during the second treatment cycle, when tumor growth became apparent in the right cerebellar area, which was far removed from the initial recurrence and immune treatment site in the left frontal lobe. BTP2 had local tumor progression during the second treatment cycle and died at 4 mo post-IT. Tumor continued to progress in BTP6, who died 1 mo following entry into protocol. BTP3, with an AODG,

did well until tumor progression was noted at 32 mo; he died at 40 mo from the start of IT. Patient BTP4, also with an AODG, experienced side effects during the second treatment cycle and withdrew from protocol. She is still alive, with no evidence of tumor progression at 62 mo from the start of IT. BTP5, with an AA, is alive at 62 mo from the start of IT. This individual is working and has no signs of tumor recurrence.

**3.1.5.3. Short-Term and Long-Term Toxicity.** Toxicity was monitored using the NCI Common Toxicity Scale at the beginning of each treatment cycle and during the 24-h period immediately following each CTL infusion. Individual patients received a range of 2–14 total CTL infusions. In general, treatment was well tolerated. The highest-grade toxicity that occurred at any point during the entire treatment period was reported, although patients may have only experienced it once or twice during that total time. Toxicity between grades 0 and 3 was observed either transiently (resolved within 24 h) or for more prolonged periods (3–5 d). The glioblastoma patients (BTP1, BTP2, BTP6; *see* Table 2) did not experience any toxicity above a grade 2. All others (with recurrent Kernohan grade III tumors) experienced grade-3 toxicity at least once during treatment. Patients experienced one or more of the following: fever, headache, nausea, vomiting, or slight neuromotor toxicity. Additionally, one patient (BTP4) had a temporary increased expressive aphasia following one infusion. Another patient (BTP3) had recurrent seizures, which were prevented by increasing his phenobarbital level. The toxicities observed early in the trial were reduced when the authors began premedicating the patients with acetaminophen and antinausea agents. Nevertheless, the transient side effects observed from intraparenchymal placement of alloreactive CTLs were tolerable.

Patient well-being over the long-term was assessed by neurological exams and Karnofsky Performance Scale ratings, which was determined for each patient prior to the first infusion of each treatment cycle. The first rating was baseline. For the Kernohan grade III tumor patients, who were the ones surviving long enough for this to be recorded, the Karnofsky scores of the treated individuals were maintained or increased during treatment, which indicated that the quality of survival was not adversely affected by treatment for prolonged periods.

During protocol review, there was concern about the possible development of a graft-vs-host reaction occurring from multiple placements of alloreactive CTLs. No symptoms indicating development of this reaction were recorded.

**3.1.5.4. Alloreactive CTL Infusate Numbers, Phenotypes, and HLA Types.** The total number of alloreactive CTLs received by individual patients ranged from  $10^8$  to  $5.2 \times 10^9$ . The wide range resulted from the varying number of treatment cycles received, the variable starting numbers of precursor donor cells (ranging from  $1-5 \times 10^8$  in the leucopaks), and the varying proliferative responses obtained by the donor and recipient lymphocytes. Donors differed by at least 2–3 HLA-AB loci (Table 1). However, the differentials in HLA type that result

in good proliferative and cytolytic responses are unknown at this time, and deserve further study. The CTLs used for therapy were generally between 14 and 21 d in culture (range 11–35 d). As a result, the phenotypes also had a wide range of CD3<sup>+</sup> (42–98%) and CD8<sup>+</sup> (30–89%) cells. In general, the CD11a/CD8 cytotoxic T-cell dual markers sorted with the CD8/CD3 markers. These results indicate that the suppressor cytotoxic T-cell population is expanded during culture. The percentage of NKH-1<sup>+</sup> cells in the CTL infusates also varied widely (7–65%), as did the percentages present in the donor leucopaks (9–53%).

To see if a humoral response developed to repeated exposure to allo-Ags, the percent reactive Ab of patients was measured at the beginning of each treatment cycle. Only patient BTP3 developed a reactive Ab to HLA-A2 Ag. A2 was present on the cells of several of his alloreactive CTL donors (Table 1). Even though BTP3 was exposed to the B8 allele multiple times, he did not develop a reactive Ab to it. Since the authors cannot rule out that his anti-A2 was not an anamnestic response, this cannot be totally attributed to the IT. None of the other patients developed a reaction to foreign alleles, even when they were exposed to them multiple times (Table 1).

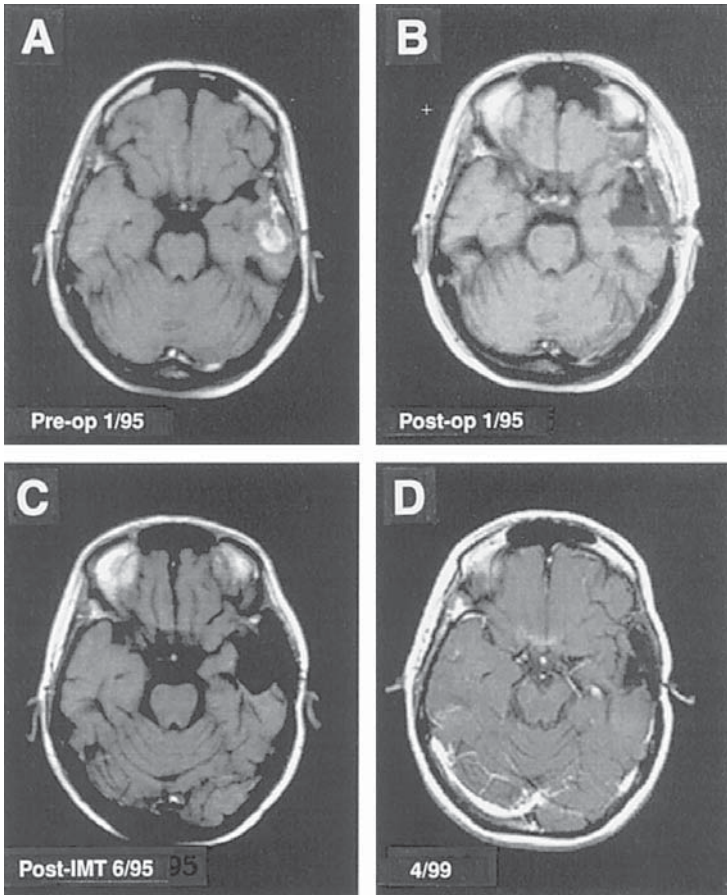
**3.1.5.5. Neuroimaging and Follow-Up.** The gadolinium-enhanced MRI of all patients, upon entrance into this study, had to show unequivocal evidence of tumor progression when measured and compared to a prior scan.

Follow-up MRI scans from patient BTP3 (AODG), who completed all five treatment cycles, are given in ref. 60. The scans span the time frame from January 1995 through December 1996. Patient BTP3 did very well following five treatment cycles. He returned to work until symptoms of recurrent tumor, confirmed by MRI scans, appeared at 32 mo. He survived 40 mo post-IT before succumbing to tumor growth.

Follow-up MRI scans from two patients, BTP4 (AODG) and BTP5 (AA), who are alive with stable disease at >62 mo post-IT, are shown in Figs. 1 and 2, respectively. These series of MRI scans, shown at approximately the same level, are the preoperative scan showing tumor recurrence, the postoperative scan, the scan subsequent to finishing immune treatment, and a recent follow-up scan.

MRI scans of BTP4 were obtained over the period from January 1995 through April 1999 (Fig. 1). The study entry preoperative MRI (January 1995) showed enhancement of tumor medial and posterior to the original operative bed. A postoperative MRI (January 1995) showed minimal enhancement, but moderately increased signal on the proton density and T2-weighted scans surrounding the operative bed. This scan was prior to the alloreactive CTL infusions of the first treatment cycle administered March 1995. The post-IT MRI scan of April 1995 (not shown) demonstrated a marked decrease in the abnormal signal on the T2-weighted images and only minimal enhancement. The June 1995 MRI scan had a persistent, mildly abnormal signal on the proton density and T2-weighted images, increased enhancement in the left temporal horn choroid

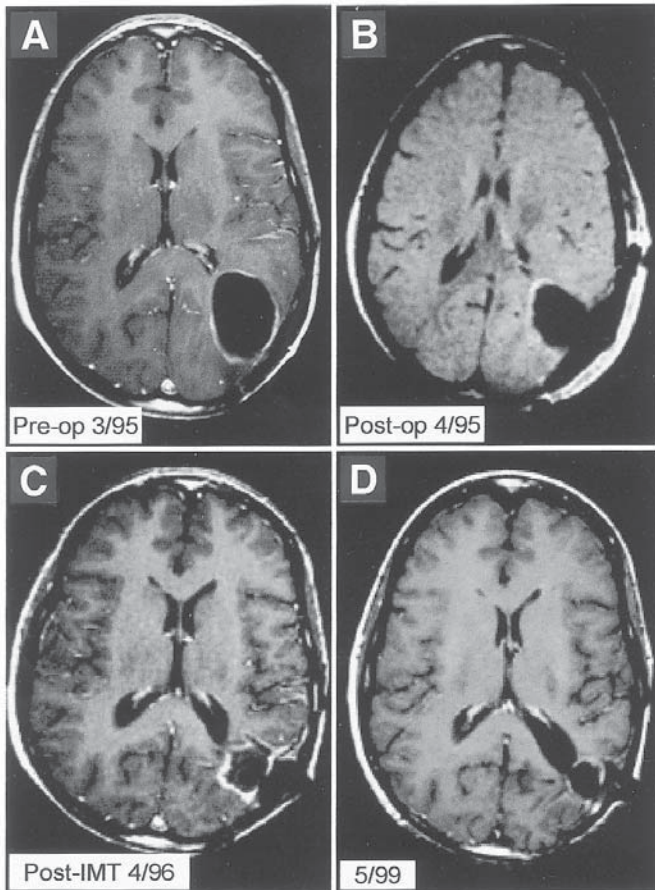




**Fig. 1.** Gadolinium-enhanced MRIs of patient BTP4 were obtained from January 1995 through April 1999. (A) The January 1995 preoperative MRI showed enhancement of tumor medial and posterior to the original operative bed at study entry. (B) The January 1995 postoperative MRI showed minimal enhancement at the margin of the resection site. Blood layered in the posterior aspect of the operative defect. (C) The June 1995 post-IT scan demonstrated minimal enhancement at the margin of the operative bed. (D) Follow-up scan, in April 1999, showed no enhancement around the operative bed and the increased enhancement of the choroid plexus in the left temporal horn, which developed during treatment.

plexus, but minimal enhancement around the operative bed. A follow-up scan of April 1999 showed that there was no enhancement at the margin of the operative bed, and that the mildly increased choroid plexus enhancement was stable. Over the follow-up period, the abnormal signal on the proton density and T2-weighted scans mildly decreased, with only a minimal amount of abnormal signal remaining. Fluid attenuated inversion recovery (FLAIR) scans, which have





**Fig. 2.** Gadolinium-enhanced MRIs of patient BTP5 were obtained from March 1995 through May 1999. (A) Performed at study entry, the March 1995 preoperative MRI showed peripheral enhancement of tumor around the original operative bed. (B) April 1995 postoperative MRI showed some remaining enhancement in the margin of the resected tumor bed. During and subsequent to treatment, enhancement at the periphery of the operative bed increased, probably related to inflammation/cytokine production. (C) Enhancement and size of the operative bed had decreased by the April 1996 post-IT MRI. (D) Follow-up MRI, performed in May 1999, demonstrated a persistent but decreased rim of enhancement around the shrinking operative bed.

T2-weighting, but in which cerebrospinal fluid (CSF) has no signal, were obtained at this patient's most recent follow-up scans in June and November 1998 and April 1999. The FLAIR images better demonstrate the abnormal signal adjacent to the CSF cavity left by surgery. The April 1999 MRI had mildly decreased abnormal signal on FLAIR images, compared to the June and November 1998 scans, but there was no other change.

Scans of patient BTP5 over the five-cycle treatment period are shown from the period between March 1995 and May 1999 (Fig. 2). The preoperative MRI scan (March 1995) demonstrated recurrent peripheral enhancement around a prior right occipital surgical cavity. The postoperative scan (April 1995) showed lessening of this enhancement. The enhancement has persisted, but has continued to decrease, as seen in a postimmune treatment MRI scan (April 1996) and in the most recent follow-up scan (May 1999). Since treatment, the size of the operative bed has slowly but steadily decreased, and the increased signal on the T2-weighted scans surrounding the operative bed has remained stable.

At 62 mo from the start of IT, recurrent BT patients BTP4 (who withdrew from the protocol during the second treatment cycle) and BTP5 (who completed all five treatment cycles) were stable without requiring further treatment. The MRI scans show that BTP5 (AA) has persistent but stable enhancement of her tumor with decreased mass effect. She is doing well, and is at work. Patient BTP4 (AODG) also has stable MRI scans. Patient BTP3 (AODG) recurred at 32 mo post-IT and succumbed to tumor at 40 mo. Thus, 3/6 patients, all with grade III recurrent gliomas, did well after being treated locally by adoptive cellular therapy with alloreactive CTLs. Follow-up on two of these patients is continuing.

### ***3.2. Intrathecal Placements of Alloreactive CTL into CSF***

Several patients were entered into the alloreactive CTL trial after obtaining a compassionate waiver. They received the biologic intrathecally.

#### **3.2.1. CASE REPORT OF INTRATHECAL PLACEMENT OF ALLOREACTIVE CTL IN AN ADULT WITH GBM**

A 43-yr-old male GBM patient received 1.5 treatment cycles of alloreactive CTLs by lumbar injection. His HLA type was A28; B44,60. The donor for his first treatment cycle was A1,3; B8,51. For his second treatment cycle, the donor was A1,3; B7,18. He received a total of  $4.6 \times 10^8$  alloreactive CTLs in two separate injections at the first cycle, and received  $15 \times 10^8$  CTL in one injection at the second treatment cycle. Within 8 h after each CTL infusion, he reacted with a sustained (>24 h) grade III toxicity by NCI Common Toxicity criteria for headache, fever, and nausea. The symptoms were described as similar to those seen with patients experiencing chemical meningitis. Adverse event reports were filed, and IT was halted. The authors made the assumption that the biologic administered into the CSF caused these potent side effects, which might not have been otherwise obtained when given directly into the brain parenchyma.

#### **3.2.2. CASE REPORT OF INTRATHECAL PLACEMENT OF ALLOREACTIVE CTL IN A PEDIATRIC PATIENT WITH EPENDYMOMA**

**3.2.2.1. Prior Therapy.** A girl was diagnosed at age 13 mo with an ependymoma in the posterior roof of the fourth ventricle. By age 7 yr, the girl had

undergone prior aggressive treatment comprised of five surgeries (four debulking and one for intraventricular shunt modification), a year of high-dose chemotherapy (cyclophosphamide, cisplatin, carboplatin, etoposide, vincristine), and a course of radiation therapy (5400 cGy). In January 1997, recurrent tumor was debulked. In April 1997, the MRI scan showed recurrence in a new, remote site in the anterior part of the left lateral ventricle. A compassionate waiver was obtained to treat the girl with alloreactive CTL and IL-2 on the trial currently accruing adults with recurrent gliomas (60). In May 1997, the girl received radiosurgery to the enhancing lesion present on the ependymal surface of the left lateral ventricle, because surgical debulking was not an option. This was followed 2 wk later with IT.

**3.2.2.2. Treatment.** Two weeks following radiosurgery, intrathecal administration of alloreactive CTLs with 60,000 IU/mL IL-2 was given, followed 3 and 9 d later with two more injections of CTL/IL-2 (cycle 1 =  $7.4 \times 10^8$  total CTL). Cisternal and lumbar injections were alternated. She underwent a second treatment cycle approx 8 wk later, consisting of two intrathecal injections of alloreactive CTLs made from a different MHC-disparate donor (cycle 2 =  $10^8$  total CTL). A third treatment cycle, given another 8 wk later, consisted of three CTL injections (cycle 3 =  $21.5 \times 10^8$  total CTL). These infusions were given through a Rickham reservoir directed toward one of two enhancing lesions.

**3.2.2.3. Toxicity Observations.** Toxicity at grades 0–2 (NCI Common Toxicity Scale) were observed during the first 24 h following administration of the alloreactive CTLs. Specifically, fever at grade 1 and headache at grade 2 were recorded at least once. All symptoms resolved within the 24-h period following CTL administration. The Lansky performance criteria, an indication of well-being over the long-term, did not go below 90 during the 5-mo period in which the three treatment cycles were given.

**3.2.2.4. Correlative Laboratory Experiments.** The patient had a ventriculoperitoneal shunt, either engaged or disengaged, when alloreactive CTLs were administered. To help determine how long the alloreactive CTLs remained in the CSF and/or if the patient produced an endogenous immune reaction to treatment in this immune-privileged site, CSF was withdrawn prior to CTL injections, for analysis of the immune cells present. CSF withdrawn before the first injection of each treatment cycle served as a baseline. On three separate occasions (at d 3, 5, and 6 following alloreactive CTL administration), CSF cells were analyzed when the shunt was operational. On two occasions (at d 1 and 3 postinfusion of CTL), CSF cytocentrifuged cells were analyzed when the shunt was disconnected. Because male donors were used as sources of precursor CTLs, fluorescence *in situ* hybridization analyses using X (patient) and Y (donor) chromosome probes could appropriately distinguish between donor and patient lymphocytes. With an operational shunt, at d 3 postinfusion of CTL, only 6% of the donor cells remained. At d 5 and 6 postinfusion, no donor CTLs

could be detected. The white cell counts increased in the CSF, from a baseline of < 1 (at d 0) to 290 (at d 3) and to 106 (at d 6). Thus, cells were present in abnormally high numbers, but they were mostly of host origin, indicating that an endogenous immune reaction was engendered. However, the *in situ* hybridization analyses performed on the fluids obtained when the shunt was disconnected showed that the donor CTL percentages remaining were much higher. The sample at d 1 postinfusion was 77% of donor origin, and the d-3 postinfusion sample was 30% of donor origin.

The patient's MHC was HLA-A2,24; B14,27; HLA-DR1,4; DQ1,3; DR53. The male donors used for precursor CTLs had HLA-AB types of HLA-A3,3; B35,57 (donor 1), HLA-A1,28; B8,60 (donor 2), and HLA-A1,2; B13,35 (donor 3). In spite of the patient having received multiple infusions of CTLs that were allogeneic, the patient's percent reactive Ab remained at baseline when tested at the beginning of each treatment cycle. Thus, the patient did not mount a detectable humoral systemic response to the alloreactive CTLs during the time monitored for such.

The alloreactive CTLs largely expressed CD3, CD8, and CD11a, all of which are markers of CTL. They also lysed targets displaying patient MHC molecules. Cytokine analyses of the CTLs showed that they produced IL-2 and interferon- $\gamma$ .

Cytogenetic analysis of the patient's tumor cells revealed complex rearrangements consistent with high-grade BTs. Abnormal clones were present in all of 28 cells examined (five were metaphases). Two derivative chromosomes showed that there were complex rearrangements involving chr 6, 7, and 9. There was loss of an entire X chr, 7q, and 17p. Half the cells examined exhibited endoreduplication.

The patient tolerated all three cycles of alloreactive CTL treatment well. Nonetheless, treatment side effects could have been greatly diminished because the ventricular shunt removed the biologic from the area where it was not only needed, but where toxicity may have been engendered. At the third treatment cycle, the authors determined that the patient needed an operational shunt. Thus, keeping the administered CTLs in the CNS was perceived as a problem at this point. Additionally, her cultured tumor cells, derived from surgical resection, were found to produce significant levels of a highly immune-inhibitory substance, transforming growth factor  $\beta$  (2337 pg TGF- $\beta$ /10<sup>6</sup> cells/24 h). Secretion of this substance would likely inhibit the endogenous immune response generated by the presence of alloreactive CTLs (61). With suboptimal conditions existing to prevent this adjuvant cellular therapy from succeeding, the authors decided to explore a new approach to her therapy, involving generation of a cancer vaccine comprised of the patient's own tumor cells transduced with antisense TGF- $\beta$  (62). This approach is discussed further in Chapter 14 of this book.

#### 4. DISCUSSION

Although the safety of intracavitary administration of alloreactive CTL has been proven, the efficacy of this treatment still awaits determination. At this time, only treatment of recurrent Kernohan grade III gliomas appears encouraging. For protracted experimental regimens such as this, the authors' recommendation would be to either enroll only those with recurrent grade III gliomas or enroll glioblastoma patients within 4 wk after completion of their radiotherapy following surgical debulking. Efficacy would be more quickly determined if the therapy is explored in phase II trials with the latter subset of patients.

Preclinical exploration of intrathecal administration of alloreactive CTL is still needed. The toxicity and safety of administration of alloreactive CTL multiple times intrathecally should be examined. As described above, the adult patient who was administered the biologic in this fashion had toxic complications reminiscent of chemical meningitis. Although the pediatric patient tolerated the intrathecal administrations well, her operational ventriculoperitoneal shunt may have removed the biologic before toxic symptoms developed. In studies by other investigators, a patient with leptomeningeal carcinomatosis, who received LAK cells and IL-2 intraventricularly, reacted with elevation of intracranial pressure, headaches, and meningismus (63). In contrast, two patients (one with meningeal gliomatosis and the other meningeal carcinomatosis) who received LAK cells intrathecally and IL-2 intravenously tolerated the treatment well (64). It will be necessary to test the safety of alloreactive CTL therapy, given with and without IL-2, at various doses intrathecally in an animal model. A dissemination of 9L gliosarcoma cells within the CSF of Fischer-344 rats is a good model for leptomeningeal disease (65) and could be used to investigate this mode of administration for alloreactive CTL.

If phase II trials are pursued, a multifactorial analysis of the MHC-I and -II types of donors and patients should be correlated with data collected on responder proliferation, cytotoxicity, and/or cytokine production in response to tumor coinubation. Proliferative and cytolytic activity will likely correlate with MHC disparities between class II and class I, respectively. It is not yet known what disparities in MHC make good mixed lymphocyte reactions or potent lytic effectors. In theory, the use of multiple alloreactive CTL donors should maximize the efficacy of this treatment. Multiple donors should increase the potential number of epitopes recognized by different alloreactive CTL preparations generated against tumor host MHC.

To optimize therapy for BT patients, the authors are preclinically testing combinations of cytokine and suicide gene therapies with local cellular IT using alloreactive CTL. Interferon- $\gamma$  upregulates MHC molecules on glioma cells (66). Enhanced expression of MHC should make tumors better targets for alloreactive

CTLs. Appropriate preclinical testing of each single modality is being, or has been, evaluated (56,66–68), so that these combination therapies can be assessed for synergism. Multimodal treatments involving IT and gene therapy should be aggressively explored, as no single modality is likely to offer meaningful cyto-reduction, which will significantly enhance survival for malignant glioma patients.

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## Autologous Vaccine and Adoptive Cellular Immunotherapy as Treatment for Brain Tumors

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*Gary W. Wood, PhD and Frank P. Holladay, MD*

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### 1. INTRODUCTION

This chapter discusses a process that combines vaccination to induce an immune response (IR) against autologous tumor-associated antigens (TAAs) with adoptive transfer of autologous cancer Ag-specific effector T-lymphocytes to treat individuals with progressing cancer. The use of this approach for the treatment of brain malignancy is described. The rationale is that the multiple genetic defects accumulating in cells during malignant transformation and subsequent tumor growth lead to production of altered protein molecules that confer immunogenicity. The host immune system can recognize malignant cells as nonself. Vaccination with autologous cancer cells and an immunologic adjuvant primes T-lymphocytes against cancer Ags, and overcomes the Ag presentation defect that prevents malignancies from being recognized during their natural progression. Cancer Ags and nonspecific Ag receptor stimuli (such as anti-CD3) can activate primed T-lymphocytes to differentiate into Ag-specific effector cells in vitro, and interleukin-2 (IL-2) stimulates these cells to proliferate. Activated effector T-lymphocytes are able to travel to sites of tumor growth, enter and reject the tumor, and potentially cure tumor-bearing animals after being infused

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into the blood stream. This strategy has been used to cure several types of experimental gliomas, demonstrating that immune privilege, immune suppression, the blood–brain barrier (BBB), Ag presentation defects, and other theoretical barriers to successful IT can be overcome. Phase I/II clinical trials using patients with recurrent malignant astrocytoma have demonstrated that cancer Ag and adoptive transfer (AT) IT is feasible, minimally toxic, and potentially efficacious. The data justify further clinical trials of cancer Ag and AT in humans with central nervous system malignancies.

## 2. BACKGROUND

Malignant transformation occurs when a normal cell accumulates genetic changes/mutations that transform it into one capable of abnormal growth and differentiation. Cells naturally accumulate genetic defects as they proliferate. Carcinogens increase the number of defects in proliferating cells. If a genetically susceptible individual lives long enough, is exposed to high concentrations of carcinogen, and/or a tissue has a high rate of cell turnover, malignant cells develop, proliferate, and spread. The genetic changes that cells undergo during malignant transformation and malignant progression produce altered protein molecules that could be recognized by the host as nonself, i.e., have the potential to function as cancer Ags (1–8). Animal data, demonstrating that spontaneous and chemically induced neoplasms express a unique Ag phenotype, are consistent with antigenicity arising from randomly occurring genetic events; but how many Ags there may be, and which genetic changes are important for cancer cell antigenicity, are unknown. Despite incredible advances in molecular technology, very few tumor-specific Ags have been defined. Whether certain Ags are immunodominant, or whether there are shared Ags that could be used in vaccines, is unknown. Nevertheless, the fact that malignant cells produce molecules that the immune system of the original host can recognize as nonself is incontrovertible and significant, because it means that IT is theoretically possible.

In this modern molecular age, if a protein has not been cloned, its very existence is open to question. It is easy to believe that, except for a few unusual experimental malignancies that express viral proteins as Ags, most malignancies, particularly those arising in humans, are nonimmunogenic. It is an absolute precondition of any form of cancer Ag-specific IT that malignant cells contain cancer Ags. Tumor protection experiments have been used to establish that histopathologically diverse experimental malignancies contain Ags that confer immunogenicity (5–8). As adjuvant strategies for augmenting cancer Ag-specific IRs have improved, (e.g., through transfection of malignant cells with cytokine genes) it has become clear that all experimental malignancies contain Ags that confer immunogenicity (9–11). Why individual malignancies are more or less immunogenic remains completely unknown.

Cancer Ags induce classical T-lymphocyte-mediated IRs that are responsible for immune protection. CD4<sup>+</sup> T-lymphocytes provide help in the form of cytokine production (e.g., IL-2) and may contribute as effectors against some Ags, but CD8<sup>+</sup> T-lymphocytes are the major mediators of protection (5–8). There are significant problems with this completely *in vivo* biological approach to understanding cancer immunology, because it is not easily amenable to molecular analysis. It is not possible to develop “magic bullets” for immunological treatment of cancer without identifying cancer Ags. More importantly, since protection experiments are not possible in humans and IRs do not develop against spontaneously arising human malignancies, it takes a leap of faith to believe that descriptive *in vivo* observations in experimental animals are relevant to human malignancies. Ultimately, it may be necessary to accept the idea that cancer Ags may be unique for each patient’s cancer and to develop IT approaches that accommodate this difficulty.

Increased understanding of the nature of Ag presentation has provided tremendous insight into the general nature of tumor Ags and the IR to these Ags. Not long ago, it was believed that cancer Ags were integral membrane proteins. That view changed dramatically when it was realized that any altered protein in a malignant cell could function as an Ag. Thus, any protein produced by genetically altered genes could be an Ag. The only requirement was that the altered cellular protein had to be able to be recognized as nonself by host T-lymphocytes. This is accomplished when the protein is processed and presented as peptides in association with major histocompatibility complex (MHC) molecules at the cell surface. Antigen-presenting cells (APCs) present cancer Ags to T-lymphocytes in two distinct ways. Malignant cells themselves express Ag proteins through the exocytic pathway in association with MHC class I (MHC-I). Cytotoxic T-lymphocytes (CTLs) recognize cancer Ags in this context on the surface of malignant cells. CTL killing has been a powerful laboratory tool for characterizing IRs to cancer Ags and for identifying and characterizing TAAs. However, CTL recognition is an efferent phenomenon. There is no evidence that malignant cells function as APCs during the generation of cancer Ag-specific IRs. Spontaneously arising malignancies fail to induce primary IRs. When an IR is induced by vaccination, malignant cells may be used as the source of Ag, but do not directly present Ag peptides to T-lymphocytes. There are probably two major reasons for this failure: malignant cells express only low levels of MHC-I; and, unlike professional APCs which are highly mobile, malignant cells are incapable of carrying Ag from sites of malignant growth, or from vaccination sites, to lymphoid tissues where IRs are generated.

The form of cancer Ag presentation that probably occurs during the generation of cancer Ag-specific IRs involves interaction of cancer cells and professional APCs. Activated APC phagocytose cancer cells, process cancer Ags through the endocytic pathway, and present those Ags as MHC-associated pep-

tides at the APC surface. Highly mobile APCs probably recirculate constantly and carry Ags from sites of Ag presentation through afferent lymphatics to the closest lymphoid tissue. It is within those specialized immunologic microenvironments that APCs and cancer Ag-specific T-lymphocytes interact to generate a primary Ag-specific IR. CD4<sup>+</sup> T-lymphocytes recognize Ag peptides in the context of MHC-II molecules and produce IL-2, which is required for proliferation of Ag-primed T-lymphocytes. CD8<sup>+</sup> T-lymphocytes recognize Ag peptides in the context of MHC-I molecules. Both CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes are primed during cancer Ag-specific IRs. It is highly likely that priming of both CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes occurs in direct association with activated APC expressing Ag peptides originally derived from cancer Ags in tumor cells.

Malignant tissues contain tumor-associated macrophages (12–14) that have the potential to be professional APCs. Theoretically, tumor-associated APCs would phagocytose dead and dying cancer cells, then travel from sites of cancer progression to lymphoid tissues, where they would initiate cancer Ag-specific IRs. Also, tumor cells produce and release proteins into the circulation. It is intuitive that individuals would develop an IR against their own tumors, and the existence of cellular and humoral IRs in humans with progressing cancer has been extensively investigated. The apparent absence of such responses clearly demonstrates that what is intuitive is not always correct.

The failure of progressing human malignancies to induce cancer Ag-specific IRs has led to the widespread belief either that they are nonimmunogenic or that there are special immunosuppressive mechanisms for preventing generation of autologous cancer Ag-specific IRs. Observations on the immunogenicity of experimental malignancies have not been directly translated into humans. It is not technically feasible to test whether IRs occur against an experimental animal malignancy in the animal in which they were originally induced because the animal is killed when the tumor is removed. It is not technically possible to perform transplantation protection experiments in humans. An alternative explanation, which allows human malignancies to be immunogenic, and IRs in humans to be comparable to those generated against cancer Ags in other mammalian species, is that the reason for the apparent nonimmunogenicity of human malignancies is inadequate antigen presentation during natural malignant progression. If human malignancies were nonimmunogenic or immune suppression prevented generation of IRs, it would be difficult or impossible to induce tumor Ag-specific IRs in cancer patients. Yet, autologous tumor Ag-specific IRs may be produced in most patients by vaccinating them with tumor cells and an immunologic adjuvant (15–19). This is particularly relevant to brain cancer, because peripheral immunization circumvents any afferent limitation imposed by the “immunologically privileged” status of the brain.

Another prevalent historical view that has inhibited the investigation of IT in humans is that immunogenicity is restricted to certain histopathological sub-



types (e.g., melanoma and renal cell carcinoma [RCC]). Rare spontaneous regressions in those diseases could lead one to such a conclusion. However, the fact that vaccination produces autologous tumor Ag-specific IRs as frequently in patients with colon or breast cancer as in patients with melanoma or RCC (15–19) demonstrates that, in humans as in experimental animals, immunogenicity is not necessarily related to histopathology. The main determinants for immunogenicity are the type and number of genetic changes that occur during malignant transformation, not the site of origin of the cancer. The chief factors determining the strength of the IR following vaccination are the number of cancer cells used to vaccinate and the strength of the immunologic adjuvant. The conclusion that can be drawn from these observations is that autologous cancer Ag-specific IRs can potentially be manipulated for therapeutic benefit in any type of human malignancy.

If malignant cells are immunogenic, why do they fail to sensitize the host even when they are multiplying within lymph nodes (LNs)? Sensitization occurs exclusively through the activities of APCs. The explanation that is most consistent with available data is that tumor-associated APC precursors are not sufficiently activated to kill, phagocytose, and degrade viable malignant cells. Although the presence of a progressing malignancy leads to local accumulation of cells that could function as APCs, malignant cells do not produce factors that activate Ag presentation. In fact, malignant cells may produce factors that suppress APC activity. It would appear that part of the process of malignant transformation involves development of a “stealth” phenotype viz-a-viz the immune system. The way that this is accomplished is likely to vary between malignancies, but the outcome is the same. The immune system fails to recognize the malignancy as nonself unless tricked to do so. The fact that some malignancies escape immune surveillance should not be interpreted as evidence that other malignancies that failed to develop escape strategies were eliminated by the immune system.

If progression of immunogenic malignancies results from deficient Ag presentation, one would anticipate that effectively overcoming that deficiency, by vaccinating the tumor-bearing individual against Ags expressed by their own cancer, would lead to an inhibitory effect on malignant progression. In theory, activated cancer Ag-specific T-lymphocytes would be released from LNs draining vaccination sites and would travel to the tumor, where they would be further activated to function as effector cells, causing tumor cell death and tumor regression. Most attempts to achieve this end in experimental animals and in humans have been unsuccessful, but there is some evidence that certain powerful vaccination strategies will slow tumor progression (9,20,21). In general, however, vaccination primes T-lymphocytes, but does not generate a sufficient number of circulating effector T-lymphocytes to bring about complete regression and cure. The inability of vaccination alone to inhibit malignant progression is con-

sistent with the general observation that vaccination, even for highly immunogenic viruses, is a preventive, not a curative, strategy.

A major breakthrough in cancer IT was achieved with the demonstration that primed T-lymphocytes from immune animals could be converted into tumor Ag-specific effector T-lymphocytes by stimulating them with Ag and expanding them with IL-2 *in vitro* (22). Tumors were rejected and animals were cured when the stimulated effector cells were returned to the circulation of animals bearing established malignancies. Although the initial studies were performed with highly immunogenic, virus-induced cancers, and therefore had limited applicability to spontaneously arising human malignancies, the general principle was subsequently extended to a wide range of experimental neoplasms (23–37). These experimental animal studies have established several important general principles about cancer IT:

1. Susceptibility is related to the ability of activated T-lymphocytes to recognize autologous tumor Ags.
2. Although some cancers are weakly immunogenic, requiring powerful immunologic adjuvants to demonstrate immunogenicity, all experimental malignancies contain Ags that can be recognized as nonself.
3. Although not all experimental malignancies have been tested for susceptibility to cancer Ag IT, the strategy has been effective against even the most weakly immunogenic, highly metastatic cancers.
4. Susceptibility to cancer Ag IT is not related to the induction method: Spontaneous, chemically induced, and virally induced cancers are equally susceptible.
5. Susceptibility to cancer Ag IT is unrelated to histopathology: gliomas are as susceptible as fibrosarcomas or melanomas.
6. Susceptibility to cancer Ag IT is unrelated to location. All tumors are neovascularized and therefore accessible to circulating T-lymphocytes. Tumors growing in the brain may potentially be treated by the same strategy as tumors growing in the lung.

The major initial limitation for the translatability of cancer Ag IT to humans was that the strategy required a large number of tumor cells that could not normally be obtained from surgical specimens. Sufficient numbers of autologous tumor cells to produce effector T-lymphocytes in bulk culture *in vitro* was only available from patients with advanced, untreatable malignancies. Therefore, the observation that Ag-specific effector T-lymphocytes could be generated by stimulating Ag-primed T-lymphocytes *in vitro* with nonspecific T-lymphocyte stimuli (such as anti-CD3, superantigen, or brystatin-1) provided another major advance in this field (35,38,39). It had long been known that mature T-lymphocytes express CD3 in association with their Ag receptors, and that anti-CD3 would stimulate T-lymphocyte differentiation. Anti-CD3 does not convert normal T-lymphocytes into effector cells capable of rejecting tumors. However, anti-CD3 does convert cancer Ag-primed T-lymphocytes into activated effector

T-lymphocytes that would attack progressing tumors. Not only did the primed T-lymphocytes acquire effector capability, but these effector T-lymphocytes also exhibited exquisite Ag specificity for the tumor that was used for priming. Two possible explanations for this specificity are that priming confers heightened sensitivity to subsequent stimulation through the Ag receptor, and/or priming increases the number of tumor Ag-specific T-lymphocytes in responding lymphoid populations. Regarding translating cancer Ag IT to humans, this observation meant that one only needed to obtain enough cancer cells for successful priming. Once primed, nonspecific stimuli may be used to produce effector T-lymphocytes. Furthermore, on a per cell basis, anti-CD3-stimulated effector T-lymphocyte populations are more powerful than effector T-lymphocyte populations generated by exposure to tumor cells *in vitro* (34,38).

Although much remains to be learned about IRs to cancer Ags, it is clear that adoptive transfer of activated cancer Ag-specific effector T-lymphocytes provides a strategy that could be relatively easily translated to humans for the treatment of a wide range of malignancies. In fact, this strategy could be implemented without defining the molecular nature of tumor Ags. Although the specific genetic changes that are responsible for malignant transformation are largely unknown and may be expected to vary between malignancies, it is widely agreed that relatively high numbers of genetic defects accumulate in all cancers. Since it is these genetic changes that are responsible for malignant cells being able to be recognized by the immune system as nonself, there is no inherent reason why human malignancies would be less immunogenic than experimental animal malignancies. In fact, one may predict that they would be more immunogenic, because a higher number of defects could accumulate in tumors that take years to develop. Moreover, higher numbers of random mutations could occur, simply because malignant cells go through many more generations in humans before they are detected. The functional capabilities of rodent and human immune systems are substantially the same, and would be expected to respond similarly to TAAs. It should be possible to apply the same general principles to humans that have proven effective for treating experimental malignancies.

The basic principles that were outlined above have been translated to humans with advanced malignancies. It is possible to vaccinate patients with their own tumor cells combined with an immunologic adjuvant. Studies performed, using melanoma, RCC, and colon cancer patients demonstrated that > 50% of patients vaccinated with tumor cells and *Bacillus Calmette-Guerin* (BCG) developed autologous tumor-specific IRs (15–18). More recently, studies using autologous tumor cells and granulocyte-macrophage colony-stimulating factor (GM-CSF), which is a more powerful adjuvant than BCG (40–43), demonstrated that nearly all cancer patients developed autologous cancer Ag-specific IRs. The authors have vaccinated more than eighty patients with their own cancer cells and GM-CSF. Patients with brain, breast, colon, lung, melanoma, ovary, and RCC

all developed autologous tumor specific IRs. Similar studies have been reported with brain tumor (BT) patients (44). These observations need to be extended to greater numbers of cancer patients with these malignancies and with other types of cancers. Nevertheless, they establish the most important fundamental principle for translating IT to humans: Human malignant cells are immunogenic in the host in which they arise. Equally important, there are a small number of studies demonstrating that cancer Ag and AT can produce clinical effects in treated patients. When T-lymphocytes from vaccinated patients were stimulated with anti-CD3 and expanded numerically with IL-2, then adoptively transferred to patients with advanced cancer, clinical responses, albeit infrequent, were observed (45). The authors and others have observed complete regressions in melanoma, RCC, and breast cancer patients. In principle, therefore, cancer Ag and adoptive transfer IT could be applicable to BTs. The following is a discussion of the application of the general principles of cancer Ag vaccination and AT to malignant brain cancer.

### 3. PRECLINICAL STUDIES IN BRAIN CANCER

The authors' intent from the outset was to use a BT model to develop an IT approach to cancer treatment that was applicable to human malignancies (i.e., to do translational cancer IT). Several requirements had to be met to achieve this goal. The obvious one was that, to have some real benefit for cancer patients, whatever strategy evolved had to be at least as effective in humans as it is in experimental animals. In fact, it probably had to be more effective in humans, because most human malignancies are relatively advanced at the time of initial diagnosis. It does not do any good to be able to cure tiny tumors growing as localized micrometastases in the lungs of experimental animals, if the larger, more widespread malignancies that are encountered in humans are too advanced to be effectively treated. There were other less obvious factors that were also important to consider when designing a translational strategy. Intracerebral gliomas were selected for these translational studies, because they provided several features that allowed a single model to be used to satisfy most of the requirements. Advantages of a glioma model for translational studies are detailed below.

The first benefit of using gliomas was that cancer Ag vaccination and AT immunotherapy could be applied to a histopathologically distinct model, thereby broadening its ultimate applicability. Another translational advantage of the glioma model is that any successful translational strategy had to be able to eliminate tumors growing in remote areas of the body. Systemic disease cannot be cured if tumor cells can metastasize to the brain, where anticancer agents cannot reach them. The strategy had to be able to eliminate tumors that infiltrated tissues in the same way that spontaneous human malignancies do. The viral leukemias and fibrosarcomas that were used in initial proof-of-principle studies are not

directly comparable to human malignancies that develop and spread naturally. In contrast, some rodent gliomas infiltrate the brain parenchyma in a manner that is somewhat comparable to human gliomas. Gliomas offer the additional advantage that, in humans, they induce a higher level of nonspecific peripheral immune suppression than most other malignancies.

There are additional translational issues that are unrelated to the model used to test susceptibility. The strategy had to have a simple design that could be replicated relatively easily in a normal clinical/hospital environment. There is little widespread value to developing a translational cancer treatment strategy that only can be delivered within the confines of highly sophisticated research centers. Finally, the strategy had to be based on sound fundamentals. Many IT strategies have proven ineffective, because they ignored available knowledge about the nature of IR to cancer Ags. Cancer Ag vaccination and AT is based on the following fundamental observations.

1. Autologous tumor cells contain the full array of Ags expressed by a particular malignancy and are the best currently available immunogen. Vaccines comprised of viable, irradiated tumor cells have invariably been used to establish the immunogenicity of experimental malignancies. One frequent objection to using cells from human surgical specimens for vaccination is that human malignancies are comprised of multiple subpopulations that could be antigenically distinct. If there were any cells that could escape the IT strategy, failure would be guaranteed. Because the genetic defects that generate immunogenicity are cumulative, subpopulations should share multiple Ags. Another objection is that cancer Ag IT requires a customized treatment approach. It may not always be possible to obtain malignant cells from the patient. The existence of a single shared Ag that could be used for cancer vaccines to treat all patients with brain cancer certainly would make life easier, but the nature of cancer antigenicity suggests that such an entity may not necessarily exist. It is important to consider that, although having to obtain the cells from each patient to be treated creates logistical difficulties, whole cancer cells containing multiple Ags actually may be superior to a single shared Ag. Cancer cells could induce a complex polyclonal IR that would be expected to produce more powerful anticancer effects. This is what the polyclonality of the immune system is designed to do.
2. Combining malignant cells with immunologic adjuvants maximizes IRs against TAAs. A variety of strategies have been successfully employed. Most animal studies used bacterial adjuvants, such as *Corynebacterium parvum* (28,30). Recently, cytokines, which have proven to be less toxic and more effective than the traditional adjuvants, have been used. The most widely used is GM-CSF, whose ability to serve as an adjuvant is related to its ability to activate professional APCs. Another strategy that is powerful, and has provided considerable insight into the immunogenicity of very weakly immunogenic malignancies, is

the transfection of tumor cells with cytokine genes, also with the intent of activating APC locally (9). This strategy probably has limited translational potential because of its current technical difficulty. Another novel strategy that takes advantage of one of the functions of adjuvants is to activate APCs in vitro, then flood the animal with additional APCs. This strategy uses vaccines comprised of cancer Ags and activated APCs (or dendritic cells) to induce cancer Ag-specific IRs (20).

3. Malignancies do not stimulate IRs during their induced or spontaneous natural development. Tumor-bearing individuals must be vaccinated to induce IRs against Ag expressed by the progressing malignancy. Immune suppression does not prevent such autologous cancer Ag-specific IRs from developing.
4. Exposure of resting Ag-specific T-lymphocytes to Ag in lymphoid tissue leads to activation changes that result in their release into the peripheral circulation. Blood would be expected to be a good source for primed cancer Ag-specific T-lymphocytes. Blood may not be the richest source, but it has the additional advantage of being a renewable resource.
5. It may be possible to generate cancer Ag-specific effector cells by multiple vaccinations with cancer cells and powerful adjuvants, but stimulating T-lymphocytes through their Ag receptor in vitro most efficiently generates high numbers of effector cells. This can be accomplished with Ag-specific or nonspecific receptor stimuli.
6. Effector T-lymphocytes injected into the blood stream of a tumor bearer will enter tumors and activate secondary immune mechanisms that lead to rejection of the tumor, elimination of malignant cells, and possible cure of the treated individual.

BTs, therefore, represent a perfect challenge for translational IT. Conventional wisdom at the time that the authors initiated studies suggested that immunological privilege, immunosuppression, the BBB, the lack of lymphatic drainage, and the brain's lack of MHC-expressing APCs precluded susceptibility of gliomas to IT. Conventional wisdom further held that malignancies growing in the brain, whether as primary gliomas or as tumors that had metastasized from distant sites, were inherently resistant to therapy, regardless of what principle the therapy was based on. If a cancer Ag IT strategy could be developed that would cure rapidly growing, highly infiltrative intracerebral gliomas, there was a good chance that the strategy would be effective against any malignancy growing anywhere in the body.

The conventional wisdom was shown to be based on faulty assumptions when the authors demonstrated that cancer Ag-specific effector T-lymphocytes, which had been infused intravenously, could enter progressing BTs and cure tumor-bearing animals (25). Other investigators (34,35) subsequently confirmed these studies, using different versions of cancer Ag IT and other BT models. Immunosuppression, if it exists as more than a laboratory artifact, did not prevent the



tumors from being rejected. Immune suppression also failed to prevent tumor-bearing animals from being immunized against tumor Ags (32,33) and did not prohibit effector T-lymphocytes from being generated in these tumor-bearing animals (32,33). The BBB is not a barrier to cells. Tumors metastasize across the BBB. Circulating mononuclear phagocytes enter the brain and become microglia. Adoptively transferred T-cells can produce autoimmune disease (e.g., experimental allergic encephalitis [EAE]) in naïve animals. Moreover, the BBB may be moot in BT immunotherapy. Most brain tumors are neovascularized, and high numbers of leukocytes leave the circulation and infiltrate progressing brain malignancies (12–14).

A valuable general lesson about IT was drawn from translational studies. The blood is an excellent conduit for cellular anticancer agents. Growing malignancies are invariably neovascularized, so infused T-lymphocytes can reach even the most remote nests of malignant cells. This observation is important primarily because it implies that T-lymphocytes can potentially eliminate malignant cells and cure the tumor-bearer. Direct evidence that adoptively transferred T-lymphocytes selectively infiltrate malignant tissue, but not normal brain, was obtained by trafficking studies using fluorescent and isotopically labeled cells (34). An interesting aspect of these trafficking studies is that, despite the fact that the tumors were rejected, few labeled cells actually entered malignant tissue. This observation was consistent with similar trafficking studies that have been performed in EAE, in which small numbers of T-lymphocytes enter and cause progressive neurological damage and ultimately death. The small number of intratumoral T-lymphocytes required to reject tumors suggests that the tumor is not solely rejected by direct T-lymphocyte-mediated killing (i.e., by CTLs). Rather, T-lymphocytes also activate local secondary effector cells (such as macrophages and natural killer cells) through their ability to produce large amounts of secondary mediators (such as interferon- $\gamma$ ).

Preclinical studies have demonstrated the susceptibility of intracerebral gliomas to IT mediated by cancer Ag-specific T-lymphocytes. Effector T-lymphocytes were produced by stimulating Ag-primed T-lymphocytes *in vitro*, either with whole autologous cancer cells (23,25) or a surrogate Ag receptor stimulus, such as anti-CD3 (38). Primed T-lymphocytes in animals were obtained either from LNs or spleen (comparable to peripheral blood (PB) in humans). Effector cells were obtained either from normal immunized animals or from immunized tumor-bearing animals (31,33). Effector cells mediated their effects either alone or in combination with systemic IL-2. Glioma cells were susceptible both when they were growing in the brain and when they were growing in extracerebral sites. Tumors that infiltrated the brain parenchyma (gliomas) and tumors that grew as localized foci in the brain (fibrosarcomas) both were susceptible (46).



#### 4. PHASE I CLINICAL TRIALS

The successes achieved in preclinical models naturally led to translational phase I clinical studies in humans. Phase I clinical trials of cancer Ag vaccination and AT were designed to determine whether the process is feasible in humans, and whether patients incur tolerable levels of toxicity during its delivery. Phase I clinical trials could also provide insight into whether cancer Ag and adoptive transfer IT may have beneficial clinical effects, but the absence of dramatic clinical effects should not be used as the basis for determining whether phase II and phase III studies are warranted.

The preclinical findings, which have characterized cancer Ag and AT immunotherapy results in all models tested, suggest that there should be limited expectations about significant anticancer effects during phase I clinical trials in patients with advanced malignancy. This may seem intuitive, because the patients who would be included in clinical trials generally have failed all other available treatments. However, failure to appreciate this concept could lead to abandonment of clinically effective immunologic treatment strategies either during or after completing phase I testing. This is a real consideration, because it affects not only the existence, but also the design, of future clinical trials. It has already had an effect on the conduct of IT clinical trials in the past.

The most famous illustration is provided by studies of tumor infiltrating lymphocyte (TIL) therapy. Significant effects were observed in a portion of treated patients (47–49), but the general interpretation of these results has been that further studies are not warranted, because the response rate was too low. The result has been that no subsequent studies have appeared, in which, for example, melanoma patients were treated with TIL at an earlier stage in their disease. The modest results obtained, using cancer Ag and AT to treat RCC patients, engendered a similar lack of enthusiasm (45). Again, the general interpretation was that the low response rate did not warrant further investigation of the strategy.

An alternative interpretation would be that the studies fulfilled the requirements of a successful phase I investigation. They demonstrated that it was feasible to generate autologous cancer Ag-specific effector T-lymphocytes from patients with advanced malignancy. They further demonstrated that adoptive transfer of high numbers of autologous T-lymphocytes could be accomplished with minimal toxicity, and that the treatment strategy could produce clinical responses. It is possible, then, to argue that the existence of some clinical responses and the lack of significant toxicity justify performing phase II and possibly even phase III studies, using poor prognosis patients at a time when they have minimal disease. This would mean that TIL therapy or cancer Ag and AT therapy would be tested on early-stage cancer patients. If this were done, survival (not response rate) would have to be the study end point, as is usually

the case in animal studies of IT. Thus, caution must be exhibited when interpreting preliminary results of studies using patients with advanced brain malignancy.

The authors' initial human cancer Ag and AT studies were designed solely to develop a strategy that was technically feasible in humans, to determine whether that strategy could be safely applied to patients, and to begin to understand the immunology of human brain malignancy. The guiding philosophy was that advances would not be achieved by trying to figure out why a strategy could not work. The authors chose to conduct investigations using patients with recurrent grade III/IV astrocytomas for the following reasons: Surgical resection is standard therapy for recurrent astrocytomas (the surgically removed tumor would provide a source of malignant cells for vaccination); using human BT patients would allow testing of the hypothesis that malignancies other than melanoma and RCC could be immunogenic, and therefore susceptible to IT; regression of the cancer and/or prolonged patient survival could not be explained by the natural biology of the disease; and patients with recurrent malignant astrocytomas may be the single most difficult group of cancer patients to treat.

The first important translational consideration was whether human astrocytomas are immunogenic. The general approach that was used to determine immunogenicity was to vaccinate patients with irradiated autologous cancer cells and an immunologic adjuvant. BCG was used as the adjuvant in early studies, but this proved to be unsatisfactory, because BCG is not a strong adjuvant and because multiple exposures frequently led to production of large painful ulcerating granulomas (18,50). Brain cancer patients vaccinated with tumor cells and BCG rarely exhibited positive skin test responses against their own cancer cells.

Recent studies have been performed with GM-CSF as the adjuvant. GM-CSF is an excellent immunologic adjuvant for human cancer vaccination for several reasons. Unlike BCG, GM-CSF is nontoxic at effective concentrations. Patients experience transient flu-like symptoms and may have erythema at the vaccination sites, but repeated immunization can be safely performed. Unlike bacterial adjuvants, which function as powerful Ags in their own right, GM-CSF, a human hematopoietic cytokine, induces no competing IRs. The result is that the only foreign Ags being presented by the patient's APCs will be those derived from the autologous malignant cells.

Data from vaccinating patients with GM-CSF and autologous tumor cells have suggested that some human astrocytomas are immunogenic (50). These studies need to be extended to larger groups of patients in order to determine the absolute percentage of patients with immunogenic brain cancers. In addition to establishing that astrocytomas are immunogenic, data demonstrated that the high level of peripheral immune suppression associated with the growth of malignant astrocytomas was not an impediment to the development of autologous tumor Ag-specific IRs in vivo, even in patients with very advanced cancers.

Recent studies by the authors demonstrated that all human BTs induce positive IRs when autologous cancer cells are combined with GM-CSF during vaccination.

Demonstrating the immunogenicity of BTs was the first requirement. However, because adoptive transfer IT is dependent on the activities of effector T-lymphocytes, it was also important to determine whether it is possible to generate activated tumor Ag-specific effector T-lymphocytes from patients that have been vaccinated with their own tumor cells. The authors initial approach to this question was to grow patients' peripheral blood mononuclear cells (PBMNC) in the presence of irradiated autologous cancer cells and low concentrations (60–120 IU) of IL-2 to determine whether effector cells could be produced. As had been reported in animal models (30,50), PBMNCs from nonimmunized individuals consistently failed to grow under those conditions. However, after patients had been vaccinated with autologous cancer cells and BCG, their PBMNCs consistently differentiated and proliferated in response to irradiated autologous cancer cells and IL-2. It is not clear, however, whether the cells that grew out had cancer Ag-specific effector activity, because it was not tested in vivo (44,50). Nevertheless, in all other systems (both human and animal models), T-lymphocytes that were generated using the same process expressed cancer Ag-specific effector T-lymphocyte activity in vitro (19,22–39,45,46).

The bulk lymphocyte populations that were generated in mixed tumor cell/lymphocyte cultures produced no significant negative side effects when administered intravenously to patients (50). They also exhibited no significant anti-cancer effects (50). None of the deposits of residual cancer decreased in size following treatment, although several patients exhibited prolonged periods during which their disease remained stable. These data could not be interpreted to mean that cancer Ag vaccination and AT immunotherapy had a meaningful clinical effect in any of the treated patients. However, the results did establish that cancer Ag IT is feasible in humans. The authors' results were similar to those obtained using a similar general approach to treat patients with stage IV melanoma or RCC (19). Although the approach was feasible, the difficulty associated with obtaining sufficient numbers of malignant cells for bulk in vitro activation limited its general applicability in humans.

The limitation imposed by tumor cell availability was overcome by treating patients with a combination of tumor vaccinations and adoptive transfer of anti-CD3/IL-2-stimulated T-lymphocytes. The authors' first series of astrocytoma patients were treated by vaccination with autologous cancer cells and BCG. The patients were vaccinated twice. All patients were vaccinated bilaterally in four sites drained by major LN chains in order to expose maximal numbers of T-lymphocytes to tumor Ags. The logic was that multiple site vaccinations would cause production of maximal numbers of primed T-lymphocytes. Primed T-lymphocytes then would be released from LNs draining the vaccination sites,

thereby producing a high density of circulating tumor Ag-primed T-lymphocytes. Patients were then leukapheresed to obtain PBMNCs as a source of cancer Ag-primed T-lymphocytes. PB was used because Ag-stimulated activation of T-lymphocytes leads to downregulation of adhesion molecules that hold naïve T-lymphocytes in lymphoid tissue. The result is that, within a short time after immunization, primed T-lymphocytes should be released into PB so that they may travel to disease sites. Leukapheresis, which is designed to remove large numbers of MNCs from the PB, can be performed on an outpatient basis. PB is a renewable resource.

The PB T-lymphocytes were then stimulated *in vitro* with anti-CD3 and IL-2 and reinfused into the BT patients, as has been described for treating animal malignancies (38), human melanoma and RCC patients (45). The technical advantages of using anti-CD3 and IL-2 were discussed in the introduction to this chapter. There also appear to be advantages to the anti-CD3-activated T-cells in relation to overall efficacy (38). No side-by-side studies have been performed to determine the relative efficacy of tumor Ag-activated and anti-CD3-stimulated effector cells in humans. However, side-by-side animal studies (34,38) indicated that, at least in the models used, anti-CD3-activated effector cells were consistently more effective on a per cell basis than tumor Ag-stimulated effector T-lymphocytes. The human data that have been obtained to date are consistent with these findings. Chang et al. (19) initially treated a series of stage IV melanoma and RCC patients by vaccination with autologous cancer cells and BCG, followed by adoptive transfer of autologous cancer cell–IL-2-stimulated LN T-lymphocytes. Although IRs were induced and the adoptively transferred cells exhibited cancer Ag-specific activity *in vitro*, the adoptively transferred cells had no clinical effect (19). In a subsequent trial (45), the same investigators immunized a comparable group of stage IV melanoma and RCC patients with autologous cancer cells and BCG. The patients were then treated with adoptive transfer of anti-CD3–IL-2-stimulated LN T-lymphocytes. Again, IRs were produced, and the adoptively transferred cells exhibited cancer Ag-specific activity *in vitro*. This time, objective clinical responses were observed in a significant proportion of the treated patients (45). The data suggested that anti-CD3-stimulated effector T-lymphocytes could be more effective than tumor cell-stimulated effector T-lymphocytes in humans as well. At the very least, the studies demonstrated that anti-CD3-stimulated effector cells could produce objective responses in cancer patients who have immunogenic tumors.

Similar observations were made using patients with recurrent grade III/IV astrocytoma. Although the initial translational human studies using irradiated autologous cancer cells and IL-2 to stimulate differentiation and proliferation of immunized malignant astrocytoma patients' peripheral blood T-lymphocytes established the proof of principle that cancer antigen vaccination and AT immu-

notherapy could be applied to patients with advanced brain cancer, no dramatic clinical responses were observed (50). However, in a subsequent series of patients that were immunized with autologous cancer cells and BCG then treated by adoptive transfer of anti-CD3 stimulated cells, objective responses were observed in 3/9 patients (51). More importantly, two of those patients have had durable long-term responses. These studies established that T-lymphocytes obtained from the peripheral blood of recurrent astrocytoma patients that were immunized with their own cancer cells could be routinely stimulated to differentiate and proliferate in response to anti-CD3 and IL-2. It further established that the strategy could produce regression of actively growing malignant astrocytomas and prolong patient survival in humans, as it did in preclinical animal studies.

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# **IV** ANTIBODY-MEDIATED IMMUNOTHERAPY OF BRAIN TUMORS

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## Anti-Idiotype Immunotherapy Strategies for Brain Tumors

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*Nai-Kong V. Cheung, MD, PhD*

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### 1. INTRODUCTION

The combination of chemotherapy, radiation, and surgery can achieve consistent and sometimes dramatic initial responses in some bulky tumors, such as neuroblastoma (1,2), which is a cancer derived from precursor cells of the sympathetic nervous system. However, minimal residual disease is often the hurdle to an ultimate cure. Immunotherapy (IT) may have the potential to eliminate microscopic residual tumors. A number of strategies have been used for active IT, such as purified antigens (Ags), tumor peptides (alone or presented by dendritic cells), whole tumor cells, and anti-idiotype vaccines. Being abundant in neuroectodermal tumors, glycolipids are potential targets for IT (3). Except for  $G_{M2}$  (4), purified glycolipid Ags (such as  $G_{D2}$ ) are poorly immunogenic in mice and humans. The immune response (IR) is usually of the immunoglobulin M (IgM) isotype, often lacking in immunological memory (5,6). Even when conjugated to a strong protein carrier, such as keyhole limpet hemocyanin (KLH),  $G_{D2}$  stimulates only a weak IgG response. It is also well known that shed gangliosides (e.g.,  $G_{D2}$ ) can inhibit cellular immunity (7), partly explaining why ganglioside vaccines may not be an ideal IT approach. Furthermore, antibodies (Abs) generally do not cross the blood–brain barrier (BBB) in substantial quantities, thereby compromising the effectiveness of humoral vaccines for brain tumors (BT). Cell-mediated immunity may provide an alternative strategy.

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Whole-cell vaccines, especially when transduced with cytokine genes (8) or co-stimulator molecules (9), can induce strong cellular immunity and suppress established tumors. However, their efficacy in human cancers is far from established. Given the complexity of Ag display and accessory peptides on tumor cells, whole-cell vaccines can be difficult to optimize. The weak/absent expression of major histocompatibility complex class I or class II (MHC-I or -II) molecules in certain tumor types (e.g., neuroblastomas and other BTs) are formidable obstacles for these vaccine strategies.

## 2. ANTI-IDIOTYPE VACCINES

Idiotopes are unique Ag determinants in the variable regions of Ig molecules. Monoclonal antibodies (MAbs) recognizing these idiotopes are termed “anti-idiotypic antibodies” (anti-Ids) (10). Those anti-Ids that recognize idiotopes within the framework region of the immunizing MAbs are termed Ab2 $\alpha$ . Of interest are anti-Ids that recognize the Ag-binding sites of the immunizing MAbs (Ab2 $\beta$ ) and mimic the original Ags. Because of this mimicry, they are potential vaccines (11). Vaccinations with anti-Ids have induced protective immunity against viral (12), bacterial (13), and parasitic infections (e.g., trypanosomiasis) (14). Anti-Id vaccines that mimic carbohydrate or glycolipid Ags offer potential advantages over natural Ags. First, unlike natural carbohydrate Ags, anti-Id vaccines can stimulate cellular IRs in addition to humoral ones (15). Second, anti-Id vaccines may be more immunogenic than the nonprotein antigens they mimic. For instance, an anti-Id Ab mimicking bacterial lipopolysaccharide (LPS) was effective in priming neonatal mice to develop a protective IR against LPS, but LPS itself could not elicit such immunity (16). Third, complex carbohydrate Ags, such as gangliosides, may be difficult to synthesize; anti-Id vaccines can be manufactured as Abs or as peptidomimetics (17).

Several anti-Ids have been raised against MAbs recognizing cell surface tumor targets. Immunization of animals with these anti-Ids can generate Abs that recognize the original tumor Ags (18–24). Some investigators (21,25–27) have also induced specific T-cell-mediated immunity. Although IRs have been generated in rodents and primates, the efficacy of such responses against tumors has been investigated in only a few preclinical models (28,29). Despite the successful production of anti-Ids against Ags of great clinical interest, such as G<sub>D3</sub> (5,30), G<sub>M3</sub> (23), HMW-HAA (22), colon Gp72 (18), GA733-2 (31), CEA (27), and leukemia Gp37 (32), anti-Ids cannot be easily optimized without appropriate animal models. Nevertheless, in a few instances, immunization with anti-Id MAbs has been shown to decrease tumor growth in mice (33).

Clinically, the potential role of an anti-idiotypic network in colon cancer was first described in patients treated with MAb CO-17-1A (34,35). In subsequent

**Table 1**  
**Anti-Ids as Tumor Vaccines**

<i>Antigen</i>	<i>MAB</i>	<i>Anti-Idiotype</i>	<i>Tumor</i>	<i>Response</i>	<i>Immune resp</i>	<i>Ref.</i>
CO-17-1A	17-1A	Anti-Id (A)	Colon D	PR 6/37	Ab3'+T	(21)
HMW-MAA	763.74	MK2-23 (B)	melanoma	Survival	Ab3'	(22)
GD3	R24	BEC2 (B)	melanoma	Survival	Ab3'	(37)
GM3	L612	TVE-1(B)	melanoma	–	–	(76)
gp37	SN2	4DC6 (A)	T-lymphoma	GPR 1/4	Ab3'+T	(77)
CEA	8019	3H1 (A)	Colon (mets)	–	Ab3'+T	(27)

(A), alum; (B), BCG; PR, partial response; GPR, good partial response; mets, metastasis; T, T-cell-mediated response; Ab3', antitumor Ag Ab response.

studies, anti-anti-Ids (Ab3) and T-cell immunity (21,26,31,36) were demonstrated in responding patients. Since then, a number of anti-Ids have been tested in clinical trials as tumor vaccines (Table 1). MK2-23, conjugated to KLH and mixed with *Bacillus Calmette-Guerin* (BCG) as adjuvants, induced Ab3 in 14/23 patients, produced three partial responses, and prolonged patient overall survival (22). Toxicity was related mostly to the BCG administration and consisted of erythema, induration, and occasional ulceration at the injection sites. BEC2 is an anti-idiotypic mouse MAb that mimics G<sub>D3</sub> ganglioside. Intradermal immunization, using 2.5 mg BEC2 (mixed with BCG) or intravenous (iv) immunization with 10 mg BEC2, induced anti-G<sub>D3</sub> Abs in a subset of patients. Combining these two immunization strategies and using BEC2 conjugated to KLH, 18 melanoma patients received intradermal immunizations on wk 0, 2, 4, 6, and 10 with 2.5 mg BEC2 conjugated to KLH and mixed with BCG (BEC2-KLH-BCG). Booster immunizations of 10 mg unconjugated BEC2 were administered iv on wk 24, 37, and 50. Four/18 patients (22%) developed IgM anti-G<sub>D3</sub> Abs. No IgG anti-G<sub>D3</sub> Abs were detected. All four responding patients developed anti-G<sub>D3</sub> IgM during immunization with BEC2-KLH-BCG; only one patient demonstrated an IgM anti-G<sub>D3</sub> titer during the iv immunizations. Thirteen of the patients remained free of melanoma (three after undergoing re-resection for local relapse); 14 patients (78%) remained alive, with a median follow-up of 28 mo (20,37). Patients generally experienced grade III toxicity at the site of immunization, which was characterized by inflammation, ulceration, and oozing. All sites healed with minimal wound care, and there were no instances of systemic BCG infection. Grade II toxicities included fever, fatigue, or malaise, and hyperglycemia. Overall, immunizations were well tolerated.



### 3. ANTI-IDIOTYPE STRATEGIES FOR NEUROBLASTOMA

#### 3.1. *Ganglioside G<sub>D2</sub>*

The ganglioside, G<sub>D2</sub>, is present in a number of human tumors, including melanoma (5), small cell lung cancer (38), BTs (39,40), osteosarcomas (41), and soft-tissue sarcomas (42). Patients with melanoma have achieved major remissions associated with anti-G<sub>D2</sub> Abs (43–47). Among patients with high-risk neuroblastomas, anti-G<sub>D2</sub> Abs have produced clinical responses, and may have the potential of prolonging clinical remissions. Because active IT is ineffective unless the host's immunocompetence has recovered, passive IT can be most useful during the hiatus when the host immune system is still recovering from chemotherapy (48). G<sub>D2</sub> is well suited for targeting therapy because it is expressed at a high density in human neuroblastoma (5–10 × 10<sup>6</sup> molecules/cell), is restricted to neuroectodermal tissues, and is genetically stable, unlike other tumor Ags, such as Ig idiotypes (49). Although it circulates in the serum, this does not interfere with the biodistribution of the specific Ab (e.g., 3F8), allowing excellent tumor localization in patients with neuroblastomas (50); it is not modulated from the cell surface upon binding to Abs; and it is expressed homogeneously in human neuroblastoma, with little heterogeneity within tumors and among patients. Three anti-G<sub>D2</sub> Abs have shown clinical efficacy in clinical trials: murine IgG3 MAb 3F8 (51,52), murine IgG2a 14G2a (47,53,54), and human–mouse chimeric MAb ch14.18 (55,56).

3F8 is suitable for targeting IT, because it mediates efficient tumor cell kill by human complement *in vitro* (57,58), and by human lymphocytes (59), neutrophils (60), and activated monocytes/macrophages (61) *in vivo*. It is not trapped nonspecifically by the reticuloendothelial system and penetrates neuroblastomas well (0.04–0.11% injected dose/g) (50,62). Neuroblastomas have poor expression of decay-accelerating factor, and are thus unable to resist complement activation and cytotoxicity (63). Complement is critical for the initiation of inflammation, which can attract leukocytes and increase vascular permeability to proteins, including passively administered MAbs. When 3F8 was tested in phase I and II studies of metastatic neuroblastoma patients (48,51,52), responses in the bone marrow (BM) were frequently observed. Acute self-limited toxicities of 3F8 treatment were severe pain, fever, urticaria, hypertension, and anaphylactoid reactions, as well as decreases in blood counts and serum complement levels; in rare patients, a self-limited neuropathy was seen (46,47,64). Since the introduction of the Ab 3F8 into clinical trials in 1986, over 100 patients have been treated with no reported deaths or long-term sequelae. When serum anti-idiotypic (Ab2), anti-G<sub>D2</sub> (Ab3'), and anti-anti-idiotypic (Ab3) responses were analyzed, anti-G<sub>D2</sub> anti-idiotypic networks were demonstrated in the clinical cohorts.

### ***3.2. Anti-Idiotypic Network After Myeloablative Therapy***

Clinical strategies that modulate the human anti-mouse antibody (HAMA) response (HAMA) in patients may have a profound influence on the idiotype network inducible by murine MAbs (65). Prior to myeloablative chemotherapy and autologous bone marrow transplantation (ABMT), patients with stage 4 neuroblastoma were imaged with  $^{131}\text{I}$ -3F8. Their serum HAMA, Ab2, Ab3', and Ab3 Abs were assayed by ELISA prior to, 3 mo, and 6 mo after imaging. HAMA and Ab2 levels remained low, in contrast to the high levels seen in patients imaged with  $^{131}\text{I}$ -3F8 without ABMT. Among the long-term survivors, all had elevated Ab3' and Ab3 levels, which were significantly higher than those who died of disease. It appears that myeloablative therapy strongly blunted the initial HAMA response to murine MAb. However, the subsequent development of Ab2, Ab3, and Ab3' responses may be associated with superior survival.

### ***3.3. Anti-Idiotypic Network in Patients Treated with Adjuvant 3F8***

Thirty-four patients were treated with 3F8 at the end of chemotherapy (66). Most had either BM (31 patients) or distant bony metastases (29 patients). Thirteen patients were treated at second or subsequent remission (group I), with 12 patients in this group having had a history of progressive/persistent disease after BMT. Twenty-one patients were treated at first remission, following N6 induction chemotherapy (group II). Before 3F8 treatment, 23 patients were in complete remission; eight in very good partial remission (VGPR), one in partial remission (PR), and two had microscopic foci in the BM. Twenty-five patients had evidence of neuroblastoma by at least one measurement of occult/minimal tumor ( $^{131}\text{I}$ -3F8 imaging, marrow immunocytology, or marrow reverse transcriptase-polymerase chain reaction for molecular markers of disease). There was evidence of response by immunocytology (6/9), by the molecular marker *GAGE* (7/12), and by  $^{131}\text{I}$ -3F8 scans (6/6). Fourteen patients are alive at the time of this writing, and 13 (ages 1.8–7.4 yr at diagnosis) are progression-free (40–130 mo from the initiation of 3F8 treatment) without further systemic therapy. None experienced late neurological complications.

Three patterns of HAMA responses were identified. In pattern I, HAMA was not detectable during the 4–6-mo follow-up period after the first cycle of 3F8, with 42% of these patients having no HAMA response, even after receiving 2–4 cycles of 3F8 over a 4–25-mo period. In pattern II, HAMA was detected, but rapidly became negative during the 4–6-mo follow-up period. In pattern III, HAMA titers were high and persistent. Kaplan-Meier analysis showed a survival advantage for those with pattern II HAMA responses (i.e., a low, self-limiting HAMA response). Patients with pattern II responses had a survival rate of 73%, vs 33% for pattern I and 18% for pattern III. When patients with pattern

II HAMA response and/or four cycles of 3F8 were analyzed as a group, their survival was significantly better ( $p < 0.001$ ) than all other groups. It appears that a threshold number of treatment cycles (four 3F8 cycles) plus a pattern II HAMA response may be necessary to maintain tumor control.

### ***3.4. Induction of Ab3' Following Anti-G<sub>D2</sub> MAbs 3F8 Therapy Predicts Survival Among Patients with Advanced Neuroblastoma***

Because HAMA was mostly anti-idiotypic (Ab2), the author postulated that the subsequent induction of an idiootype network, which includes an anti-G<sub>D2</sub> (Ab3') response, is responsible for tumor control (66). Among the 34 stage 4 neuroblastoma patients treated with adjuvant 3F8, a 40% increase in Ab3' at 6 mo posttreatment (or a 50% increase at 1-yr posttreatment) was strongly correlated with longer progression-free survival ( $p < 0.005$ ) and overall survival ( $p < 0.02$ ). Among another 24 patients treated with 3F8 plus granulocyte-macrophage colony-stimulating factor (GM-CSF) for refractory neuroblastoma, a 40% increment in Ab3' was significant for predicting improved overall survival ( $p < 0.02$ ), but not progression-free survival ( $p < 0.1$ ). Kaplan-Meier analysis of all 58 patients suggested that a 40% increment in Ab3' response was highly correlated with both progression-free survival ( $p < 0.001$ ) and overall survival ( $p < 0.001$ ).

### ***3.5. Anti-GD<sub>2</sub> Anti-Ids***

Anti-Ids can be produced using the anti-G<sub>D2</sub> Ab, 3F8, as an immunogen (67). Lou/CN rats were immunized intraperitoneally with 3F8 F(ab')<sub>2</sub> emulsified in an equal volume of complete Freund's adjuvant. Six anti-idiotypic IgG<sub>1</sub> Abs were isolated (i.e., in decreasing avidity, C2D8 > Idio-2 > C2H7 > C4E4 > A1G4 > A2A6). The cross-reactivity of anti-Id was tested against a panel of myeloma proteins or MAbs against gangliosides, surface/cytoplasmic Ags on neuroblastomas and other malignancies, as well as other carbohydrate Ags. None of the myelomas and MAbs examined showed any cross-reactivity with 5/6 anti-Ids. The exception was A2A6, which was inhibited by OKB7 (CD21). C57Bl/6 mice immunized with these rat anti-Ids showed delayed B16 melanoma tumor growth, and improved survival. Tumor immunity was G<sub>D2</sub>-specific and induced only at low anti-Id doses. Both specific IgG Ab and T-cell responses to G<sub>D2</sub> and G<sub>D2</sub>-positive tumor cells were obtained. The induced anti-G<sub>D2</sub> humoral response was specific for G<sub>D2</sub> by immuno-thin-layer-chromatography, and no cross-reactivity against other gangliosides was detected. Anti-Ids also primed T-cells for in vitro cytotoxic T-lymphocyte (CTL) responses to G<sub>D2</sub>-oligosaccharide-bearing tumors. However, to sustain these antitumor IRs, repeated immunizations were necessary. Furthermore, mice immunized with anti-Id A1G4 most effectively rejected tumors that expressed both MHC-I and G<sub>D2</sub> Ags, were less well protected against tumors expressing only G<sub>D2</sub>, and were totally unprotected against those tumors without G<sub>D2</sub> (67).

### 3.6. T-Cell Recognition of $G_{D2}$ Oligosaccharides

Carbohydrate Ags rarely provide target epitopes for CTLs.  $G_{D2}$  is expressed on several human tumors and a small group of murine lymphomas (e.g., EL4 and RMA-S). Immunization of C57Bl/6 mice with irradiated EL4 cells stimulated a specific CTL response and protected these animals from challenge with EL4 lymphomas (68). The CTL activity resided in the  $CD4^-/CD8^+$  population, was dependent on T-cell receptors, was not removed by antinatural killer cell immunoabsorption, and was restricted to  $G_{D2}^-$  and H-2<sup>b</sup>-bearing targets. CTL activity could be completely inhibited by  $G_{D2}$ -oligosaccharide-specific MAbs and their  $F(ab')_2$  fragments, but not by IgG<sub>3</sub> myelomas or Abs against  $G_{D3}$  or  $G_{M2}$ . Soluble GD2 did not inhibit specific tumor lysis. RMA-S lymphoma cells ( $G_{D2}^+$ , H-2b<sup>-</sup>, TAP2-deficient) were resistant to  $G_{D2}$ -specific CTLs. Sialic acid-containing peptides, eluted from EL4 lymphoma cells, could stabilize H-2 molecules on RMA-S cells and sensitize them to  $G_{D2}$ -specific CTL activity. Control peptides (derived from VSV-NP and  $G_{D2}$ -negative lymphomas) could also stabilize H-2 on RMA-S, but were resistant to  $G_{D2}$ -specific CTLs. These H-2-binding peptides could be purified by anti- $G_{D2}$  affinity chromatography. These findings suggest the existence of a new class of naturally occurring epitopes for T-cells, in which branched-chain oligosaccharides are linked to peptides with anchoring motifs for the MHC-I pocket. Although the presence of  $G_{D2}$ -specific T-cells can be documented after vaccination with anti-Ids, it is unknown how anti-Ids are presented to T-cells.

More recently, similar results have been obtained with 1A7, a murine anti-Id against 14.G2a (69). In cynomolgous monkeys, 1A7 induced Ab3s without clinical toxicities (70). In patients with melanoma, Ab3 responses were detected following subcutaneous immunization with 1A7 plus QS21 as adjuvant. The Ab3s appeared to mediate Ab-dependent cell-mediated cytotoxicity. Toxicity consisted of local reaction at the site of injection, including induration and pain, plus mild fever and chills (71). A human anti-Id was recently produced in rabbits, following treatment with the murine 14G2a Ab, which induced both humoral and cellular IRs (72). The potential of anti-idiotypic vaccines for inducing IRs to  $G_{D2}$ -bearing tumors merits further clinical investigations.

## 4. POTENTIAL OF ANTI-IDIOTYPIC VACCINE STRATEGIES FOR BTs

Cellular immunity is inducible by anti-idiotypic vaccines. The cytotoxic potential mediated by T-cells can be substantial, as evidenced by the spectrum of autoimmune syndromes affecting the central nervous system (CNS) (73). To safely target immune cells against BTs, an Ag should preferably be tumor-specific, but absent in the normal CNS and other peripheral normal tissues.  $G_{D2}$  is found on neurons, pain fibers, and certain lymphoid tissues. Thus, it may not

be an ideal target Ag. However, other gangliosides (39) and glycoproteins (e.g., epidermal growth factor receptor [74] or 8H9 [75]) have much more restricted distributions and are potential target candidates. In addition, strategies that can channel the anti-idiotypic IR toward a TH<sub>1</sub> pathway may be useful as well. The ability of the anti-idiotypic network to activate T-cells has been postulated to be partly responsible for the antitumor response (26). Although overt autoimmune complications are possible (because of epitope cross-reactivity or spread), one can speculate that anti-idiotypic responses are part of a self-balancing network, and should naturally revert to equilibrium. Moreover, anti-idiotypic vaccines have advantages over native tumor Ags, which may be rapidly removed by *de novo* neutralizing Abs.

Nevertheless, significant limitations for anti-idiotypic vaccine strategies for BTs still remain. The existence of the BBB is a major obstacle for the delivery of any humoral immunity. Furthermore, CNS tumors (e.g., neuroblastomas and gliomas) can downregulate MHC-I and -II Ags, thereby escaping immune recognition by T-cells. Finally, an acquired state of immune suppression (partly as a result of the BT itself) can further compromise the host IR to even the best tumor vaccines. This remains one of the greatest hurdles to overcome in designing IT strategies.

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# 10 Radiolabeled Antibodies for Therapy of Brain Tumors

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*Carol J. Wikstrand, PhD, Michael R. Zalutsky, PhD,  
and Darell D. Bigner, MD, PhD*

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## 1. INTRODUCTION

The concept of antibody (Ab)-mediated targeting of therapeutic moieties to tumors was originally introduced by Ehrlich (1) in 1906. However, it was not until the work of Bale and Spar (2,3), Day et al. (4), and Pressman et al. (5) in animal models that techniques for delivery and monitoring of Abs to tissues in vivo were established. As reviewed elsewhere (6), the original animal model paired label studies—with  $^{125}\text{I}$ -labeled polyclonal antisera to rat lymphosarcoma tissue vs  $^{131}\text{I}$ -labeled irrelevant immunoglobulin G (IgG)—established that specific tumor targeting was achievable following systemic intravenous administration of targeting Abs (4). Such feasibility studies led to early clinical trials of radiolabeled polyclonal Abs in human glioma patients. In a study of  $^{131}\text{I}$ -labeled antifibrinogen (FIB) Abs administered to patients with intracranial tumors, approx 80% of the tumors, both primary and metastatic, exhibited Ab localization as detected by immunoscintigraphy (7). Because of the nonspecificity of the target antigen (Ag), as well as the heterogeneous distribution of FIB in tumors, the utility of targeting FIB for diagnostic or therapeutic purposes was concluded to be low. In a series of studies (8–10), Day and Mahaley demonstrated the suc-

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cessful localization of immunoabsorbent-purified  $^{131}\text{I}$ -labeled antihuman glioblastoma polyclonal rabbit Ig in 11/12 human glioma patients. Localization was demonstrated by presurgical radioscintigraphy, and autoradiography and tissue  $\gamma$  counting of surgically removed tissue. Although these studies conclusively demonstrated the localization of tumor-reactive Abs at levels sufficient for scanning purposes, the absolute dose localized to tumor was not sufficient for the delivery of therapeutic levels of radioactivity.

The use of polyvalent sera (the only reagents available in the 1960s) was complicated by several factors: the induction of Abs with multiple affinities to various tumor-associated Ags; the induction of Abs to normal brain-associated Ags and the need for absorption to remove them; and the inability to produce a consistent supply of the reactive Abs of interest. Not until the development of monoclonal antibody (MAb) technology by Köhler and Milstein (11) was further work on targeted Ab therapy of brain tumors and neoplastic meningitis possible. This technology simultaneously made possible both the isolation and permanent supply of monospecific, high-affinity Ab reagents and the definition of discrete marker epitopes of defined tissue distribution.

## **2. PRECLINICAL INVESTIGATIONS: COMPONENTS, DESIGN, AND RESULTS**

### ***2.1. Components of Successful Ab-Mediated Tumor Therapeutic Approaches***

The successful *in vivo* localization of a specific Ab to a tumor-associated, and preferably tumor-specific, epitope is dependent on many factors: Ag stability (e.g., shedding, internalization), accessibility, and density within the tumor; stability and affinity of the localizing Ab; and the kinetics of transport within the tumor (e.g., tumor vascularity, vascular permeability, extracellular fluid dynamics, and interstitial pressure) (12). An added complication of Ab localization to intracranial tumors is the presence of the blood–brain barrier (BBB). However, this barrier has been demonstrated to be heterogeneous in experimental human tumor xenograft animal models and in human patients, because of the reduced integrity of tight junctions in the capillary endothelial cells of the tumor neovasculature, intratumoral variation in permeability, and altered intratumoral blood flow (13,14). Since the early 1980s, the characterization of several targetable glioma-associated and glioma-restricted epitopes have been reported, enabling the development and optimization of specific MAb targeting of central nervous system (CNS) tumors.

### ***2.2. Ag Targets and Associated MAbs***

Early work with MAbs to both CNS and systemic tumor tissues or derived cell lines resulted in the majority of reagents detecting tumor-associated Ags

exhibiting variable and unpredictable expression in normal tissues. This normal tissue cross-reactivity contributed to the early observations of very low (0.001–0.01% injected dose/g [ID/g]) or no specific tumor localization by systemically administered MAbs (15). These experiences led to a modification in therapeutic MAb approaches centered on the concept of operational specificity within a compartmental administration setting. This involved the targeting of an Ag present in CNS tumors, but not normal CNS tissue, irrespective of systemic Ag expression by restricted routes of administration (e.g., the intrathecal space for leptomeningeal disease or intratumoral/intracavitary administration to spontaneous or surgically-created cysts in primary or metastatic tumors in the CNS). Several Ag/Ab systems suitable for such approaches have been developed.

### 2.2.1. TENASCIN

The first and consequently most extensively investigated operationally specific glioma-associated Ag is tenascin, a hexabrachial molecule composed of six subunits arranged radially and joined in a central knob at their amino-terminal ends (16,17). Each identical, 200–300 kDa arm consists of a linear array of repeated sequences, beginning with a cysteine-rich amino-terminal region involved in oligomerization, a domain of 14–15 epidermal growth factor (EGF)-like repeats, a domain of 8–15 fibronectin type III (FNIII)-like repeats, and a carboxy-terminal FIB-like domain (16,17). Alternative splicing, especially within the FNIII domain, results in different-sized subunits and intact molecules, the expression of which may vary in different cell and tissue types (18,19). Tenascin is prominently expressed in fibrillary matrix and perivascular patterns in mesenchymal tumors and carcinomas, including gliomas, fibrosarcomas, osteosarcomas, melanomas, Wilm's tumors, and colon, breast, lung, and squamous cell carcinomas. In gliomas, the intensity of tenascin expression is directly correlated with the degree of malignancy, with >90% of glioblastoma multiforme (GBM) cases exhibiting tenascin deposition (17,20). The predominant localization in all grades of gliomas, however, is in and around vascular channels and has been postulated to correlate with angiogenesis (21,22). MAbs specific for human tenascin have been generated (20,23,24). BC-2, the most prominent MAb used in European clinical trials (25), binds to an epitope within the FNIII-like repeat, AD1, and, to a lesser extent, C. These repeats share 83% homology (23). MAb 81C6, used in clinical trials in the United States, binds to an epitope within the alternatively spliced FNIII-like CD region (24). Because several normal tissue-associated tenascin variants are not recognized by MAb 81C6, greater specificity is obtained with this MAb reagent.

### 2.2.2. GP240

The chondroitin sulfate proteoglycan characteristic of gliomas and melanomas, defined by its protein core as GP240, exhibits an extracellular matrix

deposition pattern similar to that of tenascin (21). As defined by two MAbs that recognize separate epitopes on the molecule (9.2.27 [26] and Mel-14 [27]), GP240 is elaborated by gliomas, the hyperplastic vascular endothelial cells within them and melanomas (21,28). The predominant localization pattern of GP240 in gliomas (28% of astrocytomas and 57% of GBM) is blood vessel-associated and fibrillar (17,21).

### 2.2.3. EPIDERMAL GROWTH FACTOR RECEPTOR AND ITS VARIANT FORM EGFRvIII

As summarized extensively elsewhere (21,29) the wild-type epidermal growth factor receptor (EGFR) is an approx 170-kDa transmembrane glycoprotein with an extracellular domain ligand-binding site for EGF and transforming growth factor- $\alpha$ , a transmembrane segment, and an intracellular domain with tyrosine kinase activity. Epithelial cells in most tissues of the body express wild-type EGFR, which is most abundant in the liver (30). Because of the variety of immunological reagents and tissue formats investigated, estimates of the incidence of EGFR expression in human gliomas vary considerably. Nevertheless, the consensus of several series is that EGFR positivity in gliomas increases with grade (i.e., 27–57% for astrocytomas, 71–94% for anaplastic astrocytomas [AA], and 68–90% for GBM, as summarized in ref. 21). A large number of polyvalent and monospecific reagents to EGFR exist (21), with the most pertinent for therapeutic applications being the murine MAbs EGFR-1 (IgG<sub>2b</sub> [31]) and MAb 425 (IgG<sub>2a</sub> [32]). Gene amplification related to increasing grade of glioma malignancy has been found to occur in 50% of all human GBMs (33). Recent studies have confirmed that approx 37–58% of GBMs have amplifications of the *EGFR* gene, with accompanying gene rearrangement (29). Greater than 50% of these cases contain the *EGFRvIII* rearrangement, an in-frame deletion of NH<sub>2</sub> terminal amino acid residues 6–273 from the extracellular domain of EGFR, resulting in a 145-kDa molecule with a unique primary sequence, characterized by an inserted glycine residue at position 6 between amino and residues 5 and 274. MAbs specific for this non-EGF-binding variant of EGFR have been characterized (35,36); and 50–52% of all human gliomas (58–61% of GBMs) express EGFRvIII as defined by murine MAb L8A4 (35,36). Both humanized (chL8A4) and single-chain variable region (scFv) immune probes of EGFRvIII (Mr-1) have been generated (37–39).

### 2.2.4. NEURAL CELL ADHESION MOLECULE

The neural cell adhesion molecule (NCAM) is a member of the Ig supergene family. Within the adult CNS, discrete isoforms of NCAM exist, probably generated by alternative splicing (21). As reviewed by Molenaar et al. (40), NCAM is expressed in medulloblastomas, other primitive neuroectodermal tumors, astrocytomas, oligodendrogliomas (ODGs), ependymomas, meningiomas, and



neuroblastomas. Although human normal and tumor tissues express the same battery of isoforms, the majority of tumor tissues express heavily sialylated embryonic NCAM molecules (41,42). MAbs specific for sialylated NCAM, used in immunotherapeutic approaches to CNS neoplasia, include ERIC and UJ13A (43).

### 2.3. Ab-Related Factors

Assuming that requisite specificity and affinity for target Ags are established, there are several factors associated with the administered Ab molecule that influence successful localization.

#### 2.3.1. PRODUCTION OF ANTI-ABS-HUMAN ANTIMOUSE AB

A single systemic administration of murine MAb is sufficient to induce a human antimouse antibody (HAMA) response in human patients, primarily against Ig-constant regions (44,45), although anti-idiotypic reactivity is detectable (46). This host response can be beneficial if Ag-reactive Ab3 (or Ab1') molecules are produced (47,48), but it is more likely to be deleterious by compromising the activity of the administered therapeutic MAb by complexing circulating MAbs, altering their pharmacokinetic properties, and/or causing immune complex hypersensitivity/allergic reactions (44).

#### 2.3.2. USE OF AB FRAGMENTS AND ENGINEERED CONSTRUCTS

The first approach to reducing the immunogenicity of administered MAbs and increasing Fc-mediated effector function in the human recipient was the creation of chimeric mouse/human Abs, with the variable region of murine MAbs inserted into a human Ig framework. Several such chimeric MAbs have been introduced in clinical trials. Although variable in ease and incidence of induction, anti-Ab responses have also been observed with chimeric molecules, primarily to the mouse variable region and the interface of the mouse variable and human constant regions (45,49).

The generation of Ig fragments by either enzymatic cleavage (Fab, F[ab']<sub>2</sub>) or by genetic engineering (scFv, [scFv]<sub>2</sub>) offers the prospects of reduced immunogenicity by mouse Ig constant regions, absence of Fc-mediated binding, rapid normal tissue clearance, and potentially higher tumor penetration (50,51). Compared to intact MAb or Fab and F(ab')<sub>2</sub> fragments, scFvs have demonstrated higher tumor:normal tissue ratios, despite a lower percent ID/g, which is a reflection of their intrinsic lower affinity and rapid clearance (50,52). Successful imaging of primary tumor and metastatic deposits in patients with CEA-expressing tumors by <sup>123</sup>I-labeled scFv anti-CEA has been reported (53). The rapid tumor uptake and normal tissue clearance of scFv are ideal parameters for short half-life nuclides, such as astatine-211 (<sup>211</sup>At) for therapeutic and <sup>18</sup>F for positron emission tomography (PET) imaging approaches (37). As is discussed in Section 3.3., F(ab')<sub>2</sub> fragments of an anti-GP240 MAb (Me1-14) are



currently in phase I/II clinical trials, and a scFv (Mr1) specific for the EGFRvIII has recently been characterized, which exhibits requisite levels of immunoreactivity following iodination to warrant further study (37).

### 2.3.3. NUCLIDES AND LABELING CHEMISTRIES

The optimal process for selection of a nuclide considers the physical aspects of the targeted tumor: size, location, and geometry. The majority of preclinical investigations of labeled immunotherapeutic MAb have used  $\beta$ -emitting radio-nuclides such as  $^{131}\text{I}$ , which have a range in tissue of several millimeters and are optimal for large tumors or those with heterogeneous uptake of labeled MAb (54,55). For tumors of less mass, such as leptomeningeal metastases and residual tumor following debulking,  $\alpha$ -particle-emitting nuclides, such as  $^{211}\text{At}$ , with a range of only a few cell diameters, would be optimal (54,55). For internalizing receptor-MAb or Ag-Ab complexes, an isotope such as  $^{123}\text{I}$ , which emits Auger electrons with a subcellular range, would be a practical approach to spare normal cells that do not internalize the agent (54).

The labeling chemistry employed is as crucial in the ultimate MAb destination as is choice of nuclide. Because conventional iodination (Choramine-T, Iodogen) methods result in extensive dehalogenation *in vivo*, presumably by the action of deiodinases acting on iodotyrosine residues (54), alternative iodination methods have been developed. The most successful have been the methods summarized by Garg et al. (56): synthesis of *N*-succinimidyl 3- $^{131}\text{I}$ iodobenzoate (SIB) or *N*-succinimidyl-5- $^{131}\text{I}$ iodo-3-pyridinecarboxylate (SIPC), followed by coupling of SIB or SIPC to the Ab by an acylation reaction. Paired label analyses of SIB-, SIPC-, or Iodogen-iodinated MAb 81C6 *in vitro*, and in localization assays in athymic mice bearing tenascin-expressing human glioma xenografts *in vivo*, established that the immunoreactive fractions of SIB- and SIPC-labeled 81C6 were higher than those obtained with Iodogen. More importantly, the tumor uptake of SIB-iodinated 81C6 was higher and induced significantly longer tumor growth delay in treated animals than did Iodogen-iodinated 81C6 (57).

In contrast to the studies investigating the extracellular matrix-localized Ag, tenascin, internalized MAb-Ag complexes require radiolabeling methods that will minimize loss of radioactivity following lysosomal processing. Because lysosomes retain positively charged compounds, SIPC, which has a positive charge on its pyridine ring, would potentially increase intracellular retention of internalized MAb nuclide. *In vitro* and *in vivo* assays, comparing the anti-EGFRvIII MAb L8A4, iodinated by Iodogen, tyramine cellobiose (TCB: a nonmetabolizable residualizing disaccharide conjugate), SIB, and SIPC, established that SIPC increased intracellular retention of activity up to 65%, compared to Iodogen and SIB (58). Although total ID/g tumor uptake was slightly higher in EGFRvIII-positive tumors with TCB-iodinated L8A4 (37 vs 33% at

24 h), the higher blood levels seen following TCB-mediated iodination resulted in tumor localization indices 3–4 × higher for SIPC–MAB than for TCB–MAB (39). This established SIPC as an effective iodinating compound for internalizing MABs.

Similar improvements for labeling with short-half-life  $\alpha$ -emitters, such as  $^{211}\text{At}$ , have also been developed. Because astatine readily dissociates from proteins in the presence of oxidants, such as chloramine-T or Iodogen, *N*-succinimidyl 3- $^{211}\text{At}$ ]astatobenzoate (SAB) was coupled to MAB by incubation in pH 8.5 borate buffer (59). SAB-mediated labeling of both antitenascin MAB 81C6 and  $\text{F}(\text{ab}')_2$  fragments of anti-GP240 MAB Mel-14 was successful in terms of yield and specific activity (59,60). In addition, tumor accumulation of the  $^{211}\text{At}$ -SAB-MABs was essentially identical to that of  $^{131}\text{I}$ -SIB–MABs for 24 h, at which time  $^{211}\text{At}$  activity was decayed to approx 10% of initial levels. Unfortunately,  $^{211}\text{At}$ - $\text{F}(\text{ab}')_2$  fragments were catabolized more rapidly than  $^{131}\text{I}$ - $\text{F}(\text{ab}')_2$  fragments, resulting in high normal tissue levels of  $^{211}\text{At}$ . This was not observed with intact Ig (60,61). In order to investigate  $^{211}\text{At}$ -labeling of an internalizing MAB, *N*-succinimidyl 5- $^{211}\text{At}$ ]astato-3-pyridinecarboxylate (SAPC) was developed for coupling to MAB L8A4. Affinity, immunoreactive fraction, rates of internalization and catabolism, and localizing properties *in vivo* were virtually identical to those for [ $^{131}\text{I}$ ]SIPC-L8A4, thus making  $^{211}\text{At}$ -labeling possible for internalizing Ag–MAB systems (62).

## 2.4. *In Vivo* Modeling Systems for Preclinical Optimization

Current development protocols test all MABs and engineered constructs in a variety of nuclide-labeling formats in athymic rodent–human tumor xenograft models, in attempts to determine relative stability (retention of label and affinity), ability to localize specifically to tumor, nonspecific binding patterns, and clearance in an intact mammalian system. Although extrapolation to the human patient is difficult (because vasculature, cell surface receptors, and mass dilution effects are uncontrollable variables), important prognostic data can be obtained from such modeling. The following summary is in no way comprehensive, but it highlights the predictive nature of these investigations.

### 2.4.1. TUMOR PERMEABILITY AND IMAGING STUDIES

Early dramatic examples of the predictive nature of athymic rodent xenograft models were the reports by Bourdon et al. (63) and Bullard et al. (64) of specific  $^{131}\text{I}$ -MAB 81C6 localization to intracranial human glioma xenografts using  $\gamma$ -camera imaging and correlative tissue counting in paired-label experiments. These studies not only established the feasibility of imaging rodents as predictions of success in human patients, but showed the utility of the intracranial tumor xenograft model as well. This model was recently used to examine the distribution of  $^{131}\text{I}$ -MAB 81C6 throughout tumor and brain around tumor (BAT)

concurrently with capillary permeability (using  $^{14}\text{C}$ - $\alpha$ -aminobutyric acid) and tumor cell proliferation (using  $^3\text{H}$ -thymidine) in a triple autoradiographic study (65). This model system allowed simultaneous localization of labeled MAb to all areas of proliferating cells and beyond the region of increased capillary permeability in the BAT, which was assumed to result from bulk flow away from the tumor edge.

#### 2.4.2. COMPARATIVE LOCALIZATION STUDIES

A primary advantage of *in vivo* rodent models is the ability to compare putative localizing reagents: parent molecule to derived fragments or engineered forms; the same targeting MAb with various nuclide/labeling chemistries; the same targeting compound by various routes to different sites. The simplest approach, paired-label analysis of reactive vs irrelevant control immune reagents, was illustrated in a large comparative study of six glioma-reactive MAbs (66). In that study, distinct localizing properties and patterns of members of a potential antiglioma MAb library were determined. The direct comparison of the localizing capacity of an intact MAb and its derived Fab fragment in both subcutaneous and intracranial xenograft sites was reported by Colapinto et al. (67), establishing the need for high-affinity fragments with faster clearance rates. The comparative localization of the mouse/human chimeric construct (ch81C6) to the parental murine MAb 81C6 ( $\mu$ 81C6), in paired-label studies of sc human glioma xenografts, directly demonstrated the superior tumor uptake of the chimeric molecule compared to the murine parent. Furthermore, there was lack of enhanced labeling of ch81C6 by the SIB reagent compared to Iodogen, which was the converse of results obtained with  $\mu$ 81C6 (68). This study highlighted the fact that, despite virtually identical affinity constants and immunoreactive fractions (IRF) *in vitro* (with the exception of a higher IRF for murine 81C6), significant differences in localization were seen, establishing the need for *in vivo* comparisons. A similar analysis with the internalizing murine MAb  $\mu$ L8A4 and its chimeric form, chL8A4, indicated no difference between the forms in terms of localization, internalization, and intracellular processing by tumor cells *in vitro* or *in vivo*. However, there was a longer blood and normal tissue residence time for chL8A4, indicating a different catabolism of the chimeric form *in vivo* (39). Other paired-label comparative studies have examined the same targeting agent in subcutaneous and intracranial tumor models (37,63). In the later study, Kuan et al. (37) demonstrated that, although systemic administration of the anti-EGFRvIII-reactive scFv MR-1 led to relatively low levels of tumor accumulation, intratumoral administration resulted in high specific localization of MR-1, compared to irrelevant control scFv, indicating that compartmental introduction of these small, rapidly cleared constructs would be advantageous *in vivo*. To examine the possible first-pass advantage of intracarotid (ic) vs intravenous (iv) administration for intracranial tumors, Lee et al. (69) simulta-

neously administered  $^{131}\text{I}$ -MAb 81C6 and  $^{125}\text{I}$ -MAb 81C6 by either ic or iv routes to rats bearing intracranial tenascin-positive human glioma xenografts. Isotope and route varied in repeat experiments. A small, but significant, advantage in terms of higher levels of percent ID/g and localization index to tumor was demonstrated over 3 d for ic-administered Ab. This observation suggested that this route of administration may well be optimal for administration of Fab or  $\text{F}(\text{ab}')_2$ , which exhibit higher blood-to-tissue transfer constants and rates of blood clearance than intact Ig (15).

### 2.4.3. HYPERTHERMIA

Manipulation of tumor hemodynamics has been intensively investigated as a means of increasing delivery of therapeutic agents to tumor. Hyperthermia is attractive, because it concomitantly decreases tumor interstitial pressure while increasing blood flow and vascular permeability (70). In a series of experiments with either labeled Mel-14  $\text{F}(\text{ab}')_2$  or ch81C6 MAb localization to D54 MG Ag-positive tumors in the legs of athymic mice, Zalutsky et al. (70) and Hauck et al. (71) have demonstrated that hyperthermia ( $42^\circ\text{C}$  being optimal) for 4 h, following administration of Iodogen-iodinated specific MAbs, increased tumor localization (in terms of percent ID/g) approx 3–4-fold for up to 96 h.

### 2.4.4. THERAPY STUDIES

Athymic rodent models also allow the comparative investigation of the therapeutic potential of labeled immune constructs. Straightforward dose escalation studies of  $^{131}\text{I}$ -MAb 81C6 or  $^{131}\text{I}$ -MAb Mel-14  $\text{F}(\text{ab}')_2$  to either subcutaneous or intracranial glioma xenografts in nude mice demonstrated prolonged survival, tumor regression, and, in the case of  $^{131}\text{I}$ -MAb 81C6, apparent cures (28,72,73). In addition, these studies described methodology for dosimetry calculations in rodent models, through a comparison of whole-body dose determination by measurement with a dose calibrator and direct tissue counting by  $\gamma$ -counter. Despite the admittedly highly permeable nature of the D54 MG glioma model used in these studies (72), it has proven useful for demonstrating the efficacy of treatment of intracranial tumors for both intact MAbs and  $\text{F}(\text{ab}')_2$ .

Perhaps the most elegant model system has been the development of an intrathecal model for neoplastic meningitis in athymic rats (13). As summarized by Fuchs et al. (51), leptomeningeal dissemination of medulloblastoma, ependymoma, adult gliomas, and metastatic tumors (such as lung and breast carcinomas and melanoma) is not suitable for conventional external radiotherapy (RT) because of CNS and bone marrow toxicity-limiting doses. Intrathecal administration of short-range  $\alpha$ -emitting nuclides coupled to specific MAbs would have the advantage of confined delivery of high radiation doses to tumor, sparing normal tissues. Initially, the tenascin-positive rhabdomyosarcoma cell line, TE-671, was established in this model, followed by D54 MG. Specific localiza-

tion to TE-671 tumor by  $^{131}\text{I}$ -MAB Mel-14 F(ab')<sub>2</sub> was readily demonstrated, and there was a significantly more rapid clearance of the nonspecifically labeled control preparations from the cerebrospinal fluid (CSF) (70). However, when the therapeutic potential of  $^{131}\text{I}$ -MAB Mel-14 F(ab')<sub>2</sub> was examined in this model, it became apparent that  $^{131}\text{I}$ -control F(ab')<sub>2</sub> preparations also resulted in modest prolongation in median survival at high doses of  $^{131}\text{I}$ , which was directly attributable to the long path length of the emitted  $\beta$ -particles relative to the small confines of the rat intrathecal space. Limiting the effective range of radiation by use of  $^{211}\text{At}$  was then investigated. TE-671 tumor-bearing rats receiving  $^{211}\text{At}$ -MAB 81C6 in escalating (4, 7, and 13  $\mu\text{Ci}$ ) doses exhibited increases in median survival, even at the lower doses, compared to identically labeled irrelevant control  $^{211}\text{At}$ -MAB M45.6. In another study at the 12  $\mu\text{Ci}$  dose, survival was increased by 113%, with 33% apparent cures and no significant toxicity (55,74).

Athymic rodent model systems have allowed: the direct comparison of localizing and therapeutic capacities of intact MABs, their derivatives, and re-engineered constructs; *in vivo* stability and activity of various nuclides and radiolabeling chemistries; and routes of administration, including clearance rates following whole-body and compartmental approaches. Direct extrapolation to human treatment is not totally possible, but efficacy and lack of toxicity in these systems have been of predictive value in human trials, as is discussed below.

### 3. CLINICAL INVESTIGATIONS

#### 3.1. Preliminary Localizing Results

The successful localization of labeled MABs and constructs in rodent models and the early polyvalent serum work of Mahaley and Day led to a series of imaging studies in human glioma patients with MABs to various Ags expressed on the tumors. Zalutsky et al. (75) reported the selective tumor localization of  $^{131}\text{I}$ -MAB 81C6 in patients with gliomas and other intracranial malignancies by  $\gamma$ -camera imaging 1–3 d following simultaneously administered doses of targeting and irrelevant  $^{125}\text{I}$ -MAB 45.6. Comparison with direct counting of biopsied tissue and blood samples allowed correlation of detected images with calculated localization indices and ID/g. The average tumor-to-normal brain ratio was 25:1, with a high of 200 seen in some samples. Localization indices of specific tumor vs blood levels were approx 5, corrected for irrelevant Ig values, indicating an immunospecific uptake by tumor. Subsequently, Zalutsky et al. (15) investigated the potential advantage of *ic* vs *iv* administration of either MAB 81C6 or MAB Mel-14 F(ab')<sub>2</sub> fragments to patients in paired-injection protocols, in which one-half of the administered dose of the given MAB was labeled with  $^{125}\text{I}$  and given simultaneously *iv* with the other  $^{131}\text{I}$ -labeled half-dose administered *ic*. In contrast to the results observed in the athymic rat model study, no difference in tumor uptake or tumor: normal brain uptake ratios was seen, indicating a lack of

tumor delivery advantage for ic administration in human patients. As a follow-up to this study, a SPECT imaging study with a broad range of  $^{123}\text{I}$ -MAB 81C6 doses was performed. Excellent images in all patients and good correlations between MAb imaging and anatomic neuroimaging studies were obtained (76). Dosimetry calculations indicated that only approx 7 Gy could be delivered to intracranial gliomas, because higher  $^{131}\text{I}$  activities delivered systemically, would deliver nonacceptable levels of radiation to the tenascin-rich liver. These results suggested that nonsystemic routes of administration would be preferable for anti-tenascin MABs. In a similar study of glioma patients with  $^{123}\text{I}$ -anti-EGFR MAB EGFR1, Kalofonos et al. (77) obtained apparently successful MAB localization in 9/12 patients. However, in 3/6 of these cases in which paired-label evaluation was performed, tumor-associated images were also obtained with the irrelevant MAB. These studies demonstrate the feasibility of localizing labeled, therapeutic intact MAB or fragments to the human CNS following systemic administration. However, the tumor doses achievable and the extra-CNS toxicity potential as a result of Ag expression by normal tissue (namely the liver) would appear to limit therapeutic efficacy using systemic administration.

### ***3.2. CNS Tumor Therapy via Systemic Administration***

The first therapeutic radiolabeled MAB trials with glioma patients were conducted with anti-EGFR MABs (77,78) and antiplacental alkaline phosphatase (77). Ten glioma patients who had previously shown positive imaging results were treated with escalating doses (40, 75, 100, or 140 mCi) of  $^{131}\text{I}$ -labeled MAB (EGFR1 or H17E2) by either iv or ic routes (77). Radiological improvement was seen in only two patients, both of whom had received ic injection. One of these patients (EGFR1-injected) survived for 3 yr. An additional four patients, who initially demonstrated clinical improvement, relapsed and died 6–9 mo after MAB therapy. In this small series, there was no discernible systemic or CNS toxicity, nor association between Ab administered or injection route and outcome. In a larger study of anti-EGFR MAB 425 therapy by systemic administration, Brady et al. (78) treated 25 patients (10 AAs and 15 GBMs) who had previously received external beam radiation following surgical debulking or biopsy with  $^{125}\text{I}$ -labeled-anti-EGFR MAB 425 administered iv or ic in multiple doses. The administered activity at each injection was 35–90 mCi (mean 50 mCi), with a total of 40–224 mCi (mean total dose, 151 mCi) for the complete regimen. At 18 mo, 48% of the patients (5 AAs and 5 GBMs) were alive, with a projected median survival of 67 wk. Treatment was well tolerated without overt immunologic or toxic reactions. Even after multiple injections, the authors could not detect HAMA responses in any of the patients. Although radiation dose delivered to tumor was not determined in either of these studies, previous localizing and imaging studies (15,76,79) suggest that the 0.001–0.004%



ID/g would be delivered by these routes to the intracranial glioma, which probably would be insufficient for therapeutic purposes.

### 3.3. CNS Tumor Therapy via Intrathecal Administration

While the efficacy of ith administration of labeled MAbs was being established in athymic rat studies, initial trials in human patients were initiated in the UK in the late 1980s. A series of 15 patients with neoplastic meningitis secondary to metastatic melanoma or primary brain tumor extension were treated with a single intrathecal injection of  $^{131}\text{I}$  MAb (i.e., either MAb UJ181.4 to the human neural-glial adhesion molecule [21], antitenascin MAb 81C6, or anti-GP240 MAb Mel-14). Injected activity varied between 24 and 60 mCi. Of six evaluable patients, four exhibited clinical response as measured by CSF and computed tomography parameters. Toxic effects of this treatment included reversible transient bone marrow suppression, headaches, and seizures. These were deemed treatable and acceptable (80). That group expanded their studies to include leukemia and lymphoma metastatic to the CNS. Administration of  $^{131}\text{I}$ -labeled MAb (629–1702 medulloblastomaq) to pediatric patients with meningeal acute lymphoblastic leukemia intrathecally by Ommaya reservoir or lumbar puncture resulted in 6/7 transient responders, all of whom relapsed by 12 wk. Despite the clinical outcome, this study and analysis of the previous data reported by Moseley et al. (80) provided valuable systemic and CNS dosimetry measurements: red bone marrow received 0.6–2.2 Gy; the dose to the subarachnoid CSF was 12.2–25.3 Gy, which was 40–140  $\times$  higher than that to whole brain (81,82).

The intrathecal administration of  $^{131}\text{I}$ -labeled antitenascin MAb 81C6 to pediatric (9) and adult (40) patients with leptomeningeal neoplasms (2 astrocytomas, 2 anaplastic mixed gliomas, 6 AAs, 28 GBMs, 1 spinal cord GBM, 7 ependymomas, 1 anaplastic ODG, 2 medulloblastomas, and 1 breast cancer) has been investigated in phase I studies. The preliminary results have been reported (16,83). Single, escalating intrathecal doses (40–100 mCi) of  $^{131}\text{I}$ -labeled antitenascin MAb 81C6 were administered to cohorts of 3–6 patients. The maximum tolerated dose (MTD) for adults was determined to be 80 mCi; the MTD for pediatric patients was not reached at 40 mCi. Hematological toxicity was the dose-limiting factor. No grade III/IV nonhematological toxicities were encountered. A partial response was observed in two patients and disease stabilization in 25/51 (49%), with nine patients progression-free at >138 wk (range, 138–324) post-treatment. Roughly one-half of the treated patients developed positive HAMA titers, but re-treatment in two patients was not associated with allergic or other side effects. Absorbed radiation doses to the subarachnoid space were estimated to be 14–34 Gy, with no evidence of neurotoxicity.

Similarly, a phase I study of intrathecal administration of  $^{131}\text{I}$ -labeled MAb Mel-14 F(ab')<sub>2</sub> to patients with metastatic melanoma (11) or neoplastic meningitis (4 GBMs, 2 ODGs, 2 medulloblastomas, 1 anaplastic ependymoma) has



been reported (16,84). A total of 20 patients (2 pediatric and 18 adult) were treated with escalating doses (40–100 mCi). Four patients exhibited complete CSF responses (two consecutive negative CSF cytologies after initial positive cytology), and 2 had partial radiographic response. The range in survival following treatment was 4–83 wk, with 3 adult long-term survivors (2 ODGs and 1 melanoma) 119–278 wk from RT, as of this writing. The average survival of melanoma neoplastic meningitis is 3 mo, so it is possible that survival prolongation did occur in this phase I study.

### ***3.4. CNS Tumor Therapy via Intratumoral Administration***

Direct instillation to the tumor bed has been investigated for glioma therapy as a means of locally concentrating the therapeutic dose, avoiding systemic targeting and toxicity, and potentially preventing HAMA induction. The most extensive series of recurrent glioma patients, treated intratumorally via one or two indwelling or removable catheters, has been reported by Riva et al. (85). Antitenascin <sup>131</sup>I-labeled MAbs BC-2 and BC-4 were administered in escalating doses of radioiodine (15–57 mCi), with 15/24 patients receiving multiple doses. Imaging studies suggested that the mean 24-h tumor-to-normal brain ratio achieved ranged from 5.5 to 32.8 (mean = 16.6), with an average %ID/g of 2.4%. On average, delivered dose was 240–270 Gy for the first two cycles, declining in seven patients to 100 Gy for the third cycle. The cumulative mean dose achieved overall was 517 Gy. Systemic toxicity was not observed, although systemic sensitization occurred, because 5/17 patients developed HAMA titers. Median survival was 68 wk, with 5/17 patients surviving at least 94 wk. Although these results were encouraging, the pattern of dose distribution within injected tumors and to surrounding BAT following intralesional injection was not determined in this study.

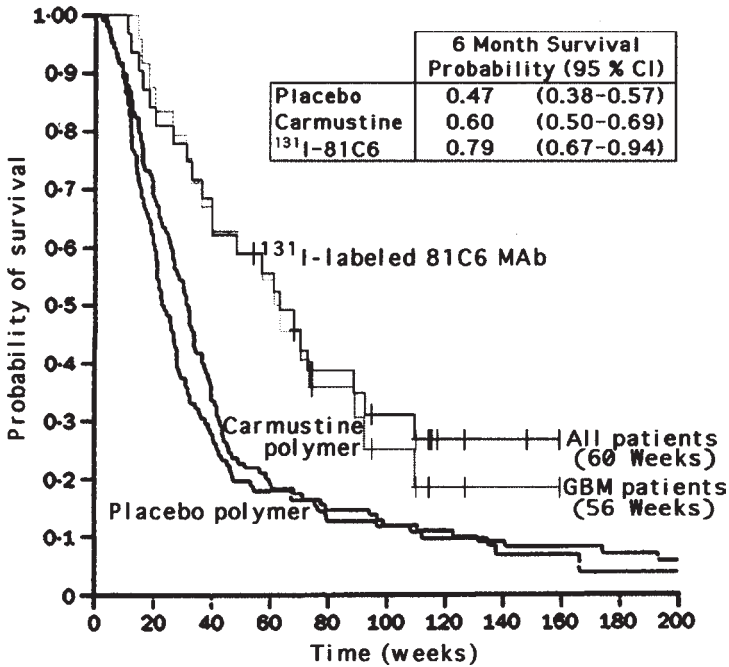
The diffusion characteristics of <sup>131</sup>I-antitenascin MAb 81C6, administered intratumorally, was investigated in a series of seven glioma patients reported by Thomas et al. (86). Following intratumoral administration of labeled Ab by one or two catheters, the distribution of uptake was evaluated by postadministration SPECT imaging and compared to pretreatment diagnostic scans using surface landmark correlations. In two patients, autoradiography and tissue scintillation counting performed postmortem was also correlated with the obtained images. Diffusion distances of 2–2.5 cm in three orthogonal planes were estimated, with a mean dose of 32.5 Gy to tumor and <0.4 Gy to normal tissue. However, even with two catheters, diffusion throughout the entire tumor was not obtained.

### ***3.5. CNS Tumor Therapy via Spontaneous or Surgically Created Resection Cavity Administration***

The lack of unrestricted diffusion throughout tumor tissue following intratumoral administration was attributed to interstitial pressure (12) and docu-

mented by Thomas et al. (86). This led to the investigation of intracavitary administration, either to gliomas recurring as spontaneous cysts or to purposefully created surgically created resection cavities (SCRC). In an extension of their earlier studies, Riva et al. (25,87) summarized results with 62 glioma patients (58 GBMs, 4 AAs) following multiple infusions of  $^{131}\text{I}$ -antitenascin MAbs BC-2 and BC-4. Thirty-one recurrent glioma patients had received initial surgery and external beam therapy, with relapse at the original site within 2–15 mo of surgery. A second surgery was performed. In 16/31 patients, significant debulking was achieved; the remaining 15 had macroscopic inoperable lesions. For 31 newly diagnosed patients who had initial surgery and external RT followed by radioactive Abs, 24/31 were considered to have minimal disease, and seven had macroscopically evident unresectable disease. Administration of radiolabeled MAb, through the reservoir of an inserted Rickham catheter, was within 10–30 d of the last RT regimen for newly diagnosed patients, and within 2 wk of second surgery for recurrent patients. For all patients the mean ID/g of tumor after 24 h was 3.1%, with a mean dose absorbed by tumor tissue of 526 Gy/cycle. For patients receiving multiple doses, cumulative dose exceeded 2000 Gy. There were only mild toxicities, consisting of headache and nausea. For all patients, median survival was 99 wk, with relapse-free intervals of 52 wk for newly diagnosed patients and 21 wk for recurrent patients. When stratified by disease status at time of treatment, the median survival for patients with minimal disease was 108 wk, and for macroscopic disease was 68 wk. As would be expected, the response rate in the minimal group was far better than that in the macroscopic disease group (70 vs 17%).

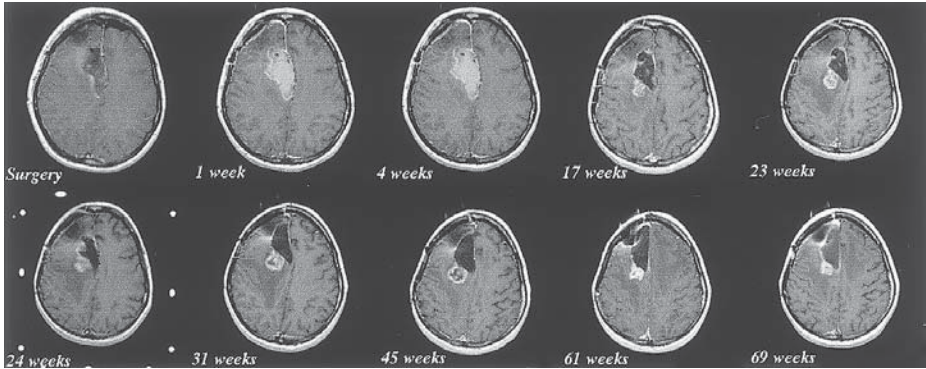
Bigner et al. and Cokgor et al. (88,89) have summarized the results of a phase I trial of 34 previously irradiated patients with recurrent or metastatic brain tumors following a single injection of  $^{131}\text{I}$ -antitenascin MAb 81C6. Cohorts of 3–6 patients were treated with escalating doses of  $^{131}\text{I}$  starting at 20 mCi, with 20 mCi incremental increases, via SCRC. Dose-limiting toxicity was reached at 120 mCi. Three of 5 patients developed significant clinical neurotoxicity, and one developed major hematologic toxicity. No patient treated with lower doses experienced neurologic toxicity. In the nine patients for whom retrospective dosimetry was performed at the determined MTD of 100 mCi, the estimated average absorbed radiation dose to the SCRC interface ranged from 65 to 1300 Gy (mean 450 Gy), and that to normal brain was 6.5 Gy (90). Figure 1 presents a Kaplan-Meier plot for the patients in that series. The median survival after radiolabeled MAb therapy for all patients was 60 wk, with a 1-yr probability of survival of 0.59. For those with GBM, the median survival was 56 wk, with a 1-yr survival probability of 0.57. For comparative purposes, these results were plotted vs those obtained in a recent study (91) of a similar group of patients receiving external beam RT and carmustine wafer implantation into resected



**Fig. 1.** Kaplan-Meier plot of the survival of patients who had recurrent disease treated with <sup>131</sup>I-labeled 81C6 MAb. Data from ref. 91 for a similar group of patients treated with external beam RT and placebo or carmustine polymer wafers is included. The 6-mo survival probability for placebo, carmustine, and all patients in the immunotherapy trial is presented.

tumor beds. The median survival times obtained with labeled MAb are twice those achieved in the carmustine study and longer than those reported for conventional surgery, high-dose brachytherapy, and stereotactic radiosurgery (92,93).

The success with recurrent glioma patients led to a phase I study of <sup>131</sup>I-antitenascin MAb 81C6 in newly diagnosed malignant glioma patients by the same investigators (94,95). To date, 48 patients have been entered into this dose-escalation study. The MTD was defined as 120 mCi. For all 42 patients for whom absorbed dose estimates were calculated, average absorbed dose to the SCRC interface was 46–9531 Gy (mean = 1435 Gy) (94). In this series, the median survival for all patients was 75 wk, and, for GBM patients ( $n = 33$ ), 69 wk (95). This latter observation suggests that the toxicity observed in the series of previously irradiated patients (at 120 mCi) probably reflects the combined effects of external beam and MAb radiotoxicity. In sum, these series have established that significantly increased survival with little toxicity is achievable following intracavitary administration of labeled MAb. Determination of tumor response was based on MRI scans, <sup>18</sup>fluorodeoxyglucose (FDG) PET scans,



**Fig. 2.** Axial T1-weighted Gd-enhanced MRI images, as a function of time in weeks after  $^{131}\text{I}$ -Mab 81C6 therapy, of a GBM patient who had received an administered dose of 5180 medulloblastomaq (140 mCi). The patient had a total resection, with a Rickham catheter placement at the time of surgery. An enhancing rim was evident 1 wk after therapy; by 17 wk, peritumoral edema around the cavity and an enhancing nodule is apparent; biopsy of the enhancing area at 24 wk revealed radiation necrosis with macrophage infiltration.

and clinical examination. Figure 2 presents a series of Gd-enhanced MRI images over time, after instillation of  $^{131}\text{I}$ -antitenascin Mab 81C6 to the SCRC of a newly diagnosed patient. At 1 wk, an enhancing rim appears, followed by the detection of peritumoral edema and nodular contrast enhancement at 17 wk. This pattern of contrast enhancement or FDG PET findings of hypermetabolic activity suggestive of tumor progression was observed in 15/97 patients in this and the recurrent patient series reviewed above (96). All 15 patients were biopsied at the imaged site of tumor progression: five biopsy samples (33%) did consist of viable tumor, with little evidence of reactive inflammation; 11/15 biopsies exhibited extensive necrosis, characterized by macrophage infiltration, gliosis, and edematous reactive tissue. In one of these cases, a small tumor focus was present. Because 2/3 of the cases radiographically suggestive of tumor progression were in fact exhibiting enhancing areas of gliosis and necrosis, we observed that the worsening of MR and PET scan images in patients treated with intracavitary radiation will require careful analysis.

Other investigations (97) of intracavitary  $^{131}\text{I}$ -Mab therapy in glioma patients have been performed with the anti-NCAM Mab ERIC. Seven glioma patients (two with spontaneous cysts and five with SCRC) were treated with a single injection of  $^{131}\text{I}$ -ERIC. One patient received a second administration upon relapse. Whole-body doses were low, and mean dose to normal brain was calculated to be 5.2 Gy. The authors used a model that calculated mean tumor dose as the sum of the activity bound to the SCRC interface and the 0.992 mm shell that represents the range for 95% energy deposition of the  $\beta$  energy from  $^{131}\text{I}$ . This was calculated to be 1843 Gy, assuming that 100% Ab binding was achieved.

Transient clinical improvement was noted, but 6/7 patients developed progressive disease within 5 mo of treatment.

The feasibility of nuclides with different emission characteristics has been reported in trials of intracavitary administration. Hopkins et al. (98) investigated the use of  $^{90}\text{Y}$ -labeled anti-NCAM MAb ERIC-1 via intratumoral injection in 15 malignant glioma patients. The aim of that study was to determine the pharmacokinetics of a pure  $\beta$ -emitter. The lack of concomitant  $\gamma$ -radiation is attractive, because it reduces the radiation dose to surrounding normal brain and medical personnel. As observed with  $^{131}\text{I}$ -ERIC, prolonged retention within the cavity was observed, with very little activity observed systemically. Dosimetry was calculated as in the study by Papanastassiou et al. (97): An estimated mean of 351 Gy delivered to tumor would be achieved if 100% binding occurred, with a 140:1 predicted dose ratio of tumor:whole brain. Again, although transient improvement was noted in 2/15 patients, the remaining patients exhibited progressive disease within a few months of treatment, developed ventricular leakage, or died of unrelated causes. No survival data could be meaningfully deduced.

A new phase I study of  $^{211}\text{At}$ -Mab 81C6 via SCRC has been initiated.  $^{211}\text{At}$  emits  $\alpha$ -particles with a tissue range of only a few cell diameters and high relative biological effectiveness (99). Similar to the previously described studies of  $^{131}\text{I}$ -Mab 81C6, dose escalation studies in glioma patients with SCRC have begun with 2 mCi  $^{211}\text{At}$ /10 mg Mab 81C6. Peak blood pool activity was only 0.02–0.04%, with greater than 98.2% of  $^{211}\text{At}$  decays occurring in the resection cavity. The calculated average radiation dose to the SCRC interface was 472 Gy, similar to that achieved with 100 mCi  $^{131}\text{I}$ -Mab 81C6. However, the cavity interface-to-normal brain dose ratios were approx 150  $\times$  higher for  $^{211}\text{At}$ -Mab 81C6, with doses to normal tissue much lower than those for  $^{131}\text{I}$ -Mab 81C6 (90), which indicated that the shorter-range emissions of  $^{211}\text{At}$  might be optimal in this setting.

#### 4. SUMMARY AND FUTURE PROSPECTS

Since the early 1980s, several investigators have made significant progress in all areas required for successful immunotargeting of human gliomas: identification of specific targetable molecules and their defining MAbs; improvement and re-engineering of MAbs and MAb constructs; characterization of the best nuclides for varying biologic target situations; development of labeling chemistries to optimize label stability and retention; and exploration of novel routes of administration and dosimetry calculation.

The clinical studies described above have already established several guidelines:

1. Specific MAbs or constructs to targetable Ags are currently available, which may provide the necessary tumor vs normal tissue discrimination. Tenascin

and GP240 have already proven to be successfully targeted. The results obtained following targeting of NCAM are not as promising, although the number of patients investigated with this system has been small. The utility of wild-type EGFR as a target in malignant glioma is still equivocal; the specificity and density of the EGFRvIII variant has been established in model systems. The behavior of these two internalizing Ag–MAB complexes in human patients will require further investigation, especially in terms of radiolabeling approaches. Additional Ags currently under study (e.g., angiogenesis-related markers, developmentally associated Ags for medulloblastoma, such as L1) and their associated MABs, fragments, or engineered constructs are yet to be characterized in animal models.

2. Current evidence suggests that selection of nuclide for the nature of the target will result in the application of shorter-path agents within the CNS. There will be movement away from isotopes that also emit  $\gamma$ -rays (such as  $^{131}\text{I}$ ), to avoid irradiation of bystander normal tissue.
3. As reviewed above, a variety of new labeling chemistries have been developed to optimize not only the targeted binding of nuclide, but also the stability of the labeled compound in *in vivo* and intracellular milieus. The selection of nuclide and labeling method can now be tailor-made to meet the constraints of the targeted Ag (i.e., matrix, cell surface, or internalizing).
4. Perhaps most significant, there has been a move to compartmental, rather than systemic, administration of immunotargeting complexes. As discussed above, in the case of tumor-associated rather than tumor-specific targeting constructs, compartmental administration reduces binding to crossreactive Ag-positive nontumor tissues and increases local concentration to tumor. For gliomas, intracystic administration, when possible, appears superior to intratumoral administration in terms of optimal dispersal to remaining tumor rim. Diffusion of all targeting complexes to solid tumor tissue is still a problem requiring resolution. The continued development of higher-affinity, smaller targeting molecules, such as scFvs, will be of use in this setting.

The clinical trials discussed above and in progress are reporting clinical responses, stabilization of disease, and significant prolongation of survival time in several settings. Such reports are most notably in newly diagnosed patients, but are also seen in the worst-case scenario of GBM patients with recurrent disease. Moreover, the lack of radiation necrosis requiring reoperation, in contrast to 30–40% reoperation rates following brachytherapy and stereotactic radiosurgery, makes MAB therapy quite promising. Survival may be even better with MAB therapeutic approaches, because second surgery may be contributing to the observed survival extensions in brachytherapy- and stereotactic radiosurgery-treated cohorts. As the results of these phase I and II trials are evaluated, the experience of the past two decades and the existing library of pertinent model systems for rapid determination of efficacy will contract the time required for future feasibility studies.



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# 11 Immunotoxin Therapy of Brain Tumors

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*Walter A. Hall, MD*

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## 1. INTRODUCTION

Current treatments for malignant gliomas, which include surgery, radiation therapy (RT), and chemotherapy, are associated with a poor prognosis (1,2). Patients with glioblastoma multiforme (GBM) have an estimated 2-yr survival of less than 20% (1). Leptomeningeal carcinomatosis carries an estimated mean survival of 2–3 months (3). Localized RT techniques, such as brachytherapy and stereotactic radiosurgery, often fail to prevent disease progression at the primary tumor site (4). Unfortunately, the lack of specificity of both RT and chemotherapy for malignant cells has resulted in central nervous system (CNS) toxicity and unacceptable side effects (1,2).

Monoclonal antibody (MAB) technology has enabled investigators to develop agents that recognize cell surface antigens (Ags) preferentially expressed by tumors compared to normal cells, particularly for hematologic malignancies (1,5,6). Neoplastic cells often overexpress growth factor receptors or carbohydrate Ags that can act as targets for cytotoxic molecules (7). Compounds designed to take advantage of the difference in Ag expression between tumor and normal cells are called targeted toxins or immunotoxins. Immunotoxins are

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molecules composed of two components, which include a protein toxin with extraordinary potency conjugated to a carrier ligand, such as a MAb, with cell-type selectivity (1,2). Growth factors, such as transferrin (TFN), have been substituted for the Ab component to create fusion proteins called oncotoxins or mitotoxins (7).

Paul Erlich first introduced the concept of immunotoxins in 1906, although their application to the CNS spans little more than 10 yr. Initial *in vitro* studies, using glioblastoma- and medulloblastoma-derived cell lines, demonstrated the profound cytotoxic effects of immunotoxins and oncotoxins (8–10). Modest therapeutic results were obtained in the first *in vivo* studies (3,11) in which immunotoxins were administered into the CNS compartment. More recently, immunotoxins have been delivered directly into tumors in animals and in patients with malignant brain tumors (BTs) in a phase I clinical trial with encouraging results (12–14). The ability to safely deliver agents to BTs with limited neurological morbidity has stimulated an interest in the generation of immunotoxins targeted to different cell surface Ags found on malignant gliomas for future use in clinical trials (15).

## 2. IMMUNOTOXIN CONSTRUCTION

### 2.1. Rationale

Conventional chemotherapy drugs rely on the difference in the proliferative rate of malignant cells compared to normal cells to achieve a cytotoxic response (1). Unfortunately, the therapeutic window between normal dividing hematopoietic stem cells and neoplastic cells is usually too narrow for chemotherapy to prove curative (2). Immunotoxins, guided by their cell-type selective carrier ligand to tumor cells, have a mechanism of action that is much different than that of chemotherapy agents (2). Factors that make tumor cells resistant to RT and chemotherapy, such as hypoxia, do not influence the potency of immunotoxins (1,2). The natural or acquired resistance, which cancer cells develop to chemotherapy, has not been demonstrated with immunotoxins.

For most chemotherapy agents that act stoichiometrically, more than  $10^4$ – $10^5$  molecules are required to kill a single tumor cell (1). In contrast, immunotoxins act enzymatically, with multiple intracellular targets, and one molecule can kill a tumor cell (1,2). Irrespective of cell cycle or cellular division, immunotoxins can inactivate 200 ribosomes or elongation factor-2's (EF-2s)/min (1,2). The concentration of the alkylating agent 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) required to kill 50% of glioma cells in tissue culture is  $10^{-3}$ – $10^{-6}$  M, compared to an intact ricin immunotoxin that has the same effect at  $10^{-13}$  M (1,10). Although exact comparisons between chemotherapy and immunotoxins are difficult, because of differences in assay techniques, the extraordinary potency of immunotoxins, by 7–10 orders of magnitude, is apparent (1,2).

**Table 1**  
**Toxins**

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Plant toxins
Ricin
Abrin
Ricin A-chain
Abrin A-chain
Abrin variant
Modeccin
Gelonin
Saporin
Pokeweed antiviral protein
Momordica charanthia inhibitor
Bacterial toxins
Diphtheria toxin (DT)
Cross-reacting material 107 (CRM107)
DAB <sub>486</sub> and DAB <sub>389</sub>
<i>Pseudomonas aeruginosa</i> exotoxin A (PE)
<i>P. aeruginosa</i> 38 (PE38)
<i>P. aeruginosa</i> 40 (PE40)
<i>P. aeruginosa</i> 4E (PE4E)
Fungal toxins
$\alpha$ -sarcin

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## 2.2. Toxins

The toxins that have been used to construct immunotoxins are natural byproducts of plants, bacteria, and fungi that all inactivate protein synthesis (Table 1). Fewer than 1000 molecules bound per cell are sufficient for complete tumor regression in vivo (16). Plant toxins that have been used in the construction of immunotoxins are ricin and abrin, or their A-chains, pokeweed antiviral protein, gelonin, saporin, modeccin, and momordica charanthia inhibitor (1,2). Bacterial toxins include diphtheria toxin (DT), cross-reacting material (CRM)107, *Pseudomonas aeruginosa* exotoxin A (PE), and three mutated forms of PE (PE4E, PE38, and PE40).  $\alpha$ -sarcin is a fungus-derived toxin. Most toxins, such as ricin and DT, contain two polypeptide chains (A and B), which are joined by a disulfide bond. The B-chain binds nonspecifically to cell surface receptors and promotes translocation and internalization of the A-chain into the cell, where the latter chain inhibits protein synthesis (1).

Plant toxins are classified as type I or type II ribosome-inactivating proteins, based on their chemical structure. Type I plant toxins, such as saporin, have a single protein chain and maintain their catalytic activity, but do not have cell-binding or translocation functions (2,7). Type II plant toxins, which include

ricin and abrin, inactivate the 60S ribosomal subunit through the cleavage of the N-glycosidic bond of the adenine residue at position 4324 of 28S ribosomal RNA (2,7). The carboxylic ionophore, monensin, potentiates the action of plant toxins through an unknown mechanism (1,2,10).

Bacterial toxins used for immunotoxin construction have been primarily from DT or PE. The cell surface receptors for DT and PE have not been characterized. The DT A chain catalyzes the transfer of adenosine diphosphate (ADP)-ribose to EF-2 (Table 2), preventing the transfer of peptidyl-tRNA on ribosomes, which blocks protein synthesis and kills the cell (1,2). CRM107 is a genetically engineered toxin that is identical to DT, except for two amino acid substitutions in the B-chain that inactivate toxin binding and increase tumor-specific toxicity (1,2). PE A has three separate domains, Ia/Ib, II, and III, which are located on one polypeptide chain. Domain Ia binds to the PE receptor present on most animal cells, and the function of Ib is unknown. Domain II mediates the translocation of the carboxy-terminal fragment into the cell cytosol; and domain III is responsible for the ADP-ribosylation of EF-2 in the cytosol (1). The COOH terminus, REDLK, is not necessary for ADP-ribosylation activity, and replacing it with KDEL increases the cytotoxicity of PE (17). The removal of domain Ia from PE results in the molecule, PE40, which retains its translocation function and enzymatic activity, but does not bind to cell surface receptors (7). The fungal toxin,  $\alpha$ -sarcin, cleaves a phosphodiester bond in the 28S ribosomal RNA near the binding site for EF-2, thereby inhibiting protein synthesis (1,2).

The recent isolation of genes that encode for many protein toxins allows for the development of chimerics composed of DNA encoding for a growth factor, cytokine, or cloned Ab variable region, plus the gene encoding for the toxin (2,7). These gene fusion products can be expressed rapidly, efficiently, and inexpensively in bacteria to yield a homogenous polypeptide chain or fusion protein known as an oncotxin or single-chain immunotoxin (7). Toxins that have been used to create fusion proteins include PE40, PE38, PE4E, CRM107, and two binding-defective DT molecules, DAB<sub>486</sub> and DAB<sub>389</sub> (7). Compared to these fusion proteins, chemical conjugates prepared using radioisotopes, protein toxins, or chemotherapy drugs (such as adriamycin [ADR]) are more heterogenous in nature (7). All of these molecules are immunogenic and will elicit an immune response, particularly with repeat administration (2).

### 2.3. Carrier Ligands

The carrier ligand is important when constructing immunotoxins and has a functional relevance similar to that of the toxin moiety of the compound. Chemical conjugation of the toxin to MAbs, polyclonal Abs, growth factors, lectins, hormones, and Ags has been performed using either a disulfide or thioether bond.

**Table 2**  
**Immunotoxin Mechanisms of Action**

<i>Toxin</i>	<i>Mechanism</i>
Plant toxins	
Ricin	Inactivates ribosomes
Abrin	Inactivates ribosomes
Ricin A-chain	Inactivates ribosomes
Bacterial toxins	
Diphtheria toxin	Catalyzes transfer of ADP-ribose to EF-2
Crossreacting material 107	Catalyzes transfer of ADP-ribose to EF-2
<i>Pseudomonas aeruginosa</i> exotoxin A	Catalyzes transfer of ADP-ribose to EF-2
<i>P. aeruginosa</i> 38	Catalyzes transfer of ADP-ribose to EF-2
Fungal toxins	
$\alpha$ -sarcin	Inactivates ribosomes

ADP, adenosine diphosphate; EF, elongation factor.

Although the function of most Ags is unknown, binding of the carrier ligand to the cell surface Ag is essential for these agents to exert their therapeutic effect. The ligand–Ag complex is internalized into the cytosol, where the toxin is later released to inhibit protein synthesis. Immunotoxins must enter the cell to be effective, compared to MAb-linked, high-energy radionuclides, which are cytotoxic after binding to the cell. The “bystander effect” that occurs with gene therapy for BTs does not occur with immunotoxins.

A number of cell surface receptors that are expressed on malignant BT cells in both tissue culture and on surgical samples, have been identified and may be potential targets for immunotoxins. These brain tumor Ags include the epidermal growth factor receptor (EGFR), a rearranged deletion-mutant tumor-specific EGFRvIII, the transferrin receptor (TR), the interleukin-4 receptor (IL-4R) (1,2,5,15,18,19), and the interleukin-13 receptor (IL-13R) (39).

Amplification of the *EGFR* gene and overexpression of *EGFRvIII* have been demonstrated in GBM cell cultures and in human glioma biopsy specimens (19–23). Laboratory techniques that have been used to demonstrate EGFR overexpression in malignant gliomas include Scatchard analysis, immunoprecipitation, competitive radioreceptor assays, Western immunoblot analysis, affinity reactions, and immunohistochemistry (19,21).

The TR, a mediator of cellular iron uptake, is expressed in greater numbers in dividing cells than in nonreplicating cells. Because of their high requirement for iron, glioblastomas and medulloblastomas have increased expression of TR, as shown by solid-phase indirect radioimmunoassay, radioreceptor assay, and immunohistochemistry (10,19,24). Normal brain tissue was not found to

express TR, although the endothelial cells of cerebral blood vessels did express TR (10,19,24). A method for the rapid detection of TR expression, using MAb-coated magnetic microspheres, has been reported (5). Flow cytometry has been used to detect TR, human leukocyte antigen (HLA)-DR Ags, and the glioma-associated Ag, GE-2, on glioma cells (2).

Previously, MAbs have been used to construct immunotoxins targeted to HLA-DR Ags, glioma-associated Ags, and TR (1,2,8–10,24). Iron-loaded TFN, as a growth factor, has been conjugated to CRM107, ricin A-chain, abrin variant, and PE, to yield potent in vitro and in vivo agents (1,2,8,9,12). TFN conjugated to CRM107 via high-flow interstitial microinfusion was the first immunotoxin used to treat malignant BTs in clinical trials (14).

IL-4 is a cytokine that causes signal transduction, upregulation of major histocompatibility (MHC) Ags and intercellular adhesion molecule-1, and inhibition of tumor cell growth (15). Recently, the high-affinity IL-4R was found in high numbers on malignant glioma cell lines using flow cytometric analysis and <sup>125</sup>iodine-IL-4 binding (15,18). Using Northern blot analysis, four malignant glioma cell lines were found to express the messenger RNA for *IL-4R* (15). In 21 patients with malignant astrocytomas who underwent surgical resection, *IL-4R* expression was demonstrated in 16/21 (76%) samples, using reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot analysis (15). In contrast, 1/6 samples of normal brain tissue was weakly positive for *IL-4R*, using the same techniques (15). In low numbers, IL-4Rs are expressed on the following normal cells: T-cells, B-cells, monocytes, basophils, eosinophils, fibroblasts, and endothelial cells (15). The overexpression of this receptor on tumor cells, compared to normal cells, provided the rationale for the creation of a recombinant toxin containing circularly permuted IL-4 and truncated PE for the treatment of malignant astrocytoma in a phase I/II trial (15).

### 3. IN VITRO STUDIES

In 1987, the first report demonstrating the in vitro efficacy of immunotoxins in a malignant glioma cell line was published (10). More than 50% of glioblastoma- and medulloblastoma-derived cell lines were killed after 18 h, using an anti-TR MAb + ricin immunotoxin at a concentration of  $5.6 \times 10^{-13}$  M in the presence of monensin (10). A 150- to 1380-fold selective toxicity was seen between target cells and nontarget normal brain cells (2,10). Using an anti-TR-ricin A chain immunotoxin, 50% of protein synthesis was inhibited (inhibitory concentration of 50% [IC<sub>50</sub>]) at concentrations ranging from  $1.9 \times 10^{-9}$  to  $1.8 \times 10^{-8}$  M, with the addition of monensin increasing toxicity 16- to 842-fold (25). In three medulloblastoma cell lines treated with an anti-TR MAb-ricin A chain immunotoxin, the IC<sub>50</sub> ranged from  $8.5 \times 10^{-11}$  to  $1 \times 10^{-9}$  M (2,26). Using an

anti-TR MAb-CRM107 immunotoxin, the  $IC_{50}$  ranged from  $3.2 \times 10^{-9}$  to  $5.7 \times 10^{-9} M$  for these same medulloblastoma cell lines (2,26). Surgical samples from GBM and medulloblastoma patients had  $IC_{50}$  values of  $10^{-12} M$  (2,26). The time to growth inhibition was after 6 h of incubation for the anti-TR-CRM107 immunotoxin, and not until 16 h for the anti-TR-ricin A chain conjugate in the one medulloblastoma cell line that was tested (2,26).

Excellent *in vitro* results in glioma cell lines have been reported when the growth factor, TFN, was used as the carrier ligand in the construction of immunotoxins. A 10,000-fold increase in tumor-specific toxicity was seen in glioblastoma and medulloblastoma cell lines when TFN-CRM107 was compared to CRM107 alone (9). The  $IC_{50}$  for TFN-CRM107 ranged from  $2.6 \times 10^{-12}$  to  $6.5 \times 10^{-11} M$  in glioblastoma cell lines, and  $3.9 \times 10^{-13}$  to  $1.1 \times 10^{-10} M$  in medulloblastoma cells (9). Immunotoxins constructed of an anti-TR-MAb and either CRM107 or ricin A chain had comparable potency, but were not as effective as TFN-CRM107 in the cell lines tested. The addition of monensin increased the efficacy of TFN-ricin A chain 5000-fold compared to the toxin alone in glioma cell lines, and was 100,000 times more potent than BCNU (27).

Three immunotoxins, constructed with human diferric TFN as the carrier ligand (and abrin variant, CRM107, and PE as the toxin moieties), were tested against the SNB19 and SF295 glioma cell lines (8). TR expression on these glioma cell lines was confirmed by direct  $^{125}I$ -TFN-binding assays. For each cell line, TFN-abrin variant and TFN-PE had comparable  $IC_{50}$  values, and were both more potent than TFN-CRM107. Monensin potentiated the effect of TFN-abrin variant 35-fold in both cell lines, and the  $IC_{50}$  values were  $4.0 \times 10^{-13} M$  (SNB19) and  $4.7 \times 10^{-12} M$  (SF295). The DAOY medulloblastoma cell line had  $IC_{50}$  values of  $1.3 \times 10^{-12} M$  for TFN-abrin variant with monensin,  $3.4 \times 10^{-11} M$  for TFN-PE, and  $1.8 \times 10^{-10} M$  for TFN-CRM107 (28). When the genetically engineered immunotoxin composed of EGF and the DT mutant  $DAB_{389}$  was tested against U87 glioma cells, the  $IC_{50}$  for  $DAB_{389}$ -EGF was  $10^{-11} M$  (2).

Three chimeric immunotoxins, IL-4-PE4E, IL-4(38-37)-PE38KDEL, and IL-4-PE38KDEL, were tested for 20 h against the A172, U251, U373MG, T98G, SN19, SF295, H638, and U87MG glioma cell lines (15,18). The  $IC_{50}$  values for the glioblastoma cell lines ranged from 5 to 180 ng/mL (~60–2000 pM) for IL-4-PE4, and were 3- to 28-fold lower for IL-4(38-37)-PE38KDEL (15). The IL-4-PE38KDEL fusion toxin, which is not circularly permuted, was the least effective (15).

The immunoconjugate constructed with an antiglioma Ag MAb (SZ39) and ADR had an eightfold increase in toxicity against glioma cells compared to ADR alone (29). ADR had an  $IC_{50}$  of  $7.08 \times 10^{-8} M$ , in contrast to  $9.16 \times 10^{-9} M$  for SZ39-ADR. ADR was 11× more toxic to nontarget K560 leukemia cells than was SZ39-ADR.



## 4. IN VIVO STUDIES

### 4.1. Efficacy Studies

DT is frequently used as a positive control in immunotoxin research, because of its extreme potency (30). Mice are resistant to the effects of DT, and humans are extremely sensitive to the toxin. Complete regression of a large human GBM grown in the flank of a nude mouse treated with intraperitoneal (ip) DT has been reported (30). Advanced ip human mesotheliomas in athymic mice were consistently cured by single ip or intravenous (iv) injections of 1.0–3.0  $\mu\text{g}$  DT (31). Survival was extended in an intracerebral nude rat model of human small cell lung cancer after iv DT administration (32). Control animals had a median survival of 15 d, compared to 19 d for animals receiving 0.1  $\mu\text{g}$  DT and 26.5 d for animals receiving 1.0  $\mu\text{g}$  DT (32). Ninety percent of the initial concentration of DT was cleared within 6 h of administration.

The first immunotoxin experiments that involved the CNS were performed in a syngeneic guinea pig model of leptomeningeal neoplasia (33). Animals were treated 24 h after the intrathecal inoculation of L<sub>2</sub>C leukemia cells with an anti-idiotypic MAb (M6) + intact ricin immunotoxin administered into the cisterna magna (33). The results were consistent with a 2–5 log cell kill of L<sub>2</sub>C leukemia cells.

In a nude rat model of human neoplastic meningitis from LOX melanoma, untreated animals developed lower-extremity paralysis at  $10.7 \pm 2.75$  d, compared to  $15.5 \pm 4.58$  d for animals that were treated 24 h after tumor cell inoculation with 1  $\mu\text{g}$  intrathecal TFN–PE immunotoxin (3). The mean delay in the onset of paraplegia by 5 d (or 31%) was statistically significant. Animals with intrathecal DAOY medulloblastoma, treated 7 d after tumor cell inoculation with 1  $\mu\text{g}$  TFN–PE, developed paraplegia at  $56 \pm 27$  d, compared to  $38 \pm 16$  d ( $p < 0.05$ ) for control animals (28). Even though TR expression was found to be decreased in vivo, a significant therapeutic response was found, which emphasizes the extreme potency of these compounds (28).

In another animal model of carcinomatous meningitis from human H-146 small cell lung cancer injected into the cisterna magna, neurological symptoms developed after a mean latency of 20 d (34). Animals received 1.5  $\mu\text{g}$  immunotoxin as a single intrathecal injection 24 h after tumor cell inoculation (34). The immunotoxins tested in this animal model were TFN–PE and two immunotoxins constructed with the anticarcinoma MAbs, MOC-31 or MrLu10, conjugated to PE. The symptom-free latency was increased by 35–46% with either MOC-31–PE or MrLu10–PE. MOC-31–PE, co-instilled with 10% glycerol, increased the symptom-free latency to 72% (34). A single bolus injection of MOC-31–PE was more effective than repeat or delayed injections.

More recently, Pastan et al. (35) reported the use of a single-chain immunotoxin, LMB-7 (B3[Fv]-PE38), constructed from the murine MAb B3 and a trun-

cated form of PE, PE38. In an athymic rat model of human neoplastic meningitis using the epidermoid carcinoma line, A431, which homogeneously expresses Ags recognized by the B3 Ab, LMB-7 was intrathecally administered 3 d after subarachnoid tumor implantation. Without treatment, the median survival of the animals was 10 d. Intrathecal administration of 10  $\mu\text{g}$  LMB-7 in 40  $\mu\text{L}$ , on d 3, 5, and 7, produced 4/10 and 8/10 long-term survivors (>170 d) in two experiments. Of the long-term survivors, 2/4 and 7/8 rats had no microscopic evidence of tumor and were considered histologic cures (35).

In a similar athymic rat model of neoplastic meningitis using the EGFRvIII-expressing human glioma U87MG. $\Delta$ EGFR, animals were treated with a single-chain immunotoxin, MR-1 [MR1scFvPE38KDEL], which was constructed from an Ab specific for the EGFRvIII mutation and a modified form of the PE (36). A dose-escalation study compared the survival of animals given three equal doses of 1, 2, and 3  $\mu\text{g}$  MR-1 immunotoxin with those given saline or 3  $\mu\text{g}$  control immunotoxin specific for the IL-2R (anti-Tac). All animals treated with saline or anti-Tac died, with median survival of 7 and 10 d, respectively. In contrast, there were 75% (6/8) long-term survivors in the group treated with three doses of 1  $\mu\text{g}$  and 57% (4/7) survivors in the groups treated with three doses of 2  $\mu\text{g}$  or 3  $\mu\text{g}$  MR-1 immunotoxin. None of the MR-1 immunotoxin-treated groups reached median survival by the termination of the study at 53 d (36).

Direct intratumoral administration of immunotoxins into a human glioma flank tumor model in nude mice resulted in a greater than 95% tumor regression by d 14, and recurrence was not seen by d 30 (12). When tumors measured 0.5–1.0 cm in diameter, animals received 10  $\mu\text{g}$  of either TFN–CRM107 or anti-TR-MAb + ricin A chain (454A12-RA) immunotoxin every 2 d for four doses (12). By d 14, 454A12-RA had caused a 30% decrease in tumor volume. A significant dose–response relationship was seen for 10, 1.0, and 0.1  $\mu\text{g}$  TFN–CRM107 injected intratumorally. Direct intratumoral injection of 1 or 10  $\mu\text{g}$  DAB<sub>389</sub>–EGF, twice a day for three doses, into U87 human glioblastoma flank tumors in nude mice significantly inhibited tumor growth, compared to control animals (2). Complete regression of small and large U251 human glioblastoma flank tumors was seen in all animals that received intratumoral administration of 250  $\mu\text{g}/\text{kg}$  IL-4(38-37)–PE38KDEL on alternate days for three or four doses (13). Ip and iv administration of IL-4(38-37)–PE38KDEL also resulted in significant antitumor activity (13).

Animals with subcutaneous human glioma xenografts which were treated with SZ39–ADR had a greater antitumor effect than animals that received a nonspecific immunoglobulin G (IgG)–ADR conjugate (29). Tumor volumes were 0.30 for animals receiving SZ39–ADR, 0.78 for those that received IgG–ADR, and 0.84 for animals treated with ADR alone. In animals with intracerebral xenografts which received ip SZ39–ADR, the median survival was 51 d, compared to 36 d for ADR alone and 33 d for phosphate-buffered saline treatment (29).

Recently, IL-13R has been used as a therapeutic target for human high-grade gliomas in animal models (37). Debinski et al. (37) found that virtually all of their studied human GBM specimens (23 samples) abundantly expressed a receptor for IL-13 *in situ*, but normal human brain has few, if any, IL-13 binding sites. Furthermore, immunotoxins targeted to IL-13R produced cures in animals bearing xenografts of human high-grade gliomas (37).

#### 4.2. Distribution Studies

Immunotoxin penetration into solid tumor has been investigated after ip, intracarotid, and intratumoral administration (12,38,39). Following an intracarotid injection of  $1 \times 10^5$  LOX melanoma cells, nude rats developed a skull base tumor with local infiltration into the brain, which caused neurological symptoms at a median of 19 d (38). With the onset of symptoms, animals received a retrograde external carotid artery injection of  $2.5 \mu\text{g}$   $^{125}\text{I}$ -antimelanoma MAb + abrin immunotoxin prior to sacrifice at 10 min. Tissue samples were taken from the tumor, ipsilateral cerebral hemisphere, contralateral hemisphere, brain stem, and cerebellum, for analysis in a standard  $\gamma$ -counter to determine the tissue immunotoxin concentration (counts  $\times$  100/total counts/g tissue). The uptake of the immunotoxin in the tumor was 4.9–9 $\times$  higher than in the ipsilateral brain tissue, demonstrating specificity for the targeted tissue (38).

Quantitative autoradiography has been used to determine the spatial distribution of immunotoxins in solid tumors (39). Immunotoxins labeled with radioactive iodine targeted to the TR were administered intravenously to animals with a subcutaneous rhabdomyosarcoma that expressed the receptor. Spatial distribution within the tumor was evaluated at 2, 6, and 24 h. DT had an uniform distribution throughout the tumor, compared to either a MAb Fab' fragment–CRM107 immunotoxin or a IgG<sub>1</sub> MAb–CRM107 conjugate, which displayed a punctate pattern on autoradiography (39). A nonbinding immunotoxin had a more homogenous pattern of distribution than either targeted toxin, but was not as uniformly distributed as DT (39). The heterogenous distribution of both targeted immunotoxins was felt to result from binding to tumor cells, which retarded penetration.

When the antihuman TR MAb (454A12)–ricin A chain immunotoxin was administered into the cerebral spinal fluid (CSF) of monkeys, a biphasic clearance pattern was seen, with an early-phase half-life of 1.4 h and a late-phase half-life of 10.9 h (40). The clearance of 454A12-RA from the CSF was 4.4 mL/h, which was twice what would be expected from clearance by bulk flow. The immunotoxin was found to be stable for up to 24 h after administration into the CSF, and the volume of distribution was 10.1 mL, or three-fourths the total CSF volume for the monkey (40).

### 4.3. Toxicity Studies

Direct delivery of immunotoxins into the intrathecal space is appealing, because of the ability to achieve high local concentrations and, potentially, to avoid systemic toxicity (2). Intrathecal immunotoxin toxicity trials (9) have demonstrated that a maximum dose of  $2 \times 10^{-9}$  M TFN-CRM107 was tolerated in guinea pigs. When this same dose was administered intrathecally in monkeys, it was nontoxic and represented a concentration of 20- to 5000-fold higher than that which was effective against glioma cells *in vitro*. Neurological toxicity, which was manifested as hemorrhagic degeneration around the central canal or a pathological cleft in the cervical spinal cord, was seen in rats that were treated intrathecally with 2.5 or 5.0  $\mu$ g TFN-PE (3).

The IL-4(38-37)-PE38KDEL immunotoxin was not associated with any toxicity in mice with subcutaneous human glioblastomas that were treated by intratumoral, *iv* or *ip* routes (13). Upon the intrathecal injection of IL-4(38-37)-PE38KDEL in monkeys, high CSF concentrations were achieved without evidence of neurological toxicity using 2 and 6  $\mu$ g/kg doses (15). No monkey demonstrated detectable levels of IL-4(38-37)-PE38KDEL in the serum after intrathecal administration. Injection of IL-4(38-37)-PE38KDEL into the right frontal lobe of rats resulted in localized necrosis with 1000  $\mu$ g/mL doses, but not at  $\leq 100$   $\mu$ g/mL doses (15).

In monkeys receiving 454A12-RA, the maximally tolerated dose yielded a CSF concentration of  $1.2 \times 10^{-7}$  M (37). In rats, the 10% lethal dose ( $LD_{10}$ ) of an antihuman-TR immunotoxin in the CSF was  $8.8 \times 10^{-7}$  M. When the antirat-TR MAb + ricin A chain (OX26-RA) immunotoxin was tested in rats, the  $LD_{10}$  was  $1.2 \times 10^{-7}$  M, which represented a concentration one-seventh that of the immunotoxin constructed with the irrelevant human Ab. Selective elimination of Purkinje cells was the dose-limiting toxicity that was seen in rats and monkeys, which was manifested clinically as ataxia and lack of coordination. The ataxia in monkeys occurred within 5 d and was reversible, with time, for the more mild form (40). No evidence of systemic toxicity was apparent, and only mild inflammation was visible in the CSF (40).

## 5. CLINICAL TRIALS

The encouraging results obtained in animal studies, particularly with intratumoral administration of TFN-CRM107 and IL-4(38-37)-PE38KDEL, provided the basis for proceeding with the development of immunotoxins for phase I/II clinical trials (12,13). Two clinical trials have been completed, which addressed the safety and efficacy of immunotoxins administered, either into the CSF for patients with leptomeningeal neoplasia or directly into malignant brain tumors by convection-enhanced delivery (14,38). The first clinical trial was for

the treatment of neoplastic meningitis, using an anti-TR MAb + recombinant ricin A chain immunotoxin (454A12-RA); and the second trial used human diferric TFN linked to the DT mutant CRM107 (TFN-CRM107) to treat BT (14,41).

### **5.1. Leptomeningeal Neoplasia**

A pilot study was performed using 454A12-RA to treat eight patients with leptomeningeal spread of systemic breast cancer or lung cancer (41). Patients received a single intrathecal dose of 454A12-RA that ranged from 1.2 to 1200  $\mu\text{g}$ . A total of 10 different doses were given during the trial. The early-phase half-life of 454A12-RA in the ventricular CSF averaged  $44 \pm 21$  min, and the late-phase half-life averaged  $237 \pm 86$  min (41). The clearance of 454A12-RA was  $2.4\times$  faster than that of co-injected technetium-99m-diethylenetriamine penta-acetic acid. There was no degradation of 454A12-RA for a period of 24 h, as determined by Western blot analysis, and bioactivity paralleled the concentration of the immunotoxin in the CSF. Bioassays of CSF from treated patients demonstrated retained cytotoxicity for 48 h after immunotoxin administration (2).

No acute evidence of chronic drug toxicity was seen in any patient that received an intrathecal dose of 454A12-RA  $\leq 38$   $\mu\text{g}$  (41). Doses  $\geq 120$   $\mu\text{g}$  caused a CSF inflammatory response that was associated with transient headache, nausea, vomiting, lethargy, and mental status changes. Corticosteroid administration and CSF drainage were necessary for these patients. Four/eight patients had  $>50\%$  reduction in their lumbar CSF tumor cell counts within 5–7 d, with a  $>95\%$  reduction seen at the highest administered dose (41). No patients completely cleared their CSF of tumor cells. Clinical and radiographic progression was seen in 7/8 patients after treatment (41).

### **5.2. Malignant BTs**

In a phase I/II clinical trial, TFN-CRM107 was delivered directly into malignant BTs using a high-flow interstitial microinfusion technique that induced fluid convection within the brain (14). Eighteen patients were enrolled in the trial. Of these patients, 10 had GBM, five had anaplastic astrocytomas, two had lung cancer, and one had an anaplastic oligodendroglioma. One patient withdrew from the study, and the two patients with metastatic disease had surgical resections after treatment and could not be evaluated for response to treatment.

Of the 15 evaluable patients, nine experienced a  $\geq 50\%$  decrease in tumor volume. Reduction in tumor volume did not occur prior to 1 mo after completing the first treatment and was not maximal in four patients for 6–14 mo. Two complete responses were seen. In one complete responder with a GBM, no tumor progression was seen for 23 mo. The second patient with an initial complete response had an anaplastic astrocytoma that recurred 5 mo after treatment. Of the 16 patients that could be evaluated by magnetic resonance imaging (MRI)

≤6 wk after treatment, 14 (88%) demonstrated a zone of necrosis or a reduction in tumor volume.

Only 2/8 patients had partial responses in the first two treatment groups, in which the TFN–CRM107 concentration was 0.1 and 0.32 μg/mL, and the total dose was 0.5–12.8 μg. In comparison, two complete and two partial responses were seen in four patients who received ≥1.0 μg/mL, or a total dose of 20–128 μg. At intermediate treatment levels, the responses correlated more with total dose level than with the concentration of the drug. Pretreatment tumor volume did not correlate with the likelihood of response. The median survival in the treatment group of nine responders was 74 wk, compared to 36 wk for nonresponders.

Intratumoral infusions of 5–180 mL were well tolerated, and there were no treatment-related deaths or life-threatening toxicities (41). Transient worsening of a pre-existing neurological deficit occurred in 3/44 infusions. These deficits resolved with steroid and hyperosmolar therapy. Four patients had seizures during treatment, and peritumoral edema was seen in three of these four patients. Peritumoral focal brain injury was seen at the higher concentrations of TFN–CRM107 (≥1.0 μg/mL) and occurred 2–4 wk after infusion. Three patients developed a hemiparesis, which resolved in two of these patients and was associated with MRI changes characterized by serpentine strips of increased signal on the nonenhanced T1-weighted scan (41). Stereotactic biopsy of these abnormal areas revealed thrombosed cortical venules and capillaries. No peritumoral toxicity was seen in patients who received 40 mL of TFN–CRM107 ≤ 0.66 μg/mL. Two/3 patients, who received 120 mL at 0.5 μg/mL (total dose 60 μg), developed peritumoral injury.

No systemic toxicity occurred, although 14 patients had transient elevation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) transaminases, and 12 experienced mild hypoalbuminemia. All patients had antidiphtheria Abs before treatment, which increased at least twofold in 6/14 patients >4 wk after treatment. Increases in Ab titer did not correlate with tumor response. No evidence of systemic toxicity was seen in any of the organs of five patients examined at autopsy. Examination of the brains at autopsy in six patients showed changes that were felt to be consistent with RT and chronic vasogenic edema and not because of the effects of TFN–CRM107 infusion (41).

## 6. FUTURE DIRECTIONS

Present immunotoxin trials are directed at treating recurrent or residual primary malignant disease in the CNS. Because of a different mechanism of action than conventional chemotherapy and RT, immunotoxins may act synergistically with these forms of treatment without subsequent cumulative side effects. Furthermore, administering a combination of immunotoxins that target different tumor Ags may result in an improved clinical response.



The immune response against mouse MAbs and protein toxins presents a potential problem, which may influence the overall efficacy of immunotoxin therapy. Abs to immunotoxins interfere with their binding ability and accelerate their plasma clearance. The generation of Abs from different animal species, the use of human MAbs or Ab fragments that bind Ags, or the creation of chimeric rodent–human MAbs, may stimulate less of an immune response (1). Immunosuppressive drugs, such as cyclophosphamide and cyclosporin A, may diminish or delay the immune response to immunotoxins and allow for repeat treatments (1). The “immunologically privileged” nature of the CNS may also offer some protection from the immune system.

Because of the large size of immunotoxin molecules and their inability to diffuse into tumor tissue in the presence of an intact blood–brain barrier (BBB), investigators have considered alternative routes of administration for these agents. Convection-enhanced intratumoral delivery appears to effectively allow immunotoxins to reach tumor cells with minimal neurological morbidity (14). BBB disruption, using hyperosmolar mannitol, may also represent another route for delivering these compounds in sufficient quantities to be therapeutically effective. Producing smaller carrier ligands (such as Ab fragments and Fabs), and using recombinant technology to generate fusion proteins, may increase tumor penetration and reduce antigenicity (1,7). The co-administration of drugs that potentiate the action of immunotoxins, such as interferon- $\alpha$ , monensin, chloroquine, and the calcium-channel blockers, verapamil and diltiazem, may enhance their effect (1).

Immunotoxins are an effective class of compounds against malignant BTs in vitro and in vivo. The extreme potency of these agents allows them to retain significant efficacy, despite the reduced Ag receptor expression that has been seen in vivo (28). There appears to be a therapeutic window of differential expression of TR, EGFRvIII, IL-4R, and IL-13R on neoplastic cells, compared to normal brain tissue, which can be exploited in the treatment of CNS tumors. Although the prognosis for malignant BTs remains poor, immunotoxins represent an exciting therapeutic modality with tremendous potential and future promise for patients with this disease.

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# 12 Antibodies to Adhesion Molecules for Immunotherapy of Brain Tumors

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*S. Ather Enam, MD, PhD*

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## 1. INTRODUCTION

The major morbidity of gliomas lies in their infiltrative growth. The cell biology of invading neoplastic cells is similar to that of migrating fetal cells. Several adhesion molecules that are downregulated after completion of fetal brain development are re-expressed in neoplasms of the nervous system. The crucial role played by adhesion molecules during neural development is repeated during glioma invasion (1).

Adhesion molecules are molecules that accumulate on the cell surface or are immobilized in the extracellular space around the cell (2–4). In vitro, almost all of these molecules modify adhesion to substrates, and are therefore called adhesion molecules. These molecules are secreted by the cell itself or by surrounding cells. Adhesion molecules can be classified as extracellular matrix (ECM) molecules, cell surface adhesion molecules (CAM), or molecules that act as receptors to ECM components or CAMs. It should be noted that these are not distinct classes of molecules, because some ECM molecules may act as CAMs or receptors on a cell surface, and some CAMs may be released and incorporated in the ECM. Besides adhesion, they provide a more complex and integral function in modifying the cellular response to external stimuli during develop-

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ment and in the mature state. These functions may include signaling for trophic effect, triggering or suppressing apoptosis, and binding growth factors, proteases, and protease inhibitors. Some individual molecules or their fragments may actually have a negative effect on the adhesion of cells to the substratum. Adhesion may or may not be the primary role of these adhesion molecules.

This chapter provides a short introduction to the adhesion molecules that seem to play an important role in the developing or mature central nervous system (CNS) and in glioma growth and invasion. This introduction is followed by a discussion on the use of adhesion molecules as targets for brain tumor (BT) immunotherapy (IT).

## 2. ADHESION MOLECULES IN NERVOUS SYSTEM

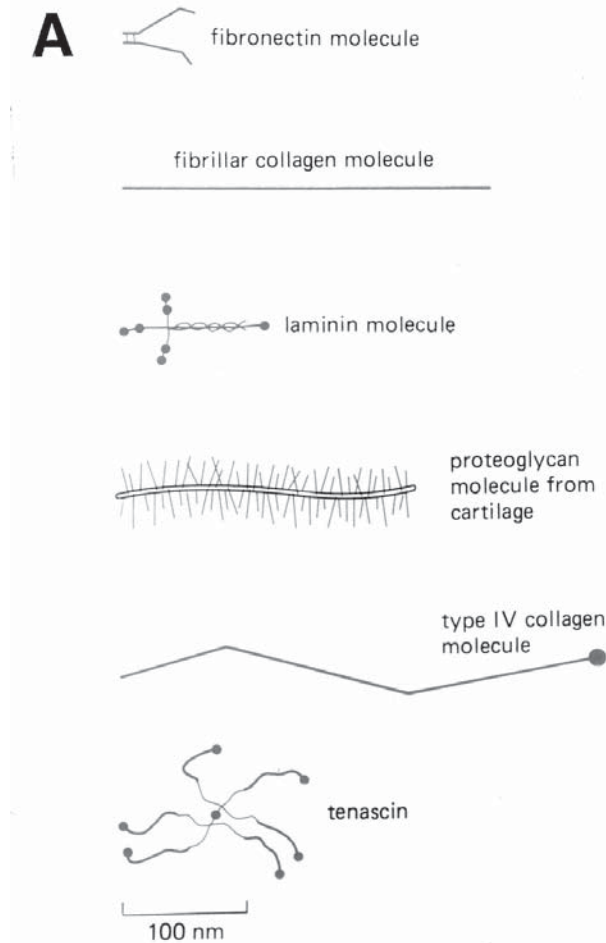
### 2.1. *Extracellular Matrix*

The ground substance, initially recognized by Golgi (5) in 1903 as a network of fibrillar and amorphous material surrounding neurons, was discredited for several decades. It was not until the 1970s and 1980s, when careful examination of the CNS ultrastructure, and use of sensitive immunohistochemistry confirmed the presence of ECM in the CNS. The ECM in the brain is scant and not well organized, except for the basement membrane around the blood vessels. Yet, by some estimates, about 17–20% of total brain volume consists of ECM (6). An interesting feature of brain ECM is the similarity of its constituents to those of cartilage (7). Some CAMs and certain trophic factors may also be incorporated into the ECM. Several of the well-known components of the ECM are mentioned here.

Most of the ECM molecules are either glycoproteins (GPs) or proteoglycans (Fig. 1A and B). Proteoglycans, which are macromolecules of glycosaminoglycan (GAG) chains attached to a core protein, form the ground substance in which collagen and other GPs are embedded. The collagen and noncollagen GPs, such as fibronectin (FN), laminin (LAM), and tenascin, have been studied in great detail. These GPs have several molecular domains with similar motif, which are repeated several times in the same molecule (e.g., FN has three such motifs, named FN type I, FN type II, and FN type III repeats). FNIII repeats are also present in tenascin and in several CAMs, including integrin molecules. Another domain important in adhesion is the immunoglobulin (Ig) domain, which is present in several CAMs that do not require calcium for binding, such as neural cell adhesion molecule (NCAM) or L1. Proteoglycans contain several other domains or repeats that have various functions. Some of these domains may bind GAG chains or lectins, or may have homology to epidermal growth factor.

#### 2.1.1. COLLAGENS

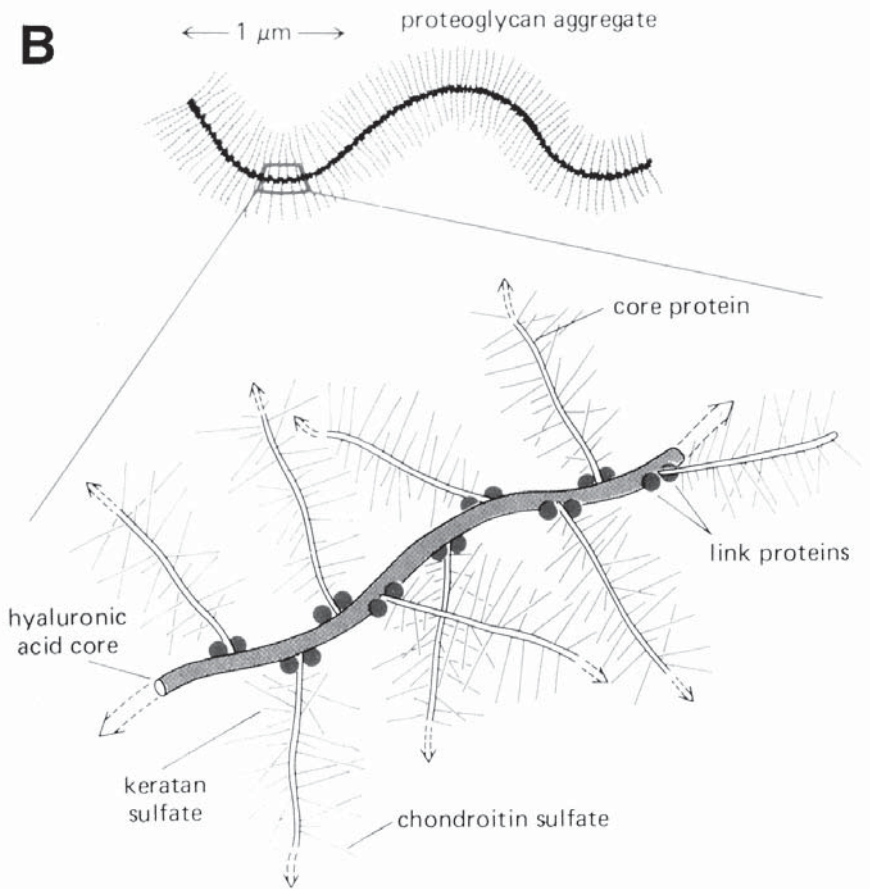
Collagen is the major GP in extraneural tissue. Three coiled chains form tropocollagen, which is the fundamental molecule of collagen. Posttranslational



**Fig. 1. (A)** ECM consists of GPs of various shapes and sizes embedded in a matrix of proteoglycans. Reproduced with permission from ref. 30.

modifications of collagen, including hydroxylation and glycosylation, make the final form of collagen. According to one recent review (3), at least 19 different forms of collagen, and 35 genes that encode for them, have been found. Mesenchymal tissues (blood vessels and meninges) of normal brain and gliomas express fibril-forming collagen (types I, III, V, VI, and VII). Collagen IV, which is classified as a sheet-forming collagen, is extensively expressed in the developing nervous system, but is restricted to synaptic basal lamina in developed brain. Although glioma cells can deposit different types of collagen *in vitro*, collagen *in situ* is restricted to the mesenchymal component of gliomas, with the exception of type IV, which may surround individual glioma cells (3).





**Fig. 1. (B)** Proteoglycans form a complex structure by linking to HA with the help of link proteins. Reproduced with permission from ref. 30.

### 2.1.2. FIBRONECTIN

FN, a dimer, was one of the first ECM molecules shown to be important for the migration and differentiation of neural crest cells (8). Few varieties of FN are produced by alternative splicing of mRNA from a single gene (3). Expressed abundantly by fetal neurons and glia, FN is restricted to mesenchymal structures in normal brain after development. *In vitro*, FN can be expressed by several glioma cell lines; *in situ* expression of FN by glioma cells is scant, somewhat restricted to the tumor blood vessels, and can be detected weakly in brain tissue infiltrated by glioma cells (9–12).

### 2.1.3. LAMININ

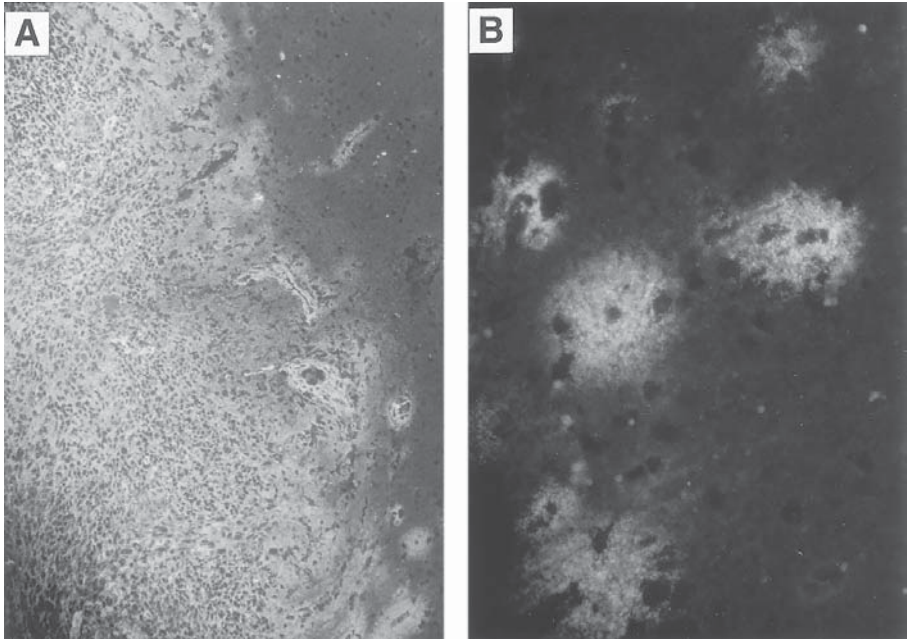
LAM is a cruciform molecule formed by disulfide bonding of three chains, each of which have a few isoforms. This gives rise to at least 18 isoforms of LAM. Expression of LAM-1 in normal human brain and in gliomas co-distributes with collagen type IV; LAM-2 (also known as merosin) is the predominant form expressed by reactive astrocytes and occasional glioma cells *in situ*, and by primary astrocytes and glioma cell lines *in vitro* (3,9,13). Expression of LAM parallels that of FN in low- and high-grade gliomas (i.e., LAM is present in the mesenchymal tissues of tumors, with only occasional variants of gliomas expressing it around individual neoplastic cells) (11,12).

### 2.1.4. TENASCIN

The identification and isolation of tenascin, a ~1900 kDa six-armed protein (also known as hexabrachion), came from several laboratories. One of the earliest discoveries was by Bourdon et al. (14), who identified it as glioma-mesenchymal ECM antigen (Ag) by the use of monoclonal antibodies (MAB). This MAB reacted with the ECM of most of the gliomas tested. Chiquet and Fambrough (15) first named it “chicken myotendinous antigen,” and, subsequently, “tenascin” (tendon nascent).

Tenascin is basically a developmental ECM molecule and is especially prominent in embryonic tissue. Expression of tenascin generally declines as the brain matures, but, in contrast to other ECM proteins, such as FN and LAM, it is found in vertebrate brain tissue throughout life (16,17). The six arms of each tenascin molecule vary in size from 220 to 320 kDa. Each arm is the product of a single gene, with heterogeneity brought in by alternate mRNA splicing. Each arm has two recurrent motifs: Centrifugally, they have a repeat of ~13 EGF-like domains; centripetally, there is a string of 8–15 FNIII domains (2,4). J1-160 and J1-180, which are related to tenascin, are dimers and trimers, respectively, of tenascin subunits (18). Besides binding to cell surface integrin receptors through RGD sequences, tenascin also binds FN and chondroitin sulfate (CS) proteoglycans, such as phosphocan and neurocan in the ECM (17,19,20).

The biological role of tenascin seems more complex than that of any other ECM protein. It has both adhesive and repulsive properties ascribable to separate molecular domains. Tenascin mediates neuron–glia interaction. It also creates inhibitory boundaries within the brain, and is the inhibitory substance within glial scars (16,21). In addition to its distribution in embryonic tissue, tenascin is extensively upregulated in granulation tissue and astroglial scars, as well as in a variety of mesenchymal tumors and cancers, such as glioma, fibrosarcoma, osteosarcoma, melanoma, mammary carcinoma, and squamous cell carcinoma (14,16; Fig. 2). In gliomas, the expression of tenascin correlates with the degree



**Fig. 2.** (A) Strong expression of tenascin by tumor cells in a xenograft model. Compare expression of tenascin in the tumor on the left side of the photograph to that in the normal brain in the upper right corner of the photograph. (B) Higher magnification shows that individual tumor cells deposit a rich amount of tenascin around it. (U251 glioma cell lines were stereotactically implanted in nude mice brain and grown for a month. Expression of tenascin was studied with the help of immunofluorescence.)

of anaplasia, although the expression may be more heterogeneous in anaplastic astrocytomas (AA) and glioblastomas (GBM) (14,16,22).

### 2.1.5. THROMBOSPONDINS

Thrombospondins are a family of at least four related trimeric proteins, consisting of three ~180 kDa subunits, which are the products of four related genes. They are expressed in both embryonic and adult brain, and their expression correlates with mitotic and migratory events in certain embryonic nervous tissues (2,3). Their expression in glioma tissues needs further investigation.

### 2.1.6. OTHER GPs

A few other ECM GPs may play a significant role in BT biology, because they are expressed much more in gliomas than in normal tissue. These include vitronectin (VN), osteopontin, and secreted protein, acidic, and rich in cysteine (SPARC). SPARC, also known as osteonectin, is developmentally regulated and plays an important role in cell migration, matrix mineralization, and angio-

genesis. SPARC is strongly upregulated in malignant tumors, including malignant glioma (23). Osteopontin, another acidic GP associated with bones and present at very low levels in normal brain, is upregulated in gliomas and correlates with the degree of malignancy. Osteopontin can be produced by glioma cells themselves (24). VN, expressed mainly by hepatocytes, is expressed at high levels by mesenchymal tissues in the brain (25). VN is also expressed in high-grade gliomas, but is undetectable in low-grade gliomas. In xenograft models, VN was preferentially detected at invading tumor borders, although it was not clear whether VN was expressed by the tumor cells themselves or by the invaded normal brain tissue (26).

### 2.1.7. GAGS AND PROTEOGLYCANS

The general structure of proteoglycans is a core protein to which GAG chains are attached (Fig. 1B). The lengths of the core protein and the GAG chains vary in different kinds of proteoglycans. Most proteoglycan names are based on their core protein, such as versican, phosphacan, or decorin, and they are classified according to the main kind of GAG chains associated with them. These GAG moieties may be heparan sulfate (HS), CS, keratan sulfate, or dermatan sulfate. These GAGs are chains of disaccharides with alternating hexuronate and hexosamine. The core proteins of proteoglycans may vary in size from 10 to 400 kDa (3). So far, CS proteoglycan and HS proteoglycan seem to be important for the CNS. Some dermatan sulfate proteoglycans, such as decorin and biglycan, which may also have CS GAGs, are expressed in the CNS and are upregulated in neural injury and degenerative processes (2,3). Most proteoglycans are present in ECM, but some may be cell-surface proteoglycans, and may serve as receptors for growth factors, as well as for ECM components. These include syndecans (which have both HS and CS chains), betaglycan, and NG2 (2).

**2.1.7.1. HS Proteoglycan.** Most of the HS proteoglycans, which include syndecans, glypicans, and betaglycan, are cell surface proteoglycans; prelecan is associated with the basal lamina (2,3). Even the cell surface proteoglycans are intimately bound to and related to the ECM. Expressed in embryonic brain, they are downregulated in adult brain, where they may persist in specialized regions. HS proteoglycan expression is increased in gliomas and correlates with the degree of malignancy (27).

**2.1.7.2. CS Proteoglycans.** The CS proteoglycans expressed in the CNS are versican, neurocan, phosphacan, brevican, aggrecan, and related molecules (2,3). Aggrecan is the major hyaluronate-binding protein in cartilage ECM. Neurocan and brevican are interesting, because their expression is restricted to the nervous system. Many CS proteoglycans, such as neurocan and phosphacan, have a high-affinity binding site for tenascin, F11/contactin, and integrin receptors (6). CS proteoglycans modulate cell interactions and play a significant role in the developing nervous system. Some, such as aggrecan-related Ag CAT-301, are associ-

ated with synapses, and some form part of the astroglial axon barriers. Experimental findings suggest that CS proteoglycans may act as repulsive molecules, and thus permit cell division and migration. The overall expression of CS proteoglycans is not increased in gliomas compared to normal glial cells (27), and may actually show a progressive reduction as anaplasia increases in gliomas (28). However, there are a few exceptions. For instance, a splice variant of versican, V2, although decreased in glioma ECM, is increased in the tumor vessels (3). Brain-enriched hyaluronan binding (BEHAB), which is a truncated form of brevican, is developmentally regulated and absent from the adult brain, but is expressed in the gliomas. NG2, which is an integral membrane CS proteoglycan, is downregulated in adult brain, but reappears in vascular proliferations of gliomas (3,29).

**2.1.7.3. Hyaluronic Acid.** Known as hyaluronan, hyaluronate, or hyaluronic acid (HA) is different from other GAGs, because of a relatively simple, but much larger molecular structure and the absence of any covalent bond to a protein (30). HA is a ubiquitous component of the ECM, and organizes the matrix. This acid organizes large aggregates of proteoglycans by binding different proteoglycans to itself with the help of link proteins (Fig. 1B). These aggregates are the main component of ECM in the neuropil and form the pericellular network, as first described by Golgi (5). Hyaluronate plays an important role in cell migration and proliferation in the developing CNS. In gliomas, HA is expressed at the leading edge of the tumor. The overall increase in the expression of HA in gliomas correlates with the degree of anaplasia (3,6,27).

## 2.2. Cell Surface Adhesion Molecules

It should be reiterated here that the distinction between a CAM and an ECM molecule is somewhat arbitrary. For example, certain CS proteoglycans may act as cell surface receptors, and a form of NCAM is released and incorporated into the ECM.

Based on their molecular structure and physiological role, most of the CAMs can be grouped into six classes (31). Four of them, which involve protein–protein interaction, are Ig superfamily, cadherins, integrins, and receptor protein tyrosine phosphatases (RPTPs). The other two, which involve binding to carbohydrates, are hyaluronate receptors and selectins. This classification, based on molecular homology, is also indistinct. Ig domains may occur in other adhesion molecules, such as perlecan, which is a proteoglycan; and some Ig superfamily adhesion molecules and certain integrins may contain FNIII repeats (4). RPTPs differ from other CAMs by virtue of a catalytically active site in the cytoplasmic domain. The adhesion molecules may bind to themselves (homophilic binding) or bind to other CAMs or ECM molecules (heterophilic binding). From what is currently known, it appears that hyaluronate receptors are involved only in cell–ECM interaction, integrins in both cell–ECM and cell–cell interaction, and the rest of the CAMs in only cell–cell interaction.

The classification of CAMs into the six groups mentioned above is not comprehensive. CD24 (e.g., a GP that does not traverse the cell membrane, but is covalently linked to the membrane lipid bilayer) is expressed transiently by the developing brain and is upregulated in malignant glioma (32,33).

### 2.2.1. IG SUPERFAMILY

NCAM is the prototype of this class of CAMs. Other molecules in this class include contactin, and intercellular and vascular adhesion molecules. They have multiple Ig domains; NCAM has five. Some members of this group may be important in tumor biology. Increasing the expression of NCAM in rat glioma cells by transfection decreases their invasiveness (34). L1 (NgCAM), which may act as a receptor for CS proteoglycans and bind to other CAMs in the nervous system, is upregulated in gliomas (35).

### 2.2.2. CADHERINS

Cadherins are a group of calcium-dependent adhesion molecules that consist mostly of E-cadherin, P-cadherin, and N-cadherin. Their significance in gliomas needs further investigation.

### 2.2.3. INTEGRINS

Integrins are a large family of heterodimeric GPs composed of  $\alpha$ - and  $\beta$ -subunits. In most cases, integrins bind to RGD sequences, which is found and repeated in several adhesion molecules. Currently, at least 16  $\alpha$ - and 8  $\beta$ -subunits are known. Both  $\alpha$ - and  $\beta$ -subunits are transmembrane GPs with large extracellular domains. Only limited combinations of these subunits exist as functional receptors. The  $\beta$ -subunits of integrin are used for subclassification of these molecules. The  $\beta_1$  and  $\beta_3$  subfamilies are more involved in cell–matrix interaction and are expressed on most of the cell types; the  $\beta_2$  subfamily is more involved in cell–cell interaction and is somewhat restricted to leukocytes (31). Integrins  $\alpha_3\beta_1$  and  $\alpha_v\beta_3$  are upregulated in astrocytomas and glioblastomas, and occasionally upregulation of  $\alpha_2$ ,  $\alpha_5$ ,  $\alpha_6$ , and  $\beta_4$  can also be found in gliomas (31,36).

### 2.2.4. HYALURONATE RECEPTORS

Although several ECM proteins bind to hyaluronate, two proteins act as the major cell surface receptors for hyaluronate: CD44 and receptor for hyaluronic-acid-mediated motility (RHAMM). Both are expressed by normal glia, as well as by glioma (3). CD44 has more affinity for hyaluronate and is the major hyaluronate receptor in mammalian cells, but RHAMM may play a larger role in the motility of cells. CD44 exists as a family of more than 20 GPs, because of alternative splicing of 10 variant exons and posttranslational glycosylation (3). The smallest form without the variant exons is the standard isoform (CD44s); the remaining isoforms are larger in size and can be identified numerically. CD44s



is expressed by normal astrocytes, as well as in all gliomas. The variant forms (CD44v), when studied with the help of polyclonal antisera that reacted to most of the variant epitopes, were found to be expressed by most gliomas (and present in all the glioblastoma), and expression correlated with the degree of malignancy (37,38).

### 3. ADHESION MOLECULES AS TARGETS FOR IT

During the initial efforts to use radiolabeled MAbs for radioimmunotherapy (RIT), it was soon realized that systemic administration of radiolabeled MAbs in therapeutic amounts for primary CNS neoplasms would cause significant systemic toxicity (39,40). Furthermore, the access of MAbs to the brain was inadequate, because of the blood–brain barrier (BBB) and high interstitial pressure in the tumor mass, as posited by Bigner et al. (41). Following other examples, such as intrapleural or intrapericardial administration of radioimmunoconjugates (RICs) for the treatment of malignant effusions, direct application of RICs to CNS malignancies was first reported by Kemshead et al. (42). Compared to external radiotherapy (RT) with focal beam radiation or brachytherapy, which can be given only once, local RIT can be repeated several times. If conjugated to yttrium-90 ( $^{90}\text{Y}$ ), RICs may even be administered in the outpatient setting, cutting the cost and making it more acceptable to the patient (43). Direct delivery of RICs to tumors results not only in improved irradiation of the tumor tissue, but also decreases the amount circulating in the blood and reaching other organs, thus improving the therapeutic advantage. Although intrathecal or intratumoral administration of RICs may have fewer systemic toxic side effects, they can cause more neurotoxic effects (41,44).

Adhesion molecules, particularly those that incorporate into the ECM, provide reasonably good targets, because they are relatively more accessible, compared to intracellular Ags and have a slower turnover. Several MAbs against adhesion molecules have been tested in preclinical and clinical trials and are discussed below.

#### 3.1. ERIC-1

NCAM, which belongs to the Ig superfamily of CAMs, is expressed equally on both neural cells and glioma cells (43,45). The logic of using NCAM as a target was to achieve maximum binding of RICs to the wall of the resection cavity, thus bringing the cuff of tissue most likely to harbor glioma cells within the range of the radiation (43). In the UK, Kemshead's group used MAb ERIC-1, which is an IgG<sub>1</sub> isotype, to deliver radiation by conjugating it to either iodine- $^{131}\text{I}$  or  $^{90}\text{Y}$  (43–57). After injection into the resection cavity, Hopkins et al. (46) found that the RIC diffused exponentially from the edge of the resection cavity. The peak dose delivered this way was within one-fifth of a centimeter deep to



the cavity margin; the mean dose at a distance of 2 cm from the edge was only ~5% of the total (46). Injecting directly into the resection cavity produced better pharmacokinetics than injecting intrathecally. When injecting into the resection cavity, tumor clearance was slow, with a maximum blood activity of ~14% of the injected dose; intrathecal injection showed a peak maximum blood activity of 40% within 48 h of injection (43). In a pilot study of seven patients using  $^{131}\text{I}$ -ERIC-1, in which 36–59 mCi was delivered to the tumor, the side effects were cerebral edema, radionecrosis, and seizures (47), but the number of patients in the study was too small to reach any conclusion about the effectiveness. Switching to  $^{90}\text{Y}$  as the radiation source conjugated to ERIC-1, the toxicity seemed to be lower (e.g., cerebral edema that responded to steroids and transient myelosuppression). In the study by Hopkins et al. (43) on 15 patients who received a total of 23 injections (10.8–24.9 mCi) into the resection cavity, the median survival was approx 24 wk after treatment.

### 3.2. BC-2 and BC-4

Although using NCAM as the Ag target for RICs concentrated the radiation to tissue in the wall of the resection cavity, this Ag is not selective for the tumor cells. On the other hand, tenascin is an Ag that seems to be more selective for tumors, and its expression is more consistent than other cell surface tumor-associated Ags (48). It is localized in the intercellular space on cell surfaces, in the basement membrane of tumor vessels, and in the cytoplasm of some tumor cells. It inversely correlates with the degree of dedifferentiation (49). Tenascin's presence is scanty in the adult CNS, but it is expressed strongly by gliomas (i.e., as much as 99% of glioblastomas in one study [41]). Two groups have chosen to use MAbs against tenascin for RIT: Riva et al. (50 in Italy used BC-2 and BC-4 MAbs, and Bigner et al. (41) in the United States have used MAb 81C6.

BC2 and BC4 MAbs bind to different epitopes on tenascin (50). BC-2 binds to an epitope within FNIII repeats.  $^{131}\text{I}$ -conjugated BC-2 and BC-4 were used in both phase I and phase II trials (50). That study, which started in 1990, had a total of 105 patients with malignant gliomas who received RICs, with a mean dose of 54 mCi. Sixty-two patients (58 glioblastoma multiforme [GBM] and four AAs: 31 with newly diagnosed tumors and 31 with recurrent lesions) received multiple courses (up to six) of the MAb through an indwelling catheter. The median survival after RIT was 23 mo, and the overall response rate, which included either partial or complete remission or no evidence of disease during the period of observation, was 51.6%. The time-to-relapse after surgery and RT was 12 mo (4–25 mo) if RIT was given immediately after initial surgery and RT, and 5 mo (2–15 mo) if RIT was given only after relapse. The most important factor that seemed to dictate outcome with RIT was the residual tumor burden during surgical resection prior to RIT. In the subgroup that received gross total resection (40/62 patients), the response rate was 70%, and the median survival

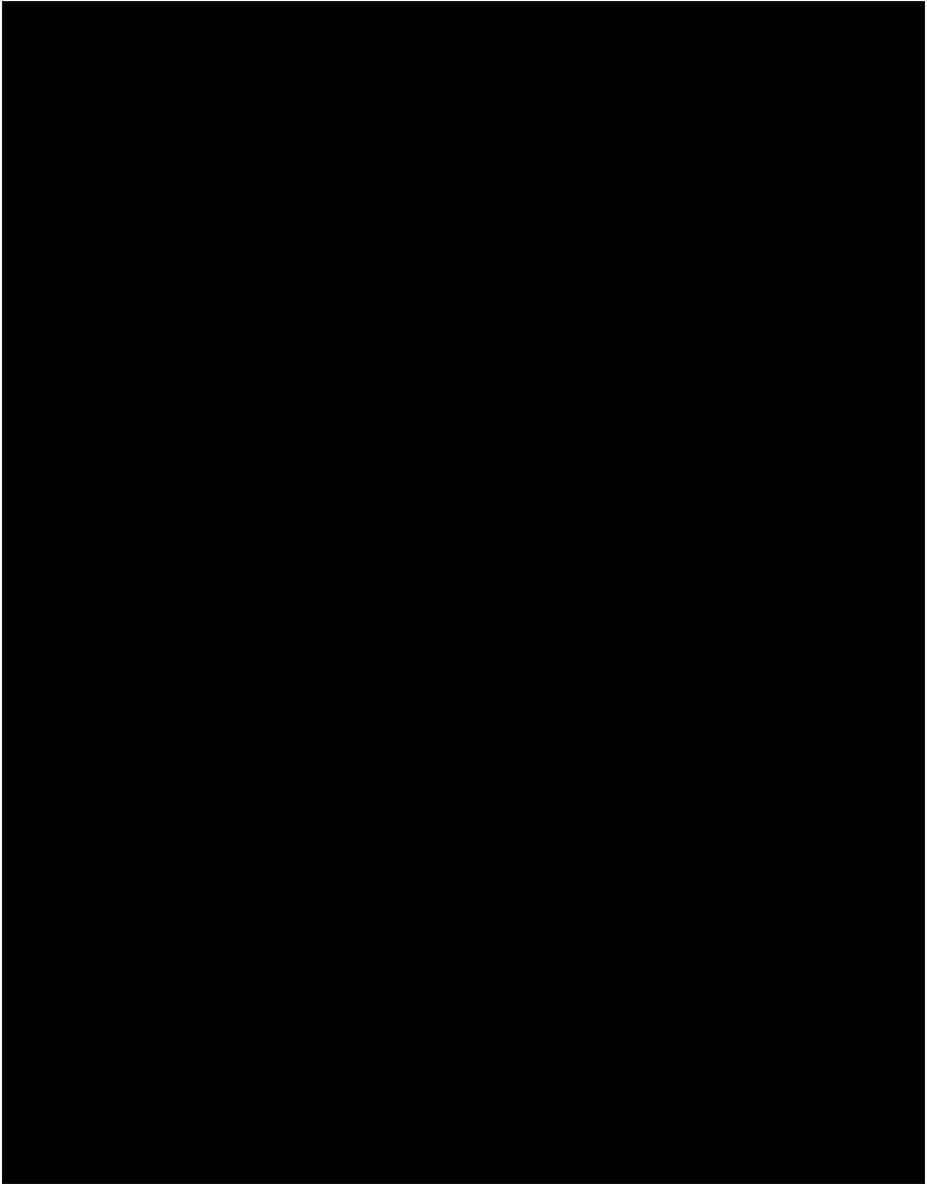
was 27 mo. In those who had macroscopic evidence of disease after surgery (3.5–54.3 cm<sup>3</sup> of tumor mass remaining), the response rate was only 18%, and the median survival was 17 mo. Of those patients who had a gross total resection, 50% continued to have no evidence of disease (Fig. 3) during the period of observation (median, 13 mo; range, 7–38 mo). The majority of side effects observed were minor, such as transient headaches lasting <24 h, which were preventable with nonsteroidal anti-inflammatory drugs (NSAIDs) and steroid premedications. Human antimouse antibody (HAMA) production was the only significant side effect. It appeared to be dose-dependent and was observed in all patients who had three or more courses.

### 3.3. 81C6

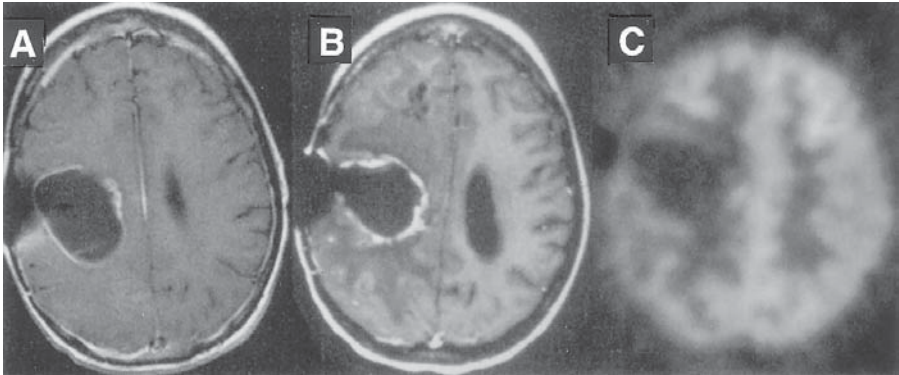
81C6 is an antitenascin IgG2b MAb, and its tumor-localizing capacity has been shown to be superior to other antiglioma Abs (41). BC-2 and BC-4 bind to all major kinds of tenascin, 81C6 binds to a particular variant of tenascin that possesses extra alternatively spliced forms of FNIII repeats (41). This gives 81C6 a greater specificity. 81C6 does not bind to normal brain, but more than 90% of biopsy specimens of GBM or AA react with 81C6 MAb (51). Preclinical trials with this MAb were promising. Significant delay in tumor growth, prolongation of survival, and, in a few cases, apparent cures were observed when <sup>131</sup>I-labeled 81C6 was used in subcutaneous glioma xenograft models using athymic mice or in intracranial xenograft models using athymic rats (41).

A phase I trial evaluated the safety of <sup>131</sup>I-labeled 81C6 after gross or near-total resection in 34 patients (36 treatments) with either recurrent malignant glioma (six AAs and 26 GBMs) or metastatic melanoma (two patients) (41). Patients were given 20, 40, 60, 80, 100, or 120 mCi to find the maximum-tolerated dose (MTD). No toxicity was noticed in doses up to 80 mCi, but at higher doses two patients developed major hematologic toxicity. Meningitis or encephalitis did not occur in any patient, but at 120 mCi some clinical manifestations of neurologic toxicity (such as hemiparesis, monoparesis, aphasia, transient focal seizure, and headaches) developed in five patients. The neurologic toxicities were either self-limiting or responded to steroids, except in one case of hemiparesis, which developed 13 d post-treatment. 100 mCi or lower doses were not associated with any neurologic toxicity (11 patients). No other significant side effects, such as hepatic, renal, thyroid, or other toxicity, were observed. HAMA developed in 57% of cases, but did not cause any evident toxicity. That phase I study suggested that approx 450 Gy radiation could be delivered safely to the wall of the resection cavity, with whole-brain irradiation delivering as low as 700 cGy.

In 14/33 (42%) assessable treatments, stabilization of disease was observed (Fig. 4; 41). The median survival after RIT was 60 wk for GBM and AA patients combined, and 56 wk for GBM patients alone ( $n = 26$ ) (Fig. 5). This improved



**Fig. 3.** Example of response to RIT. A patient with left frontal GBM (A) underwent gross total resection, then received five cycles of BC2 and BC4 MAb conjugated to  $^{131}\text{I}$  over 12 mo. Repeat computed tomography scans during follow-up showed no evidence of disease (B). The scan in (B) was taken after 15 mo, and shows a catheter in the resection cavity for delivery of the RIC. Reprinted by permission from ref. 50.

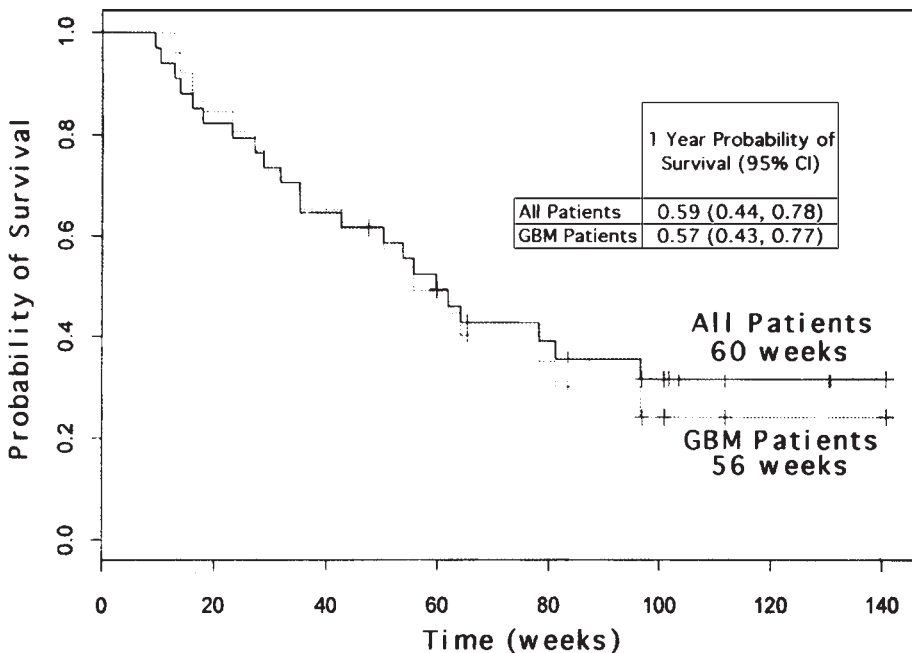


**Fig. 4.** Another example of response to RIT using MAb to tenascin (81C6). Surgically created resection cavity before (A) and 24 mo after (B) injection of MAb. The rim of contrast enhancement in (B) resulting from postsurgical changes and reaction to the RIC did not change and correlated with a hypermetabolic ring on PET scan (C). Reproduced with permission from ref. 41.

survival after RIT is very promising, compared to other adjunctive rescue measures taken at recurrence. In a multi-center trial of 222 patients, local delivery of 1,3-bis-(2-chloroethyl)-1-nitrosourea in polymers increased the median survival from 23 wk to 31 wk after surgery for recurrence (52). Stereotactic radiosurgery and high-dose brachytherapy increased the median survival by 41 wk and 46 wk after treatment, respectively (53). The efficacy of RIT needs the test of randomized trial. RIT also seems safer than other methods. Whereas 50–64% of patients undergoing brachytherapy required reoperation for symptomatic radionecrosis (54,55), none of the patients undergoing  $^{131}\text{I}$ -81C6 treatment required reoperation for treatment of radionecrosis (41).

A phase I trial using  $^{131}\text{I}$ -81C6 for intrathecal injection of 40–100 mCi in 31 patients with leptomeningeal carcinomatosis or BTs with subarachnoid communication showed a partial response in one and stabilization in 13 (42%). Twelve patients were alive during the median follow-up of ~46 wk, and five were progression-free with a median follow-up of 58 wk. Because of hematologic toxicity, the MTD in this study was 80 mCi (41).

In another recent phase I study (56), 42 patients with newly diagnosed malignant gliomas (32 GBMs and 10 AAs) were treated with escalating doses of 20–180 mCi of  $^{131}\text{I}$ -labeled 81C6 MAb via surgically created resection cavities. At doses >120 mCi, seven patients developed acute neurologic symptoms, one developed subacute symptoms, and four patients had delayed neurologic findings. There was no major hematological toxicity. MTD was reached at 120 mCi. In a preliminary report, median survival for all patients was 79 wk (and 69 wk for the 32 patients with GBM), which was significantly better than the 40 wk



**Fig. 5.** Kaplan-Meier analysis of survival data of patients with recurrent malignant gliomas who were treated with RIT using 81C6 MAb. The 1-yr survival probability for all patients was 0.59 (95% CI, 0.44–0.78), and for those with GBM was 0.57 (95% CI, 0.43–0.77). Reproduced with permission from ref. 41.

obtained with conventional therapy. Encouraged by the minimal toxicity and significant increase in median survival observed in this study, a phase II trial has been initiated (56).

### 3.4. Mel-14

MAb Mel-14 was developed against melanoma cells, and initial analyses suggested that it was specific for melanoma CS proteoglycans. However, it has also been found to be selective for selectin, a leukocyte cell surface adhesion receptor that shares a domain with versican, a CS proteoglycan (57,58). Mel-14, an IgG<sub>2a</sub> MAb, has a high Fc receptor affinity. Therefore, its tumor uptake is highly variable, and it caused significant hematologic toxicity in animal studies (59). In the analysis of Mel-14 affinity, the CS proteoglycan that bound to Mel-14 was identified as GP-240. In addition to melanomas, Mel-14 was found to react with malignant gliomas (51). Mel-14 reactivity was detected in 2/7 (28%) astrocytomas and in 28/49 (57%) GBMs (51).

Preliminary results of a phase I study, using Mel-14 conjugated to <sup>131</sup>I for intrathecal injection in leptomeningeal metastases (glioma or melanoma), or for

injection into surgically created resection cavities open to the subarachnoid space, have been reported (59). In leptomeningeal metastasis, 3/11 patients showed complete responses and two showed partial radiographic responses, with a slight increase in median survival from 3 mo to more than 5 mo. Only one developed major hematologic toxicity (59). In one patient with metastatic melanoma, who received  $^{131}\text{I}$ -Mel-14 into the BT resection cavity but later died because of systemic metastases, there was no evidence of recurrent tumor at the treatment site of autopsy (59).

### 3.5. Bispecific Abs

Development of bispecific MABs created by the hybridization of two hybridoma cell lines, each specific for one Ag, has provided promising results in vitro. A parental hybridoma (BC-2), which produced MAB against tenascin, and a hybridoma (CBT3G), which produced MAB against the CD3 Ag on T-cells, were fused, then selected in vitro to produce a hybridoma cell line that would provide a bispecific MAB. This new bispecific MAB induced and targeted T-cells to tenascin-positive cells and created significant cytotoxicity against tenascin-positive tumor cells in vitro (49). A similar strategy was used earlier by Obukhov et al. (60) to produce bispecific MABs against NCAM and one of the T-cell Ags, either CD3 or CD16. Bispecific MABs against NCAM and CD16 were more effective than bispecific MABs against NCAM and CD3 in causing cytolysis of U251 human glioma cells in vitro. Studies in animal models and preclinical trials, using bispecific MABs have not been reported.

## 4. TROUBLE SHOOTING

Circulation of MABs in the blood may result in the development of HAMAs, in addition to other toxic systemic side effects. Development of HAMAs is a common problem with IT and can potentially occur in any immunocompetent patient who receives murine MABs. Development of HAMAs precludes repeated injection of the MAB. There is evidence that the Fc fragment of MABs may be the cause of hematologic toxicity and considerable variation in tumor uptake (51,61). Using chimeric Abs, created by combining human Fc fragments and the Fab fragments from murine MABs, should reduce HAMA formation, systemic toxicity, and variation in tumor uptake, if injected systemically. Chimeric Abs have been prepared for 81C6 and Mel 14 (59). Doubling of tumor uptake was noticed by using chimeric Mel-14 in an animal model, without sacrificing its specificity and affinity (62). Similarly, chimeric 81C6 Abs improved localization in human glioma xenograft models, as well as prolonged serum half-life (63).

As better control is achieved in the local recurrence of gliomas, distant recurrences are becoming more frequent. On average, recurrences more than 2 cm

away from the original tumor site were found in 21% of cases following RT or brachytherapy, and the incidence of multifocal glioma was found in 7.5% of cases (64). In a phase I trial of  $^{131}\text{I}$ -81C6 IT, recurrences more than 2 cm away from the original tumor were present in 3/32 patients (9%), and multifocal failures were found in 4/32 (12%). The pattern of failure in the IT trial suggests that the MAb did not penetrate enough beyond the resection cavity. This was confirmed by dosimetric analysis of intracavitary delivery of the RICs. At a distance of 2 cm from the edge of the cavity, the mean dose of radioactivity was only ~5% of the total (46). The method of delivery of RICs must be modified or supplanted by other means to better target the invading glioma cells. Absorption within the cavity wall is a function of dose administered, volume of the resection cavity, and MAb clearance from the resection cavity. Studies in which antitenascin MAbs were administered intratumorally, prior to the operation, have shown that labeled MAbs spread adequately to all the viable neoplastic tissue within the main tumor mass (65). Spread of the RICs beyond the main tumor mass is needed to prevent distant recurrences. The use of either monomeric or dimeric Fab fragments, or the use of hyperthermia, which has controversial benefits (66), may aid in the distant spread of RICs. Furthermore, intratumoral injection can be supplemented with intrathecal or intravenous low-dose injection to improve the delivery of the MAb to cells that have invaded far beyond the main tumor mass.

The Ag heterogeneity of glioma cells also needs to be considered. Anti-NCAM MAb (ERIC-1) was used with the intention of decreasing the loss of RIC from the cavity where it was initially injected, by maximizing the binding of the radionuclide to the wall even when minimal residual tumor was left (46). This strategy, however, also limited delivery of the MAb to the periphery of the tumor mass and to cells that had invaded beyond. The benefit of using a MAb to an Ag that is preferentially expressed by tumors (such as tenascin) is that the MAb probably will accumulate in those areas where there is a higher population of tumor cells. These Abs to tumor-associated Ags may even attack solitary tumor cells that have migrated into the brain parenchyma. In such cases, however, the dosage of the MAb must be adjusted according to the residual tumor at the site of injection, because, if there are not enough tumor cells to capture MAb, a high residue of MAb in the cavity will eventually diffuse systemically and cause toxicity.

## 5. FUTURE DIRECTIONS

Significant improvements are required if RIT is to achieve the status of a primary adjunctive modality in the treatment of gliomas. Surgery and RT will continue to play major roles in the treatment of BTs for some time. IT and gene therapy may play important adjunctive roles. To further define the use of IT as an adjunctive treatment, several clinical studies using adhesion proteins as Ags



are underway. One is a pilot study incorporating a single injection of RIC during the period between surgery and RT. A second is a randomized trial in which surgery is followed with labeled or unlabeled antitenascin Abs (81C6), along with RT and chemotherapy. The third study (41,43) is a phase I trial on newly diagnosed malignant BTs treated with RICs before external beam RT.

A review of the CAMs and ECM molecules shows the complexity inherent in the numerous GPs and in particular proteoglycans. Many of the known proteoglycans have not been studied with respect to gliomas, and some of these known and unknown Ags may become potential targets for IT. Among the known adhesion molecules that are associated with gliomas, BEHAB is a good candidate for IT because it is absent from the adult brain but is expressed in gliomas, particularly in the invasive kind (67). Also, alternate splicing and variation in glycosylation create several isoforms of CD44 and NCAM. Considering the rich expression of CD44 and NCAM in gliomas, discovery of an isoform of CD44 or NCAM which is expressed exclusively in tumors can be another potential target for IT. However, IT need not be directed only to the glioma cells. Theoretically, injury to the vascular basement membrane in gliomas by RICs should cause necrosis in the tumor mass and decrease the size of the tumor. Attention should also be given to integrin receptors. For example, the  $\alpha_5\beta_1$  integrin receptor is expressed significantly more in gliomas than in normal brain. It upregulates the antiapoptosis protein, Bcl-2, inside the cell (68,69). An Ab that can block activation of this integrin receptor may thus induce apoptosis in tumor cells. Furthermore,  $\alpha_5\beta_1$  also acts as a FN receptor, which probably plays a key role in glioma cell invasion. Thus, a MAb to  $\alpha_5\beta_1$  may also potentially retard invasion (9). Several other integrins, such as  $\alpha_v\beta_3$  (VN receptor), and other CAMs, such as intercellular adhesion molecule 1 and lymphocyte function-associated antigen 3, are expressed several times more in the glioblastoma parenchyma and/or glioblastoma endothelium, compared to normal brain (69). Each of these needs careful examination when choosing an appropriate IT target.

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# V TUMOR VACCINES AND OTHER STRATEGIES

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# 13 Cytokine-Based Immuno-Genetic Therapy for Brain Tumors

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*Roberta P. Glick, MD, Terry Lichtor, MD, PhD,  
and Edward P. Cohen, MD*

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## 1. AN IMMUNE PRIMER

The function of the immune system is to protect the body. Leukocytes (white blood cells) and a number of accessory cells distributed throughout the body perform this defensive function. Lymphocytes are the key cells controlling the immune response (IR). They specifically recognize foreign material and distinguish it from the body's own components. But this is an old concept, and a new model by Fuchs and Matzinger (*1*) is presented below.

There are two chief types of lymphocytes: B-cells, which produce antibodies (Abs), and T cells, which have a number of functions, including helping B-cells to make Abs, recognizing and destroying virus-infected cells, activating phagocytes to destroy pathogens, and controlling the level and quality of the IR.

The essential role of T-lymphocytes is to recognize antigen (Ag) presented by antigen-presenting cells (APCs) through specific cell-surface T-cell receptors. APCs are a group of cells that are capable of taking up Ags, partially degrading them, and presenting them to T-lymphocytes in a form they can recognize. B-cells recognize Ags in their native form, but T-cells only recognize Ag peptide derivatives of complex Ags that have become associated with major histocompatibility complex (MHC) molecules. Thus, MHC molecules present Ags (i.e., peptides) to T-cells. MHC class I (MHC-I) molecules are found on all

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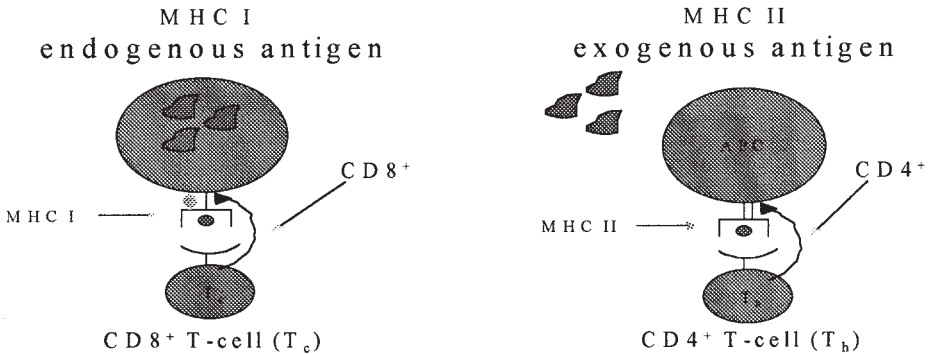
nucleated cells and platelets. MHC-II molecules (Ia Ags) required for helping B-cells or making Abs are expressed on B-cells, macrophages, monocytes, APCs, and some T-cells. CD8<sup>+</sup> cells (i.e., cytotoxic T-cells/cytotoxic T-lymphocytes [CTLs]/killer T-cells) are MHC class I-restricted, meaning they only recognize Ag presented in the context of MHC-I molecules; while CD4<sup>+</sup> cells (i.e., helper T-cells) are MHC-II-restricted (Fig. 1). Ags synthesized within a cell, such as viral polypeptides, associate preferentially with MHC-I molecules and present Ag directly to CD8<sup>+</sup> cells (direct pathway). In contrast, Ags that are taken up by an APC are partially degraded (processed) and returned to the cell surface associated with MHC-II molecules, which are recognized by CD4<sup>+</sup> cells (indirect pathway) (Fig. 1).

Recently, Fuchs and Matzinger (*1*) described a new model for the immune system: the “danger” model. According to this model, the immune system distinguishes between dangerous and harmless entities, and this distinction is made by the APCs. Tumor cells are not immunogenic, and therefore do not act as APCs. They do not activate T-cells directly. However, it should be possible to immunize against tumor Ags by priming APCs when tumors are small, or immediately after resection. The authors’ laboratory and others have recently used this strategy in the development of new types of tumor vaccines against brain tumors (BTs).

Immunity may be cell-mediated (e.g., by T-cells, natural killer [NK] cells, and phagocytes) or humoral (e.g., involving Abs and complement). Cytokines are a group of molecules, other than Abs, produced by lymphocytes, which are involved in regulating the immune system. They include the interleukins (ILs), the interferons (IFNs), tumor necrosis factors, and colony-stimulating factors (CSF). This chapter discusses the preclinical studies and clinical applications of cytokine-based immuno-gene therapy for BTs.

## 2. INTRODUCTION

The current prognosis for patients with primary and metastatic brain cancers remains poor (*2*). Malignant gliomas are the most common primary BT. Despite treatment with surgery, radiation, and chemotherapy, the 2-yr survival rate remains less than 20%. Patients with malignant melanoma, one of the most common tumors to metastasize to the central nervous system (CNS), survive approx 3 mo after symptoms first appear (*3*). Twenty to 30% of all patients with malignant melanoma develop cerebral metastasis. Breast cancer is the second leading cause of cancer-related death in American women. In 1997, more than 40,000 women died of the disease, with brain metastases occurring in 15–30% of these patients. The 2-yr survival for patients with intracranial breast cancer metastases remains poor, despite optimal conventional therapeutic treatments. Thus, new



**Fig. 1.** Direct vs indirect recognition of Ag peptides. T-lymphocytes recognize short Ag peptides presented in a groove formed by the external domains of MHC-I and -II molecules. Tumor cell Ags on the surface of the tumors are recognized by cytotoxic T-cells (CD8<sup>+</sup> T-cells) via MHC-I presentation. Alternatively, the tumor Ags can be ingested by macrophages, which can then express the Ags and stimulate CD4<sup>+</sup> T-cells via MHC-II presentation.

and innovative forms of effective treatments are urgently needed for both primary and metastatic malignant CNS tumors.

One emerging strategy in the treatment of tumors involves stimulation of an immunologic response against the neoplastic cells. The hope is that the immune system can be called into play to destroy malignant cells. However, in most instances, proliferating tumors do not provoke antitumor cellular IRs. The precise mechanisms that enable “antigenic” neoplasms to escape host immunity are not completely understood. Tumor cells appear to escape recognition by the immune system, although neoplastic cells have weakly immunogenic tumor-associated Ags (TAAs). Successful methods to induce immunity to TAAs could lead to tumor cell destruction and prolong the survival of cancer patients. A variety of strategies have been used to increase the antigenic properties of tumor cells. In some instances, objective evidence of tumor regression has been observed in patients receiving immunizations only with tumor cell immunogens, suggesting the potential effectiveness of this type of immunotherapy for malignant neoplasms.

Some of the molecular gene therapy techniques used to augment the IR to tumor cells include:

1. Genetic modification of cells to secrete immune-augmenting cytokines, such as IL-2, granulocyte-macrophage CSF (GM-CSF), INF- $\gamma$ , and IL-12.
2. Genetic modification of cells to express foreign (allogeneic) MHC determinants.
3. Genetic modification of cells to express co-stimulatory molecules (e.g., B7) required for T-cell activation.
4. Genetic modification of cells to express TAAs.

5. Use of tissue specific promoters.
6. Modification of delivery techniques, e.g., systemic vs intracerebral immunization.

### 3. PRECLINICAL AND CLINICAL APPLICATIONS

#### 3.1. *Direct Administration of Cytokines*

Cytokines have been used in systemic therapy for tumors outside the CNS. In particular, systemic biologic therapy with IL-2 has been shown to result in significant antitumor effects in patients with advanced metastatic melanoma or renal cell cancer (4,5). IL-2 has no direct toxic effect on cancer cells. Its antitumor activity is mediated by modulation of the host's immunologic response to the neoplasm. IL-2 is required for the growth of CTLs (CD8<sup>+</sup> cells), and stimulates these cells to engage in cytotoxic activity. It also enhances the activity of NK/lymphokine-activated killer (LAK) cells, which possess nonspecific antitumor activity; the CD8<sup>+</sup> cells provide Ag-specific antitumor functions. Activation of both of these cell types probably is required for the optimum opportunity for tumor rejection. Another cytokine, IFN- $\gamma$ , induces the expression of MHC-I determinants and augments the sensitivity of tumor cells to CTL-mediated lysis. IL-12 activates APCs for tumor rejection. Thus, the weakly antigenic neoplastic cells may become immunogenic and stimulate antitumor IRs, as a result of the presence of these cytokines.

Use of IL-2 in the treatment of patients with high-grade gliomas has been actively pursued, particularly since the observation that such patients may have both decreases in IL-2 production and IL-2 receptor expression (6). However, significant antitumor effects have not been found utilizing cytokines in patients with gliomas (7-9,11). Clinical immunotherapeutic attempts against gliomas have involved the systemic intrathecal and intratumoral injection of autologous lymphocytes and/or administration of various cytokines that may enhance the IR (7-11). Systemic administration of cytokines along with LAK cells has been attempted with limited success (7-9,11). Some of the failures observed in the clinical trials using systemic IL-2 for patients with gliomas may result from adverse effects of the therapy. IL-2 causes an increase in vascular permeability that leads to accumulation of fluid in body compartments and possibly intracerebral (ic) edema. Because the administration of IL-2 does not result in neutropenia or immunosuppression, patients are not susceptible to opportunistic infections, which are frequently observed following treatment with chemotherapeutic agents known to suppress the immune system. However, the CNS toxicity that commonly occurs with this form of treatment limits the amount of IL-2 that can be administered (8).

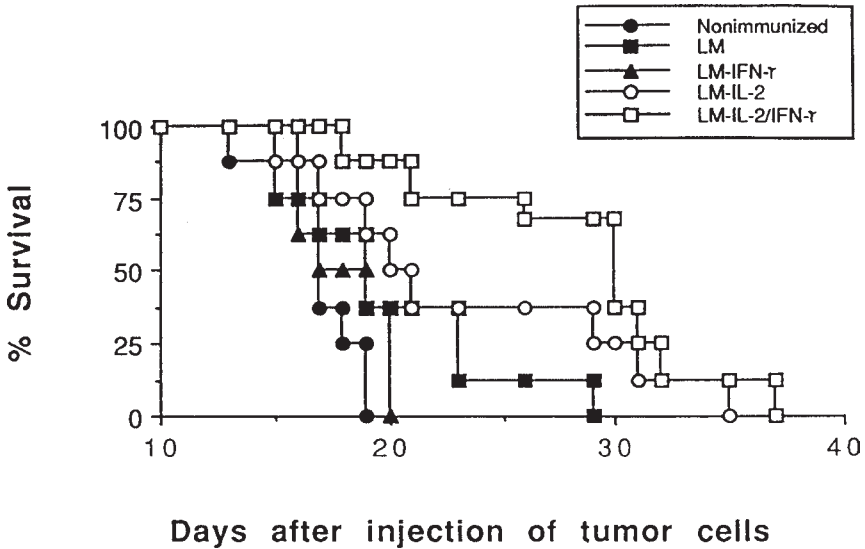
IFN- $\gamma$  has been found to inhibit the *in vitro* growth of glioma cell lines (12). However, many of the findings established with BT cell lines have not been

observed in vivo, and most of the in vivo studies have involved the treatment of tumors in immunodeficient nude mice (13). The relevance of these models to human disease is uncertain.

### 3.2. Cytokine-Based Gene Therapy

The use of gene therapy for BT treatment has included new methods for the delivery of cytokines. Transduction of genes specifying various cytokines into malignant cells, and cytokine secretion by the tumor cells themselves, have also resulted in augmentation of the immunogenic properties of brain neoplasms. Transfection of the human *IFN- $\gamma$*  gene into human glioma cells led to an inhibition of growth when the tumor cells were exposed to LAK cells (14). Significant inhibition of growth of a nontransfected human glioma cell line in nude mice was observed when an IL-4-secreting cell line was implanted subcutaneously with the glioma cells (13). A similar effect was also observed when the tumor and IL-4-secreting cells were injected intracerebrally. Rats injected subcutaneously with 9L gliosarcoma cells transduced with the *IL-2* gene demonstrated tumor eradication, but only minimal effects were seen when the cells were injected intracerebrally (15). In other studies (16), it was found that GM-CSF-transduced nonreplicating (irradiated) melanoma cells administered as a subcutaneous (sc) vaccine, but not as an intracranial injection, afforded some protection from intracranial challenge with wild-type melanoma. In contrast, direct intracranial injection of nonreplicating (irradiated) melanoma cells secreting IL-2 was protective, but injection into one flank with IL-2-producing cells was not. Furthermore, combination therapy with both sc injections of nonreplicating melanoma cells genetically engineered to secrete GM-CSF, and local administration of IL-2 into the brains of mice with intracranial melanomas achieved a synergistic antitumor response (16). These findings provide a basis for the application of cytokine delivery for the treatment of BTs, both as a systemic vaccine and/or via local administration.

In a study by Sampson et al. (17), irradiated B16 murine melanoma cells, producing murine IL-2, IL-3, IL-4, IL-6, IFN- $\gamma$ , or GM-CSF, were used as sc vaccines against tumors within the brain. Under conditions in which untransfected B16 cells had no effect, cells producing IL-3, IL-6, or GM-CSF increased the survival of mice challenged with viable B16 cells in the brain. Vaccination with B16 cells producing IL-4 or INF- $\gamma$  had no effect, and vaccination with cells producing IL-2 decreased survival time. GM-CSF-producing vaccines were also able to increase survival in mice with pre-established tumors. The response elicited by GM-CSF-producing vaccines was found to be specific to tumor type and to be abrogated by depletion of CD8<sup>+</sup> cells. Unlike the immunity generated against sc tumors by GM-CSF, however, the effector responses generated against tumors in the CNS were not dependent on CD4<sup>+</sup> cells. These data further sup-



**Fig. 2.** Graph showing the survival rate of mice injected ic with a mixture of glioma cells and fibroblasts (LM cells) engineered to secrete cytokines. The C57Bl/6 mice (8/group) were injected ic with a mixture of  $10^6$  cells of one of the cell types and  $10^5$  GL261 glioma cells. The median lengths of survival were as follows (in days): mice with nonimmunized glioma cells,  $16.9 \pm 1.9$ ; glioma plus LM cells,  $20.0 \pm 4.5$ ; glioma plus LM-IL-2 cells,  $23.4 \pm 6.8$ ; glioma plus LM-IFN- $\gamma$  cells,  $18.0 \pm 1.8$ ; glioma plus IM-IL-2/IFN- $\gamma$  cells,  $28.1 \pm 5.8$ . Probability values were: nonimmunized vs LM-IL-2,  $p < 0.025$ ; nonimmunized or LM vs LM-IL-2/IFN- $\gamma$ ,  $p < 0.005$ ; LM-IL-2 vs LM-IL-2/IFN- $\gamma$ ,  $p < 0.05$ .

port the proposal that cytokine-producing tumor cells are very potent stimulators of immunity against tumors within the CNS, but effector responses in the CNS may be different from those obtained against sc tumors (17).

Besides cytokine-producing tumor cells, another method utilizing genetic engineering techniques by which cytokines can be delivered for intracranial tumors involves the use of replication-competent herpes simplex viruses (18). In recent studies, it was reported that treatment of intracranial gliomas in mice using a herpes simplex virus, modified for the expression of *IL-4*, resulted in a significant prolongation of survival. Furthermore, an inflammatory reaction was seen in the brains of the treated animals, which was composed primarily of CD8<sup>+</sup> and CD4<sup>+</sup> T-cells, suggesting a specific cytotoxic antitumor response.

The use of genetically engineered allogeneic cells as a delivery vehicle for cytokines may also be an attractive alternative method of cytokine therapy. The authors' laboratory introduced the gene for *IL-2* into a mouse fibroblast cell line (LM) expressing defined MHC determinants (H-2<sup>k</sup>). The immunotherapeutic properties of these allogeneic IL-2-secreting cells were tested in C57Bl/6 mice

**Table 1**  
**Antiglioma Cytotoxic Activities of Spleen Cells**  
**from C57Bl/6 Mice Injected Intracerebrally**  
**with a Mixture of Glioma and Cytokine(s)-secreting Cells**

<i>Cell types</i>	<i>% Cytolysis at E:T ratio of 100:1</i>
Glioma	2.2 ± 1.3
Glioma + LM	10.3 ± 3.9
Glioma + LM-IL-2	19.4 ± 1.6 <sup>a,b</sup>
Glioma + LM-IL-2/IFN- $\gamma$	40.5 ± 12.4 <sup>b,c,d</sup>

C57Bl/6 mice received a single intracerebral injection of glioma cells ( $10^5$ ) together with one of the modified fibroblast cell types ( $10^6$  cells). Three wk after the injection, mononuclear cells from the spleens of the immunized mice, obtained through Ficoll-Hypaque centrifugation, were used for  $^{51}\text{Cr}$ -release assays. All assays represent the mean  $\pm$  standard deviation of triplicate determinations.

<sup>a</sup> $p < 0.005$  relative to  $^{51}\text{Cr}$  release for spleen cells from animals immunized with glioma.

<sup>b</sup> $p < 0.05$  relative to  $^{51}\text{Cr}$  release for spleen cells from animals immunized with glioma + LM cells.

<sup>c</sup> $p < 0.025$  relative to  $^{51}\text{Cr}$  release for spleen cells from animals immunized with glioma.

<sup>d</sup> $p < 0.05$  relative to  $^{51}\text{Cr}$  release for spleen cells from animals immunized with glioma + LM-IL-2 cells.

with GL261 gliomas in both sc and ic models (19–21). The survival of mice with an ic malignant glioma, treated by injection into the tumor bed of allogeneic fibroblasts genetically modified to secrete IL-2, was significantly prolonged, compared to untreated control mice injected with glioma cells alone (Fig. 2; 20,21). Furthermore, using a chromium-51 ( $^{51}\text{Cr}$ ) release cytotoxicity assay, a significant and specific anti-glioma immunocytotoxic response was detected in the animals treated with the allogeneic IL-2-secreting fibroblasts (Table 1). The intracranial implantation of the modified fibroblasts was without detectable toxicity, and the animals exhibited no neurologic deficit. In prior experiments, immunity to melanoma and prolongation of survival were seen in mice with peripheral melanomas treated with allogeneic fibroblasts genetically engineered to secrete cytokines (22,23). Based on these experiments, the authors extended studies to the treatment of ic metastatic malignant melanoma, and found that mice with an ic malignant melanoma treated by injection into the tumor bed of allogeneic fibroblasts genetically modified to secrete IL-2 survived significantly longer than untreated control mice injected with melanoma cells alone (21). A specific and significant antimelanoma immunocytotoxic response was found in the treated mice. Like other allografts, the fibroblast cells were even-



tually rejected, preventing the possible neoplastic growth of the vaccine itself in the tumor-bearing mice.

The use of allogeneic, rather than syngeneic, cells was based on evidence that allogeneic MHC determinants augmented the immunogenic properties of the tumor vaccine (24–28). Hammerling et al. (24) transfected genes for allogeneic MHC Ags into mouse fibrosarcoma cells. The cells' immunogenic properties were increased as a result. Hui et al. (25) found that immunization with a k haplotype tumor, modified by the introduction of the gene for *H-2K<sup>b</sup>*, led to an antitumor cellular IR in k haplotype mice. Ostrand-Rosenberg et al. (26), Fearon et al. (27), and Gattoni-Celli et al. (28) have reported analogous results.

Because the fibroblasts in the authors' previous studies were allogeneic in C57Bl/6 mice, presentation of TAAs may have followed Ag uptake by APCs of the host, with the tumor Ags processed and presented to CTLs of the host by APCs. Conceivably, the immunogenic properties of the allogeneic cells would be enhanced if the fibroblasts were modified to form syngeneic *H-2K<sup>b</sup>* class I determinants. The self-MHC-I determinants may provide a mechanism for the direct presentation of Ag peptides to CTLs of the tumor-bearing host, further enhancing the cells' immunogenic properties. To investigate this question, a plasmid (pBR327H-2K<sup>b</sup>) was used to introduce expression-competent genes for *H-2K<sup>b</sup>* determinants into the fibroblasts. In initial studies of mice with either sc gliomas or melanomas, the authors found that intratumoral administration of a cellular vaccine consisting of fibroblasts which expressed both syngeneic and allogeneic MHC determinants prolonged survival and induced an antitumor IR (29,30). Using this allogeneic–syngeneic vaccine in the treatment of ic gliomas in mice, the authors found a further prolongation of survival, complete regression of tumor in some cases, and an increased immunocytotoxic response in the immunized mice (29).

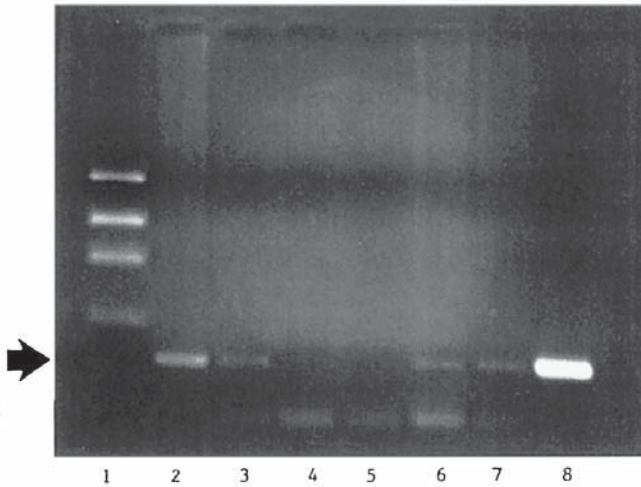
Two possible mechanisms may explain the IR of mice treated with the allogeneic–syngeneic cells. Large numbers of CTLs with specificity toward TAAs may have been generated in the micro-environment of allograft recognition and rejection. The immunogenic properties of tumor cells transfected with genes specifying allogeneic determinants are supportive of this interpretation. In addition, MHC-I genes that share identity with the tumor-bearing host may present tumor-associated T-cell epitopes directly to CTLs. Fibroblasts can act as efficient APCs (31,32): They form B.7.1, a co-stimulatory molecule required for Ag-specific T-cell activation, as well as MHC-I determinants. The authors' Ab depletion <sup>51</sup>Cr-release studies demonstrated that a specific CD8<sup>+</sup> immunocytotoxic response against gliomas was stimulated by immunization with the allogeneic–syngeneic IL-2-secreting cells. Thus, these results suggest that the IR could be augmented by genetic modification of one cell to form syngeneic MHC and to secrete immune-augmenting cytokines.

Genetic modification of a cell line, rather than cells from a primary neoplasm, has other important advantages for use in BT treatment. Modification of neoplastic cells taken directly from tumor-bearing patients may be difficult. A primary tumor cell line, required for genetic modification, must be established. The establishment of a cell line from a primary neoplasm is not always possible. Furthermore, it is conceivable that a subpopulation of the primary tumor, selected for its capacity to grow in vitro, may not reflect the tumor cell population as a whole, especially because tumors such as gliomas are known to be heterogeneous. In addition, the use of tumor cells as the vehicle of cytokine delivery is of concern, since the cells themselves may grow into a tumor. Thus, the advantages of using an allogeneic fibroblast cell line are that it is reliable, readily available, stably transfected, and not tumorigenic. Like other allografts, the cells are rejected. Furthermore, the number of cells can be expanded as desired, for multiple rounds of therapy. In addition, the slow, continuous release of cytokines, and the eventual rejection of the allograft, may be useful advantages in the treatment of BTs, in which long-term secretion of high concentrations of certain cytokines may be associated with increased morbidity and mortality.

### ***3.3. Safety and Toxicity***

The toxic effects of cytokines in the CNS may limit the quantity that can be administered (4,33,34). Neurologic side effects have been seen in animals injected intracranially with syngeneic cytokine-secreting cells. The co-implantation into the rat brain of syngeneic (RG-2) glioma cells and fibroblast cells, modified by retroviral transduction to secrete IL-2 or IFN- $\gamma$  resulted in short-term, cell-mediated antiglioma responses. However, the survival of the tumor-bearing rats was not prolonged (35), and the animals died from secondary CNS side effects, including severe cerebral edema. Most of the systemic toxicities of IL-2 therapy can be avoided by the introduction of the gene for *IL-2* directly into the tumor mass, resulting in high local concentrations of the cytokine. If not for the CNS toxicities, this form of treatment is particularly advantageous in primary gliomas, because these tumors usually recur locally and are rarely metastatic.

The toxicity of a cellular-based cytokine gene therapy for BTs is likely to depend in part on the survival of the genetically modified cells in the CNS. The authors investigated the survival of an allogeneic IL-2-secreting vaccine in the CNS by two different means. First, polymerase chain reaction (PCR) analysis was used to detect the presence of the neomycin resistance gene, a component of the modified retrovirus used to engineer the cells for IL-2 secretion, in brain sections, at various time intervals after implantation (Fig. 3). As a second means of assessing the survival of the modified cells in the CNS, the authors attempted to recover the modified fibroblasts from cultures derived from brain sections

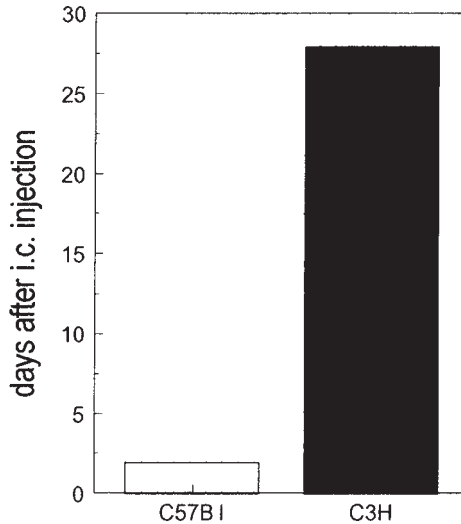


**Fig. 3.** PCR analysis for the survival of modified fibroblasts in the CNS. PCR analysis was performed for the presence of neomycin gene in brain sections taken at various time intervals (0–60 d) after implantation of modified fibroblasts into CNS in allogeneic and syngeneic mice. DNA sequences for the neomycin gene were found on d 8 and 14, but not 28 or 60 postimplantation, in allogeneic mice, and up to 55 d in syngeneic mice. Lanes displayed are as follows: lane 1, low mass molecular marker (Gibco); lane 2, 8 d following injection into allogeneic mice; lane 3, 14 d following injection into allogeneic mice; lane 4, 28 d following injection into allogeneic mice; lane 5, 60 d following injection into allogeneic mice; lane 6, 55 d following injection into syngeneic mice; lane 7,  $10^3$  LM-IL-2 cells; lane 8, pZipNeo plasmid.

taken at various time intervals after implantation (Fig. 4), and found, by both assays, that the survival of allogeneic cells in the CNS was less than 28 d. Presumably, the cells, like other allografts, were rejected. The cells were well tolerated, and the animals did not demonstrate any significant neurologic toxicity (36), which suggests that cytokine-secreting allogeneic cells may serve as a useful vehicle for the safe delivery of cytokines into brain neoplasms.

### 3.4. Blood–Brain Barrier and Delivery to BTs

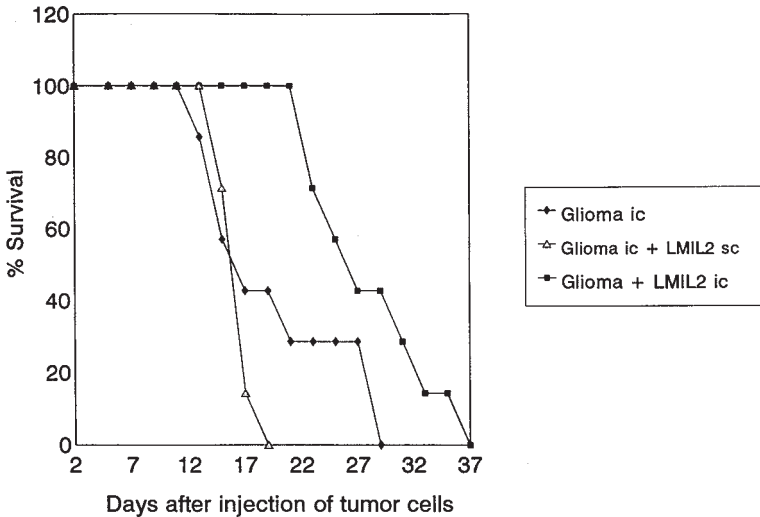
One of the major concerns related to the immunologic treatment of BTs is the effect of the blood–brain barrier (BBB) on the development of a host IR in the CNS. Studies using IL-4-secreting plasmacytoma cells implanted into the brains of nude mice, along with human glioma cells, demonstrated a dramatic eosinophilic infiltrate in regions of necrotic tumor, suggesting that an IR can take place against a tumor of the CNS *in situ*. The response, however, was not T-cell-dependent (13). The authors found that a specific and significant systemic immunocytotoxic response (as measured by  $^{51}\text{Cr}$  release assays) was present



**Fig. 4.** In vitro recovery analysis for the survival of modified fibroblasts in the CNS. The recovery of modified fibroblasts in cultures derived from brain sections of the implanted mice, taken at various time intervals following implantation (0–60 d) in allogeneic (C57B1/6) and syngeneic (C3H) mice and grown in the presence of neomycin. Allogeneic cells could only be recovered for up to 3 d after ic implantation; syngeneic cells were recovered for up to 28 d postimplantation.

in animals with intracranial gliomas treated with allogeneic IL-2-secreting fibroblasts administered intracerebrally (20,21). Thus, the secretion of IL-2 by the cellular immunogen (37,38), or an immunogenic derivative of the cells, may have altered the BBB, enabling tumor immunogens to reach the spleen and lymph nodes in the periphery. Furthermore, there is a recent report (39) that sc injection of irradiated GM-CSF-transduced glioma cells can induce a potent IR against intracranial gliomas, both as a vaccination against subsequent intracranial glioma cell implantation, and as a treatment of established intracranial gliomas.

Another problem related to immuno-gene therapy for CNS tumors has been the determination of the most optimal route of delivery. In recent studies, the authors compared the effect of direct ic vs systemic administration of a tumor vaccine on the survival and IRs of mice with ic gliomas or melanomas (19). Using allogeneic fibroblasts genetically engineered to secrete cytokines, the authors found that only direct ic injection into the tumor bed resulted in significantly prolonging survival (Fig. 5), despite a significantly elevated systemic immunocytotoxic response against the tumor Ags by  $^{51}\text{Cr}$ -release assay in both the sc and ic immunized animals (Table 2; 19). Subcutaneous administration of IL-2-secreting cells, in addition to tumor cells, was not effective in the



**Fig. 5.** The survival of mice with ic glioma injected with either ic LM-IL-2 cells or sc LM-IL-2 cells. Median survival time (d): nonimmunized,  $19.0 \pm 6.1$ ; LM-IL-2 injected sc,  $16.1 \pm 1.0$ ; LM-IL-2 injected ic,  $27.9 \pm 4.7$ . *p* values: nonimmunized or injected with LM-IL-2 cells sc, vs animals injected with LM-IL-2 ic, *p* < 0.005.

treatment of mice with ic glioma or melanoma (19), which was a surprising result, because the authors observed significantly elevated systemic immunocytotoxic responses by  $^{51}\text{Cr}$ -release assay in the subcutaneously immunized animals (Table 2). Thus, immunization by sc injection was not as effective as injection of the vaccine directly into the tumor bed itself.

An additional challenge for the delivery of immuno-gene therapy is that of tissue specificity and selectivity. Retroviruses are selective because they integrate only into dividing cells, but adenoviruses may be advantageous, because they can infect nondividing cells. The use of cell-type-specific enhancers may add to the efficacy of delivery of these viruses, as well as to their specificity. Because glial fibrillary acidic protein (GFAP) is specific for cells of glial origin, and the majority of primary BTs are of glial origin, this is one candidate gene for enhancing tissue specificity. Using genetic engineering techniques, a replication-incompetent adenovirus was constructed that contained a fragment-enhancer region of the *GFAP* gene coupled to a tetracycline transactivator (40). This produced a delivery system that was glial-specific and repressible. This novel type of virus delivery system should be useful for a variety of gene therapy approaches for CNS tumors, including the delivery of cytokines. Further studies are enthusiastically awaited.

**Table 2**  
**Antiglioma Cytotoxic Activities of Spleen Cells**  
**from C57Bl/6 Mice Bearing Intracerebral Gliomas Treated**  
**Either Subcutaneously or Intracerebrally with LM-IL-2 Cells**

<i>Cell types</i>	<i>% Cytolysis at E:T Ratio of 100:1</i>
Glioma	−0.3 ± 0.6
Glioma + LM-IL-2 (sc)	24.9 ± 3.2 <sup>a</sup>
Glioma + LM-IL-2 (ic)	17.8 ± 2.6 <sup>a</sup>

C57BL/6 mice received  $10^5$  glioma cells ic and either  $10^6$  LM-IL-2 cells ic or sc. The animals receiving LM-IL-2 cells sc received additional injections after 1 and 2 wk. Two wk after the initial injection of glioma cells, mononuclear cells from the spleens of the immunized mice, obtained through Ficoll-Hypaque centrifugation, were used for the  $^{51}\text{Cr}$ -release assays.

<sup>a</sup> $p < 0.005$  relative to  $^{51}\text{Cr}$  release for spleen cells from animals injected with glioma cells alone.

### *3.5. Combination Therapy*

In addition to investigating the optimal route of delivery, the most efficacious combination of cytokines is currently being examined. Several laboratories have recently reported their results using GM-CSF alone (39) or in combination with IL-2 (16). Subcutaneous injection of irradiated GM-CSF-transduced glioma cells induced a potent IR to intracranial gliomas, both as a vaccination against subsequent intracranial glioma cell implantation and for treatment of established intracranial gliomas (39). In addition, the combination of IL-2 intracranially and GM-CSF subcutaneously was synergistic (16). Thus, these studies and others suggest that tumor vaccines consisting of tumor Ags in the presence of multiple cytokines may be effective in the treatment of CNS tumors, both as a systemic vaccine and via local administration. Certainly, the most effective means of administration of cytokines in the treatment of glioma has not been determined.

Based on the studies reviewed in this chapter, it is likely that immunotherapy using cytokine-secreting tumor vaccines in combination with traditional surgery, radiation therapy and/or chemotherapy may provide improved treatment and prolong life for patients with malignant ic tumors. Cytokine-based tumor vaccines, as a means of targeted immuno-gene therapy is a promising new treatment for malignant BTs.

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# 14 Downregulation of Transforming Growth Factor $\beta$ as Therapeutic Approach for Brain Tumors

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*Habib Fakhrai, PhD,  
Svetlana Gramatikova, PhD,  
and Rohangiz Safaei, PhD*

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## 1. INTRODUCTION

Brain tumors (BTs) are responsible for significant morbidity and mortality in both pediatric and adult populations. Approximately 20,000 new cases of glial tumors are diagnosed in the United States each year, and recent evidence suggests a rising frequency, although it is not clear whether the increasing incidence results from improved diagnosis or from a true increase in cases (1–3). Although gliomas rarely metastasize outside of the central nervous system (CNS), they are frequently diffuse within the brain (4,5). Surgery and high-dose radiation still provide the best standard therapy (2), but this approach is noncurative, and prognosis for the patients with this disease remains poor. Exploration of new therapeutic approaches for these tumors is therefore essential.

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Gliomas suppress host immunity by secreting active forms of immunosuppressive molecules, such as transforming growth factor  $\beta$  (TGF- $\beta$ ), which is known to inhibit T-cell activation, and it has been shown that blocking its production can restore some normal immune activation process (6,7). This review further explores the possibility of utilizing TGF- $\beta$  antisense gene therapy in the treatment of cancer. Mechanisms such as TGF- $\beta$  signaling, the stage-dependent effect of TGF- $\beta$  on growth inhibition or stimulation, and the role of TGF- $\beta$  in the suppression of the host immunity are discussed.

## 2. LYMPHOCYTE THERAPY FOR GLIOMAS

Several investigators (8–11) have demonstrated the efficacy of adoptive immunotherapy in the rat 9L gliosarcoma and RT-2 astrocytoma tumor models. Such investigations have shown that *in vitro* activated cytotoxic T-lymphocytes (CTLs) can eradicate intracranially implanted 9L and RT-2 tumors (8). In these studies, CTLs generated against either 9L or RT-2 tumors were effective against both glial tumors (10). However, this approach was efficacious only if splenocytes of immunized animals were used as the lymphocyte source of the *in vitro* activated CTLs (8,9,11). The safety and efficacy of this approach are currently under clinical investigation (*see* Chapters 6–8 in this volume).

## 3. GENE THERAPY FOR CANCER

IL-2 is an important cytokine in the generation of antitumor immunity (12). In response to engagement of T cells with tumor antigens (Ags), a subset of immune effector cells, designated as helper T cells (CD4<sup>+</sup>), secrete small quantities of IL-2. The secreted IL-2 acts locally at the site of T cell-Ag engagement, and activates killer (CD8<sup>+</sup>) T-cells. These activated killer T-cells, together with lymphokine-activated killer (LAK) and natural killer (NK) cells, then mediate the systemic destruction of tumors.

To manage or cure cancers, many investigators have used this rationale to administer different cytokines systemically, in both animal models and clinical trials. It was observed (12,13) that intravenous, intralymphatic or intralesional administration of the cytokine, IL-2, resulted in clinically significant responses in some cancer patients. However, severe toxicity (hypotension and edema) limited the dose and efficacy of intravenous and intralymphatic IL-2 administration (14,15).

### 3.1. Cytokine Gene Therapy in Animal Tumor Models

To circumvent the toxicity and other associated difficulties of systemic IL-2 administration, several investigators have used vaccines composed of cytokine gene-modified tumor cells to generate systemic antitumor immunity in both animal studies and human clinical trials.

Recent studies in animal models have demonstrated that cytokine gene therapy may be an important way to deliver immunostimulants to hosts and mobilize their immune defenses against tumors. This approach employs molecular biology techniques to introduce and express cytokine genes in carrier cells (tumors or fibroblasts), which are then admixed with irradiated tumor cells and used as vaccines for inducing systemic antitumor immunity (16,17).

Several animal tumor models have been developed to study the effects of different cytokines on the management of cancer. In these studies, transfer of genes for *IL-2* (16–18), interferon  $\gamma$  (*IFN- $\gamma$* ) (19), *IL-4* (20,21), *IL-7* (22–24), and granulocyte-macrophage colony-stimulating factor (*GM-CSF*) (25) have all significantly reduced or eliminated tumorigenicity in a variety of murine tumor models. In some studies, treated animals developed persistent and elevated levels of tumor-specific CTL activity, in part mediated by CD8<sup>+</sup> CTL (16,17).

However, this approach has not been uniformly successful. As different gene therapy experiments have shown, a single cytokine that is efficacious in one tumor model may be completely ineffective in another. Immunosuppressive molecules that affect either the production levels of cytokines or their receptors may limit the efficacy of cytokine therapy (7).

### 3.2. Gene Therapy for Gliomas

Utilization of gene therapy for the treatment of BTs is still at an early stage of development. In one approach, several investigators (26,27) have used herpes simplex virus thymidine kinase (*HSV-TK*) gene therapy, a form of suicide gene therapy, to treat tumors of different histologic origins. In these studies, the *HSV-TK* gene was transferred to the tumor cells *in situ*, resulting in partial gene modification of the tumor, which was then followed by systemic administration of ganciclovir (GCV). Expression of the *HSV-TK* gene in tumor cells enabled them to efficiently convert the nontoxic GCV into cytotoxic GCV-phosphate. This resulted in the death of gene-modified tumor cells and their nonmodified neighbors. The phenomenon that causes cell death in nonmodified neighboring cells is known as the “bystander effect” (26,27). However, despite this bystander effect, some resistant tumor cells survived and grew into large, fatal neoplasms (28). The procedure of *in situ* gene modification with the *HSV-TK* gene (26) has been used in different clinical trial phases for malignant gliomas. However, it is still too early to evaluate the outcome of its application.

Some glial tumors express insulin-like growth factor (*IGF*) (29,30), which may keep them at an immature stage of development, and thus enable them to escape immune surveillance. In one study, rat C6 glioma cells, which produce IGF, were modified with an antisense gene to block their production of IGF (30). Immunization of Wistar rats with the antisense-modified C6 tumor cells resulted in induction of antitumor responses that rejected subsequent challenges

with lethal doses of unmodified parental tumor cells (29,30). However, interpretation of studies with the C6 glioma model is complicated by the fact that this glioma cell line was induced in random-bred Wistar rats, so there is no truly syngeneic rat strain within which to implant C6 tumors. Therefore, studies using the C6 tumor model of immunotherapy in immunocompetent animals should be interpreted with this in mind (31).

#### 4. PRODUCTION OF TGF- $\beta$ IN BTS

TGF- $\beta$  includes a group of multifunctional proteins that regulate the growth and function of many normal and neoplastic cell types (32–34) of both epithelial and mesenchymal origins (35).

TGF- $\beta$  is a potent inhibitor of T-cell activation (32–34). Elevated levels of TGF- $\beta$  have been detected in tumors of different histologic origins, including glioblastoma (36–40), chondrosarcoma (36), and cancers of breast (41–43), colon (44), prostate (45), bladder (46), pancreas (47), as well as small and nonsmall cell lung cancers (48). The high level of secretion of active TGF- $\beta$  then results in suppression of patients' immune responses and prevents detection of tumors by their immune system.

Five isoforms of TGF- $\beta$  (TGF- $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ ,  $\beta_4$ , and  $\beta_5$ ) have been identified in this family of growth factors, of which TGF- $\beta_1$  and TGF- $\beta_2$  are the best-characterized molecules (49). Although these isoforms differ in their sequence and in their receptor-binding patterns (50), they exhibit similar in vivo properties.

Most cell types, including T- and B-lymphocytes, produce inactive forms of TGF- $\beta$ , which cannot bind to high-affinity TGF- $\beta$  receptors (32,35,51–53). To become active, TGF- $\beta$  must first be cleaved at a multi-basic peptide sequence to yield a 112-amino acid molecule, which is then dimerized to form the active homodimer molecule (54,55).

#### 5. EFFECTS OF TGF- $\beta$ ON IMMUNE SUPPRESSION

TGF- $\beta$  suppresses immunity by inhibiting T-cell activation in response to Ag stimulation. Additionally, TGF- $\beta$  has antagonistic effects on NK cells, as well as on the induction and proliferation of LAK cells (56–58).

Incubation of peripheral blood lymphocytes (PBLs) with the supernatant of glial cell cultures makes PBLs nonresponsive to activation. Addition of IL-2 to mitogen-activated human T-cells in the presence of TGF- $\beta_2$  does not restore the normal proliferative response of the T-cells (59). However, neutralization of TGF- $\beta$  by antisera during the activation process restores normal T-cell activation (59,60). Furthermore, TGF- $\beta$  becomes ineffective after T-cell activation. These observations indicate that secretion of TGF- $\beta$  by tumors may suppress immune effector cells during the activation phase following detection of tumor Ag. This thereby inhibits antitumor responses in tumor patients.

### ***5.1. Mechanisms of Immune Suppression in Different Tumors***

Advanced gliomas are associated with systemic suppression of host immune competence (8,9,61), which is characterized by T-cell lymphopenia and impaired T-cell responsiveness to a variety of specific and nonspecific stimuli (61). Immunologically, the brain is partially privileged. This makes the brain an excellent site to investigate whether peripherally activated immune effector cells can migrate through the blood–tumor barrier and concentrate in an intracranial tumor.

Tumors in general escape immune surveillance by using different mechanisms. Some tumors do so by expressing lower levels of major histocompatibility complex (MHC) class I and class II (MHC-I and -II) molecules. Other tumors escape by increasing their expression of immunosuppressor molecules, such as TGF- $\beta$ . A third group of tumors, such as glioblastomas, utilizes a combination of different mechanisms to escape immune surveillance. Gliomas express MHC-I and, to a lesser extent, MHC-II molecules. They also express tumor-associated antigens (TAA), which are not present in normal tissues (62). However, although the levels of MHC-I molecules in glioblastoma seem to be unaffected, the majority of these tumors express significantly lower levels of MHC-II molecules. The reduced levels of MHC-II molecules may be an additional factor in allowing gliomas to escape immune surveillance.

It has recently been shown (63) that the lower expression levels of MHC-II molecules on glioblastomas may be the result of the suppression of IFN- $\gamma$  by TGF- $\beta$ . The diminished levels of MHC-II molecules on glioblastoma cells has also been shown to be associated with a reduced expression of human leukocyte antigen-DR (HLA-DR). In conjunction with ex vivo secretion of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , and IL-10, this can be indicative of monocyte deactivation in glioblastoma patients, but not in patients with low-grade astrocytomas (64).

Other investigators (65,66) have shown that even in the presence of IFN- $\gamma$ , the expression of the *HLA-DR* gene can become markedly reduced when TGF- $\beta$  is present. Young et al. (66) have shown that the levels of TGF- $\beta$  production in metastatic tumors are higher than in primary tumors. Furthermore, the high levels of TGF- $\beta$  resulted in decreased production of IFN- $\gamma$  and consequently decreased expression of MHC-II molecules.

### ***5.2. TGF- $\beta_2$ Suppresses Expression of High-affinity IL-2 Receptors on T-cells***

Secretion of IL-2 by helper T-cells (CD4<sup>+</sup>) in the microenvironment of Ag–CTL engagement results in CTL activation (67). Advanced gliomas are frequently accompanied by progressive immunosuppression (IS), as measured by various functional assays (55,68,69). IS in glioma patients is demonstrated by the depression of mitogen responsiveness of their peripheral T-cells in vitro



(49,50,70–73), which may partially result from decreased expression of high-affinity IL-2 receptors (IL-2Rs). Gliomas and tumors of other histologic origins which produce biologically active TGF- $\beta$  show suppression of high-affinity IL-2Rs (74). Surgical removal of the tumor results in the reduction of circulating TGF- $\beta$  levels, and thus partially reverses the IS. However, immune suppression reappears in patients before clinical signs of tumor recurrence are detected by neurological exam or radiographic means (72).

## 6. EFFECTS OF TGF- $\beta$ ON DIFFERENT STAGES OF TUMOR PROGRESSION

TGF- $\beta$  has multiple effects on the immune system, most of which are inhibitory and are mediated through signal transduction pathways (39). For example, TGF- $\beta$  controls the expression of different genes that are essential for the progression of cell cycle and mitosis.

The effects of TGF- $\beta$  vary significantly during different stages of tumor progression. For example, Jennings et al. (75) showed that TGF- $\beta$  inhibits the growth of some tumors and stimulates that of others in culture, and that growth inhibition and/or stimulation of tumors by TGF- $\beta$  is tumor-stage-dependent. Production of high levels of TGF- $\beta$  in advanced tumors induces an increase in tumor vascularization and growth, as well as IS of patients. In contrast, TGF- $\beta$  induces apoptosis in near-diploid tumor cells (75,76).

In the authors' studies, TGF- $\beta$  antisense gene modification of tumor cell lines resulted in growth stimulation in some tumors and inhibition in others. These observations suggest that extreme caution must be exercised in using TGF- $\beta$  antisense gene therapy for *in situ* modification of tumors. To effectively use this approach, new methods must be developed that can distinguish between tumors that are growth-inhibited and those that are stimulated by TGF- $\beta$ .

In the spontaneously arising SMA560 astrocytoma animal tumor model, Ashley et al. (77,78) demonstrated that increased levels of TGF- $\beta_1$  production by tumors resulted in prolonged survival. This may have been caused by increased tumor cell apoptosis and reduced cerebral edema, which are side effects of the enhanced TNF- $\alpha$  release induced by local production of TGF- $\beta_1$ .

In breast cancer patients, Auvinen et al. (41) demonstrated a more favorable response to therapy when the tumors produced high levels of TGF- $\beta_1$  and TGF- $\beta_2$ . Patients whose tumors produced only TGF- $\beta_2$ , without producing TGF- $\beta_1$ , had a significantly better prognosis. In another study (42), measurement of TGF- $\beta_2$  plasma levels, before and after 4 wk of tamoxifen therapy, revealed that patients with higher concentrations of TGF- $\beta_2$  had more favorable prognoses than those whose tumor did not produce high levels of TGF- $\beta$ .

In contrast, Merzak et al. (79) demonstrated that, in glioblastoma tumors, there is a positive correlation between TGF- $\beta$  levels and the degree of tumor

invasiveness. They also found that high levels of TGF- $\beta$  were inversely correlated with survival, suggesting an important role for TGF- $\beta$  in the malignant progression of gliomas. The relationship between TGF- $\beta$  levels and survival has also been shown in tumors of other histopathologic origins. For example, Friedman et al. (44) showed that levels of TGF- $\beta_1$  production in colorectal carcinoma correlated well with disease progression and metastasis, and this phenomenon was independent of nodal status and the degree of differentiation of the primary tumor. Their results also demonstrated that the recurrence frequency was 18-fold higher in patients whose tumors produced high levels of TGF- $\beta_1$  compared to those with low levels of this growth factor.

### **6.1. Effect of TGF- $\beta$ on Expression of Different Genes**

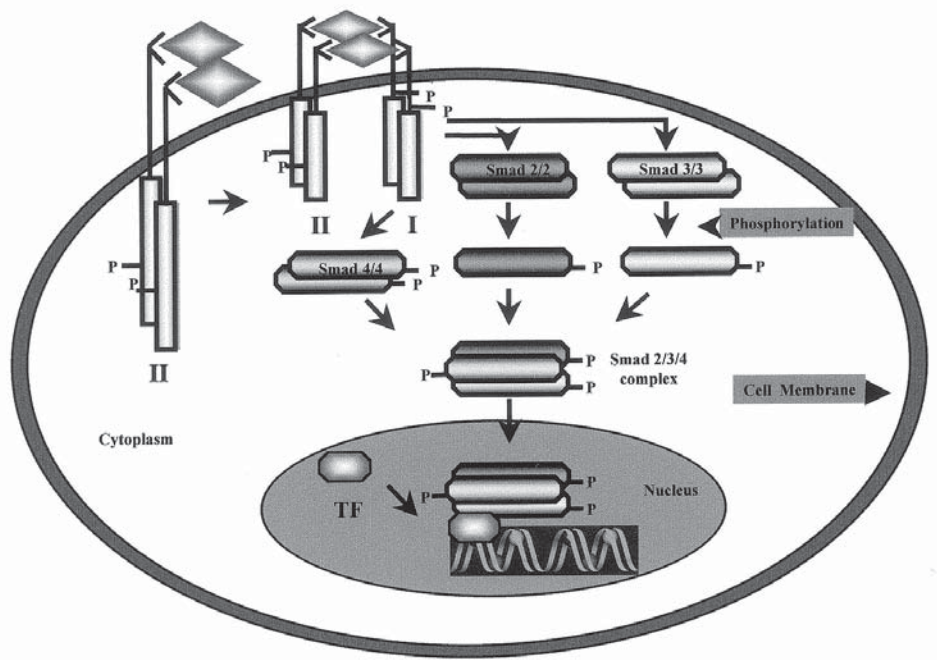
The growth of neoplastic tissue, including BTs, depends on the continuous formation of new blood capillaries. A direct relationship has been observed between blood vessel formation and tumor progression (80). In a different study, Hsu et al. (81) showed that, in contrast to the less malignant astrocytomas, glioblastoma multiforme (GBM) is associated with intense angiogenesis, and that the angiogenesis may be correlated with high levels of TGF- $\beta$  production by the tumor.

Glioblastomas also show frequent loss and gain of chromosomes, resulting in the loss of tumor suppressor genes or the activation of proto-oncogenes. For example, it has been shown that, in contrast to the less malignant astrocytomas, GBM is associated with frequent loss of tumor suppressor genes that are located on chr 10 (81,82). Other investigators have also demonstrated a relationship between the chromosome number in glioma cells and the degree of growth responsiveness to TGF- $\beta$ . For example, Jennings and Pietenpol have shown that near-diploid gliomas of any grade are growth-inhibited by TGF- $\beta$ . This growth inhibition then switches to growth stimulation, upon transition of tumor cells from being near-diploid to becoming hyper-diploid ( $n = 57$ ) (83,84). The stage-dependent effects of TGF- $\beta$  on tumors may be the consequence of mutational accumulation or deletion of tumor suppressor genes. In this case, the loss of autocrine growth inhibition by TGF- $\beta$  can be attributed to the possible loss or mutations of TGF- $\beta$  receptors (85). TGF- $\beta_2$  secreted by glioma cells is also known to decrease the expression of TNF- $\alpha$ -induced cell adhesion, resulting in diminished leukocyte-endothelium interaction (86).

### **6.2. TGF- $\beta$ Signal Transduction**

TGF- $\beta$  exerts a wide range of effects on a large variety of cell types (87). These effects are mediated by TGF- $\beta$  signal transduction, which turns expression of different genes on or off. It is estimated that TGF- $\beta$  signal transduction affects the expression of more than 20 genes (88).

TGF- $\beta$  signal transduction is mediated by the *SMAD* gene family (89). It is initiated by binding of the TGF- $\beta$  molecule to the intrinsically phosphorylated



**Fig. 1.** Schematic view of the TGF- $\beta$  signal transduction pathway. TGF- $\beta$  makes a complex with TGF- $\beta$ RII. The complex then recruits the nonphosphorylated TGF- $\beta$ RI, resulting in its phosphorylation. The phosphorylated TGF- $\beta$ RI then mediates the phosphorylation of dimeric SMADs 2–4, which causes them to become monomeric, followed by heterotrimeric complex formation. The heterotrimeric complex then migrates to the nucleus and interacts with different transcription factors to regulate gene transcription.

TGF- $\beta$  receptor II (TGF- $\beta$ RII), which then recruits the nonphosphorylated TGF- $\beta$  receptor I (TGF- $\beta$ RI). As a result of this complex formation, the TGF- $\beta$ RI becomes phosphorylated by the TGF- $\beta$ RII, which then phosphorylates homodimeric members of the *SMAD* family (*SMAD2*, *SMAD3*, and *SMAD4*). The phosphorylated *SMAD*s become monomeric, then form a heterotrimeric complex within the cytoplasm (Fig. 1). The complex is then translocated into the nucleus (87). Upon accumulation in the nucleus, it combines with different transcription factors, to regulate the expression of different genes (87). Complex formation with transcription factors depends on the site at which the different *SMAD* molecules are phosphorylated (88). In this way, a small set of signal transduction molecules can influence the expression of a great number of genes.

### 6.3. Effect of TGF- $\beta$ on Different Cytokine Receptors

IL-2 secretion, as well as the expression of IL-2-induced high-affinity IL-2R, are important elements for initiation and maintenance of T-cell proliferation and

function (89–91). Immune suppression mediated by TGF- $\beta_2$  appears to partially result from impairment of IL-2R function and suppression of IL-2-induced high-affinity IL-2R expression (92). Such impairment of high-affinity IL-2R has been shown to inhibit T-cell activation by IL-2, and therefore reduce the efficacy of IL-2 gene therapy (93).

Signaling through IL-2R results in the phosphorylation of JAK1, JAK3, STAT3, and STAT5 molecules. TGF- $\beta$  affects IL-2 signal transduction by preventing JAK3 and STAT3 phosphorylation, resulting in inhibition of cell cycle progression from G1 to S phase (94).

Evidence suggests that the immune suppression in patients may be the result of the effects of TGF- $\beta$  on the high-affinity  $\gamma$  chain of the cytokine receptor that is shared by IL-2, IL-4, IL-7, IL-9, and IL-15 (95–97). These cytokines, which possess overlapping functions, are important for the growth and differentiation of T- and B-lymphocytes, NK cells, macrophages, and monocytes.

## 7. TGF- $\beta$ ANTISENSE GENE THERAPY FOR CANCER

Different *TGF- $\beta$*  gene therapy approaches are being studied for the treatment of various diseases. These approaches take advantage of the IS properties of TGF- $\beta$ , and are designed to either enhance or eliminate local IS. For example, in organ transplantation, a localized IS is more desirable than a systemic one. Experimental evidence suggests that expression of TGF- $\beta$  at the transplantation site prevents or delays organ rejection.

*TGF- $\beta$*  gene therapy is also used in diseases, such as cancer, where the overcoming of IS is essential. In these studies, two different approaches for antisense gene modification have been used. One is to block TGF- $\beta$  secretion by *in situ* gene modification of the tumor with antisense gene transfer technology. However, because several investigators have shown that blocking TGF- $\beta$  *in situ* may result in enhanced tumor growth (especially in near-diploid and early-stage tumors), this approach is less desirable. The second method is to gene-modify tumor cells *ex vivo*. The gene-modified cells are subsequently irradiated and used as a subcutaneous vaccine. The advantage of this approach is that it acts locally by blocking IS at the site of injection, and it does not interfere with the growth-inhibitory effects of TGF- $\beta$  in some tumors. Based on the above reasoning, the authors utilized the *ex vivo* method of TGF- $\beta$  antisense gene therapy in preclinical and clinical studies (6,7).

The authors hypothesized that genetic modification of glioma cells to block their secretion of TGF- $\beta$  may render them more immunogenic and suitable for gene therapy. The authors then demonstrated the efficacy of this approach by genetically modifying rat 9L gliosarcoma cells with an expression vector containing the *TGF- $\beta$*  antisense gene (6). Like human gliomas, rat 9L gliosarcoma cells secrete active TGF- $\beta$  in their culture medium. Intracranial implantation of

as few as 3000 of these tumor cells in Fischer 344 rats resulted in over 99% fatality after 5–6 wk. In one study, the authors intracranially implanted  $5 \times 10^3$  unmodified, parental 9L cells into rats. The animals were then immunized subcutaneously 4 $\times$  (on a twice/wk schedule for 2 wk), with either  $TGF-\beta_2$  antisense-modified 9L cells or  $TGF-\beta_2$  antisense-modified 9L cells that were also genetically modified to secrete IL-2. The control groups of animals were immunized with parental 9L or with 9L cells that were modified with an empty vector. After 12 wk, the numbers of tumor-free animals were significantly greater in the two experimental groups that had received either the  $TGF-\beta_2$  antisense-modified tumor cells or the combination  $TGF-\beta_2$  antisense and IL-2 gene-modified tumor cells. All the animals were tumor-free in these two groups for the duration of the study (24/24, or 100% tumor-free survival).

In contrast, most animals in the control groups developed tumors, and had to be euthanized within 5 wk of starting the study. Survival in the control groups was 4/25 (16% tumor-free survival,  $p < 0.01$ ). There were no differences between the survival of animal groups immunized with either unmodified irradiated tumor cells (2/10 tumor-free) or vector-modified irradiated tumor cells (2/15 tumor-free).

The efficacy of  $TGF-\beta$  antisense gene therapy has also been demonstrated in two other tumor models, the C6 glioma (7) and a murine ovarian teratoma (MOT) (98). It is of interest to note, although  $TGF-\beta$  antisense gene modification alone was efficacious in the glioma tumor models, the combination of both  $TGF-\beta$  antisense and IL-2 gene expression was required for efficacy in the MOT model. Other investigators (99) have demonstrated the efficacy of this approach in a rat prostate tumor model.

### ***7.1. TGF- $\beta$ Antisense Gene Therapy in Clinical Trial***

The authors used the above evidence and rationale to obtain FDA approval for a phase I clinical trial. In this trial, samples of primary glioma tissues were obtained at the time of clinically indicated surgery and used to establish primary tumor cultures. Upon establishment, the tumor cells were genetically modified to express a human  $TGF-\beta$  antisense molecule, which was approx 930 base pairs long. Once sufficient numbers of gene-modified tumor cells became available (after approx 12–16 wk of selection in culture), the patients were inoculated subcutaneously on a monthly basis with either  $5 \times 10^6$ ,  $1 \times 10^7$ , or  $2 \times 10^7$  cells/injection.

The aims for this clinical trial were as follows:

1. Evaluate the safety of subcutaneous injections of irradiated autologous tumor cells that were genetically modified to block their  $TGF-\beta_2$  secretion in patients with GBM. The eligible participants in this phase I clinical trial were glioblastoma patients who had undergone surgery and radiation therapy.

2. Monitor tumor progression and humoral/cellular immune responses in the patients injected with TGF- $\beta_2$  antisense, gene-modified tumor cells, and compare them with those of historical and traditionally treated controls.
3. Evaluate the effects of repeated inoculations with irradiated, gene-modified tumor cells by monitoring and comparing the nature of immune infiltrates in needle biopsies obtained from the injection sites at 24 h after each inoculation.
4. Evaluate, by immunohistologic procedures, the nature of immune infiltrates and the cytokine profiles in the original tumor biopsy (and at recurrence, if BT samples were available).

### 7.2. Eligibility

Patients with suspected GBM on the basis of clinical presentation and radiologic evaluation were eligible to participate in this study. Pathological confirmation of GBM was required prior to treatment. These patients, despite being treated with radical surgery and radiation therapy, have a median life expectancy of 36–38 wk.

### 7.3. Conclusion

Two patients at UCLA Medical Center were treated with this protocol. In these patients, injection of  $5 \times 10^6$  or  $1 \times 10^7$  cells did not cause any acute toxicity. Furthermore, the authors found that successive inoculations resulted in activation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. On immunohistochemistry analysis, these CD4<sup>+</sup> and CD8<sup>+</sup> cells were capable of migrating through the blood–tumor barrier and infiltrating the tumors (Fakhrai et al., manuscript in preparation). However, there was no significant increase in survival in the two treated patients, compared to historical controls with GBM. The preliminary results obtained from these patients at UCLA suggest that TGF- $\beta$  antisense gene therapy, once developed, may be a useful adjuvant tool for clinicians to manage brain cancer.

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## Dendritic Cell Immunotherapy for Brain Tumors

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*Stéphane Vandenabeele, PHARM.D.,  
Linda M. Liau, MD, PH.D.,  
and David Ashley, PH.D.*

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### 1. INTRODUCTION

Dendritic cells (DCs) represent <1% of circulating white blood cells in the body. They were first visualized in the skin in 1868, when Paul Langerhans described the so-called “Langerhans cells of stellate morphology” in the epidermis. It was not until 1973 that Ralph Steinman first isolated DCs (1,2) on the basis of their striking morphological features. This novel cell type of bone marrow (BM) origin is also found in mouse peripheral lymphoid organs (3). DCs do not exhibit the endocytic capacities of macrophages and cannot become macrophages upon culturing (4). Furthermore, they are at least 100-fold more effective than other antigen-presenting cells (APCs), such as B-lymphocytes and macrophages, at stimulating T-lymphocytes (5). It was only in 1985 that epidermal Langerhans cells (LCs) were shown to acquire, upon culture, all the characteristics of splenic DCs (6).

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The current working model is that DCs found in lymphoid organs are the mature form of precursors (such as the LCs) found in nonlymphoid organs, such as skin, lungs, and gut. Altogether, migration experiments and progress made in the isolation and definition of this rare population of leukocytes led to the unveiling of a highly regulated network of professional APCs, which displayed unique properties and played a strategic role in the immune system. Indeed, the DC network, scattered throughout all tissues and organs ensures the immune surveillance of the body and captures the characteristic features of microbes: antigens (Ags). In short, the function of DCs is to induce immunity, when required, by controlling T- and B-lymphocyte function.

Consequently, DCs have become the target for manipulation of the immune response at the early phase of immunity. Also, the possibility of generating large numbers of DCs from blood progenitors *in vitro* makes their use in clinical immunotherapy (IT) feasible.

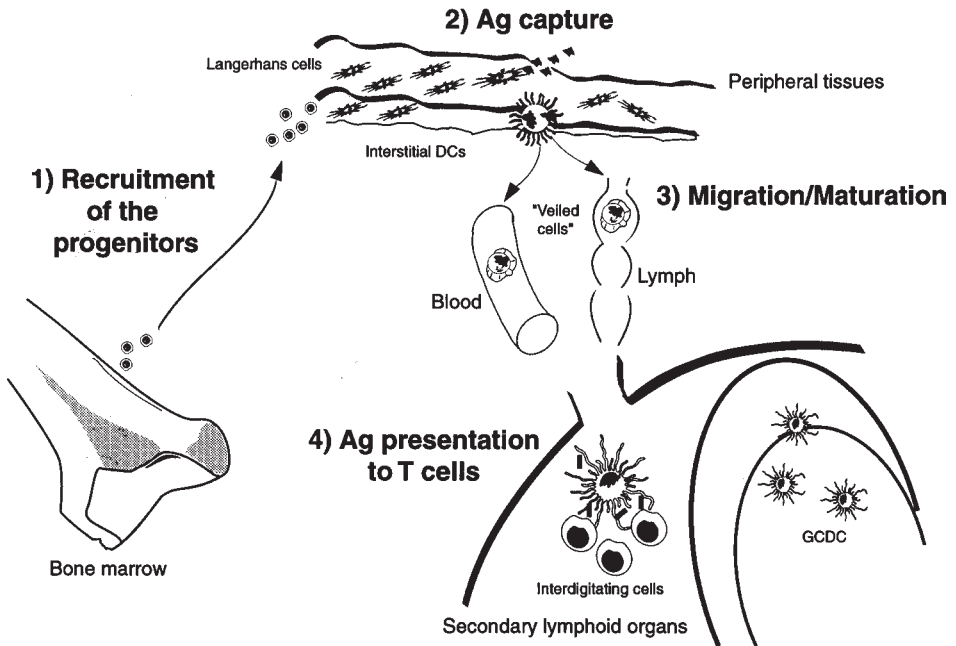
## 2. DCs: PROFESSIONAL APCs

Priming of naïve T-lymphocytes requires two steps: first, recognition of peptide–major histocompatibility complexes (MHCs) by the T-cell receptor; and, second, stimulation by surface molecules, such as CD80 (B7.1) and CD86 (B7.2), expressed on APCs. Because of their tissue distribution, their particular capacities for uptake and processing/presentation of Ags, and their expression of high levels of co-stimulatory molecules, DCs are the only APCs able to prime naïve T-lymphocytes. In contrast, B-lymphocytes and macrophages are only able to stimulate previously activated T-lymphocytes. Therefore, DCs are considered “professional APCs” and are also termed “nature’s adjuvants.”

DCs are organized as a network present in all tissues and organs at trace levels. This network is dynamic, because DC populations are constantly renewed and migrate from site to site, while modulating their functional properties (Fig. 1). This review excludes from consideration follicular DCs, a separate group of cells whose origins are not clearly established and which localize and present unprocessed Ag–antibody complexes to B-cells (reviewed in ref. 7).

### 2.1. Immature DCs: Sentinels in the Periphery

DC progenitors are recruited from the BM via the blood stream to enter the epithelial and interstitial zones of nonlymphoid organs. There, they differentiate into LCs in the epithelium and interstitial DCs in the interstitium. At that stage, they express CD1a and are considered immature DCs, because they are not yet able to prime T-lymphocytes (8–10). In relatively quiescent tissues, such as skin or muscle, DCs can develop and stay around for weeks. In contrast, in stimulated tissues, such as the gut or the respiratory tract, DC populations are renewed every 3–4 d (11). The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) and



**Fig. 1.** The dynamic dendritic cell (DC) network. 1) DC precursors are recruited from the bone marrow and differentiate into immature DCs in peripheral tissues. 2) These immature DCs display extremely effective antigen-capture capacities and a typical dendritic morphology to cover an extensive surface area. Langerhans cells of the epidermis are the best models of immature DCs. 3) Following intrusions by parasites, virus, bacteria, or tumor, immature DCs capture the antigens and migrate via blood or lymph to home into the T-cell areas of the draining lymphoid organs. 4) While they migrate, DCs mature. They process antigens and acquire the expression of costimulatory molecules CD40, CD80, and CD86 to allow an optimal presentation of the antigens captured in the periphery to T cells and the induction of immunity. [This figure is an adaptation of a figure published in *Hematologie* (1999) 5, 546–548.]

the chemokine macrophage inflammatory protein 3 $\alpha$  (MIP-3 $\alpha$ ) are expressed by inflamed tissues and recruit DC progenitors that carry their receptors (12).

This constant renewal of DC populations helps spin an immune web ready to collect intruders. To do so, immature DCs develop a stellate morphology to cover extensive surface areas, and they display effective Ag-capture capacities. Indeed, they can take up parasites, bacteria, and apoptotic cells by phagocytosis (13–15). Moreover, small amounts (only nanomoles) of Ags are sufficient, because immature DCs carry surface receptors mediating endocytosis, such as the mannose receptor (16), the molecule DEC-205 (17), and receptors to the Fc fragment of immunoglobulins. Finally, DCs can ingest extracellular fluids by macropinocytosis (16). Until DCs capture an Ag, MHC-II products are constantly degraded and recycled; hence, the analogy by Banchereau and Steinman,

comparing immature DCs to “idling motors in neutral gear” (8). However, as soon as an Ag is captured, a gear is engaged, and MHC-II molecules are efficiently loaded with the Ag.

## ***2.2. Migration to Secondary Lymphoid Organs***

Once having captured an Ag, DCs undergo functional changes during trafficking from the periphery to the draining lymphoid organs. These changes enable optimal presentation of the Ag to T lymphocytes. Migrating DCs, called “veiled cells,” because of their sheet-like processes reach the lymph nodes (LNs) via the lymphatic vessels and the spleen via the blood stream. The capture of the Ag gives a migration signal to DCs (8), leading to downregulation of anchor molecules (e.g., E-cadherin), which mediate the adhesion of DCs to keratinocytes of the epidermis (18). In addition, they become sensitive to chemokines expressed within the T-cell zones of lymphoid organs (12). This migration can be inhibited by blockade of the multi-drug receptor by verapamil or specific blocking antibodies (19).

DC maturation is initiated under the influence of cytokines released by the inflamed microenvironment, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), GM-CSF, and interleukin 1 (IL-1) (8); microbial products, such as lipopolysaccharide (LPS) (20); or maturation signals delivered by endothelial cells, while DCs enter the lymphatics (21). In contrast, IL-10 blocks DC maturation (22). This maturation process results in three main functional changes. First, Ag capture is shut down. Second, MHC-II products are effectively loaded with Ag peptides and not constantly degraded. Subsequently, the MHC-II–Ag peptide complexes are translocated from the endosomal compartments to the cell surface, where they may remain stable for days (23,24). This gives DCs the time necessary to encounter rare specific T-lymphocytes and activate them. Third, DCs downregulate CD1a; upregulate the co-stimulatory molecules, CD40, CD80 (B7.1), and CD86 (B7.2); and acquire the maturation markers, CD83 and DC-LAMP (25). They also upregulate adhesion molecules, such as CD54 and CD58, which allow prolonged and intimate contact with T-cells. Consequently, when they reach secondary lymphoid organs, DCs are frozen in an optimal state for presentation of the peripherally captured Ag to specific T-cells.

## ***2.3. Induction of Immunity***

### **2.3.1. CONTROL OF T-CELL FUNCTION**

Within the T-cell zones of lymphoid organs, mature DCs (initially termed “interdigitating cells” (1), because of their many elongated processes) present MHC-II-restricted Ags to CD4<sup>+</sup> T-cells. This encounter affects both DCs and T-cells. Indeed, a dialogue starts when the CD40 ligand, borne by T-cells, binds

CD40 on DCs. This results in the upregulation of CD80 and CD86 by DCs, which triggers CD28 and CTLA-4 on T-cells and subsequently stimulates them. Moreover, DCs then produce IL-12 and skew T-cell differentiation toward the helper T-cell type 1 or cell-mediated/cytotoxic pathway (26).

DCs are also able to stimulate CD8<sup>+</sup> T-cells and induce cytotoxic responses *in vitro* and *in vivo*. However, the nature of the Ag presentation pathway used by DCs to cytotoxic T-cells is not clearly elucidated. Unlike CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells recognize Ags associated with MHC-I molecules on the surface of every cell of the body. The conventional model of Ag presentation requires an intracellular origin for an Ag to be presented by MHC-I molecules. This pathway is usually termed the “endogenous” pathway, in contrast to the “exogenous” pathway, where extracellular Ags are presented in association with MHC-II molecules to CD4<sup>+</sup> T-cells. Nevertheless, it has been shown that exogenous Ags can also reach the endogenous pathway, be associated to MHC-I molecules, and be presented to CD8<sup>+</sup> T-cells. This phenomenon has been called “cross-priming,” and would be at the origin of antiviral and antitumor responses *in vivo* (reviewed in ref. 27). It has recently been shown that DCs were capable of cross-priming (15). In this study, immature DCs, but not macrophages, were shown to phagocytose virus-infected apoptotic cells and trigger specific cytotoxic T-lymphocyte (CTL) responses against the virus. Thus, the capacity of DCs to induce cytotoxic responses against exogenous Ags can be utilized when inducing antitumor immunity.

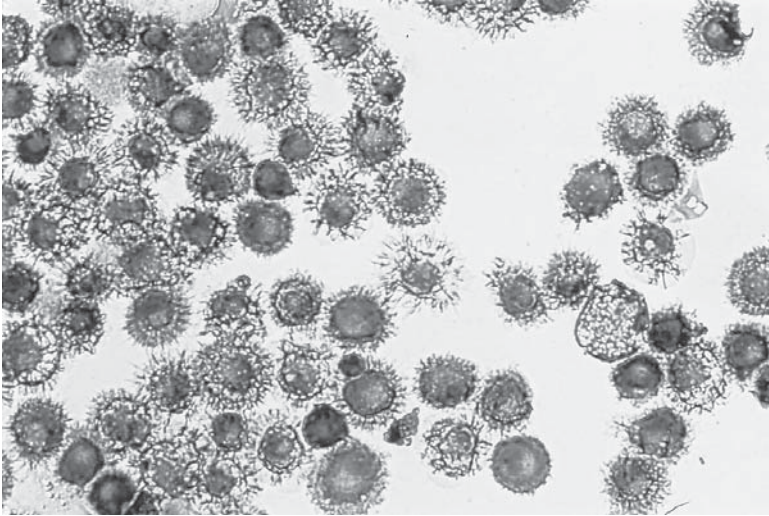
Finally, DCs play a critical role in the induction of tolerance. Indeed, DCs have been shown to express very high levels of MHC–self-peptide complexes (28) and to play a critical role in the deletion of self-reactive T-cells in the thymus (29). Moreover, some DCs are able to negatively regulate the cytokine output of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (30). Again, such data may have importance in future DC-based cancer IT.

### 2.3.2. CONTROL OF B-CELL FUNCTIONS

DCs play an essential role in the induction of T-cell-dependent humoral responses. Some DCs are localized within B-cell germinal centers (31), and can induce the proliferation and differentiation of naïve B-cells *in vitro* (32,33).

## 3. IN VITRO GENERATION OF DCs

It is now possible to generate large numbers of DCs *in vitro*, using proliferative and nonproliferative progenitors isolated from peripheral blood, cord blood, and BM (34). The DCs generated *in vitro* display all the characteristics of DCs described *in vivo* (Fig. 2). Regarding clinical applications, a critical issue is the generation of DCs without nonhuman proteins such as fetal calf serum (FCS),



**Fig. 2.** Photomicrograph of human dendritic cells (DCs) generated in vitro from CD34<sup>+</sup> hematopoietic progenitors.

which is often used as source of proteins in cell culture. In addition, it is important to know when the introduction of an Ag, such as a tumor Ag, would be most suitable for optimal capture by the DCs.

### ***3.1. In Vitro Generation of Human DCs***

#### **3.1.1. CD34<sup>+</sup> HEMATOPOIETIC PROGENITORS**

DCs can be generated from CD34<sup>+</sup> hematopoietic progenitor cells (HPC) isolated from peripheral blood, cord blood, or BM. In the presence of GM-CSF and TNF- $\alpha$ , CD34<sup>+</sup> HPCs proliferate and differentiate into DCs identical to LCs in morphology, phenotype, and function (35–37). The expansion can be enhanced by addition of stem cell factor (SCF) or FLT-3 ligand. For example, in the presence of FCS, it is possible to obtain  $3 \times 10^7$ – $10^8$  DCs from  $10^6$  CD34<sup>+</sup> HPCs, after 10–12 d of culture. After 14 d of culture, CD34<sup>+</sup> HPCs are usually fully mature. However, it is possible to induce further activation and secretion of IL-12 by triggering CD40 (26).

CD34<sup>+</sup> HPC-derived DCs can also be obtained without nonhuman proteins in culture. A recently described system (38) used TGF- $\beta_1$  (in addition to GM-CSF, TNF- $\alpha$ , and SCF) in serum-free media. In this system, CD34<sup>+</sup> HPCs proliferated and differentiated normally into DCs (38).

#### **3.1.2. PERIPHERAL BLOOD MONOCYTES**

DCs can be generated from monocytes isolated from adult peripheral blood. After 4–7 d of culture with GM-CSF and IL-4, monocytes differentiate into

immature DCs without proliferation. Like LN DCs, they exhibit a veiled morphology; have migratory abilities; downregulate the monocyte/macrophage marker, CD14; and acquire the LC marker, CD1a (39–41). Moreover, they display very efficient Ag-capture capacities. Injected subcutaneously in the chimpanzee, they behave like LCs and migrate to the T-cell zones of the draining LNs (42). However, these immature DCs are not fully differentiated into the DC pathway. Indeed, cultured with M-CSF, they lose the properties of DCs and become adherent macrophages. To ensure an irreversible commitment toward the DC pathway, two more days of culture with TNF- $\alpha$ , with a monocyte-conditioned medium, or by triggering CD40, is required (43,44).

Monocyte-derived DCs can also be obtained without nonhuman proteins in the culture. Human serum and human plasma can be used as substitutes for FCS (45). In addition, culture in the presence of a monocyte-conditioned medium, free of nonhuman proteins, allows full maturation of DCs.

Recently, monocyte-derived DC differentiation has been obtained within 2 d of culture in a system that mimics the trafficking of DCs from tissues to enter the lymphatic vessels (21). This differentiation of DCs is optimal after capture of particulate Ags and reverse transmigration through a layer of endothelial cells.

### ***3.2. In Vitro Generation of Mouse DCs***

DCs can be generated from mouse BM and blood in the presence of GM-CSF, but not G-CSF or M-CSF (46–48). The addition of TNF- $\alpha$  late in the culture further enhances DC development. The DC progenitors are only selected on the basis of no expression of MHC-II molecules and no adherence to plastics. In semi-solid cultures, DCs were shown to arise from a precursor common to macrophages and granulocytes.

In addition, lymphoid-related DCs can be generated in vitro, from low-CD4<sup>+</sup> mouse thymic precursors in the presence of IL-1, IL-3, IL-7, FLT-3 ligand, SCF, and TNF- $\alpha$  (49). These low-CD4<sup>+</sup> thymic precursors were shown to give rise to DCs and T-cells in vivo, but not macrophages or granulocytes (50). These lymphoid-related DCs may play a regulatory, rather than stimulatory, role on T-cell functions (30).

## **4. DC-BASED CANCER IMMUNOTHERAPY**

Tumors express specific Ags that can be recognized by T-lymphocytes in vitro (51). Although these tumor-associated Ags (TAA) are immunogenic in vitro, a variety of mechanisms exist by which tumors can evade immune detection and rejection. Such mechanisms may include loss of expression of TAAs (52–54), secretion of inhibitory substances (55), or expression of the T-cell killing molecule Fas-ligand by tumor cells (56). All such mechanisms result in escape from immunity.



Because DCs are crucial for generating primary immune responses and are considered “nature’s adjuvants,” they may provide a way to overcome such escape from immunity. Recent progress in the understanding of the requirements for recruitment and differentiation of DC precursors, and the sequential events occurring from Ag capture to T-cell stimulation, has made DC-based IT feasible. Therefore, strategies are being developed to enhance the number of DCs in vivo, or to grow DCs ex vivo and load them with TAAs before reinfusion into patients (Fig. 3). Results in animals have been encouraging, and many clinical trials are now ongoing for different types of cancers.

### ***4.1. Preclinical Studies***

#### **4.1.1. MANIPULATING DCs EX VIVO**

Many preclinical studies have examined ex vivo DCs generated from mouse BM or purified epidermal LCs loaded with different sources of TAA. All the different sources of Ag listed below have induced specific cytotoxic responses, protection against tumor challenge, and long-term survival in the case of pre-established tumors.

1. Tumor extracts, obtained by sonicating tumor cells, have subsequently been introduced into DCs using liposomes.
2. Synthetic or acid-eluted peptides represent the most commonly used source of TAA in pre-clinical studies (57–59).
3. Whole-tumor proteins have the advantage of containing both MHC-I- and also MHC-II-restricted Ags (60,61). Therefore, whole proteins may trigger CD4<sup>+</sup> T-cell help, which has been shown to be critical for induction of CTL responses (62).
4. Tumor RNA or DNA can successfully be introduced into DCs by viral vectors. This method has been shown to be more effective than physical methods, such as liposomes, electroporation, or CaPO<sub>4</sub> precipitation (63). Adenoviral vectors, which have been rendered replication-deficient, are the most commonly used (64,65), although they may generate antiviral responses (66). TAA mRNA has also been introduced into DCs by replication-deficient retroviral vectors (67).
5. Fusion between DCs and tumor cells has also been attempted, using a tension-active compound, like polyethylene glycol (68,69). However, the tumor cells must proliferate in order to allow successful fusion.

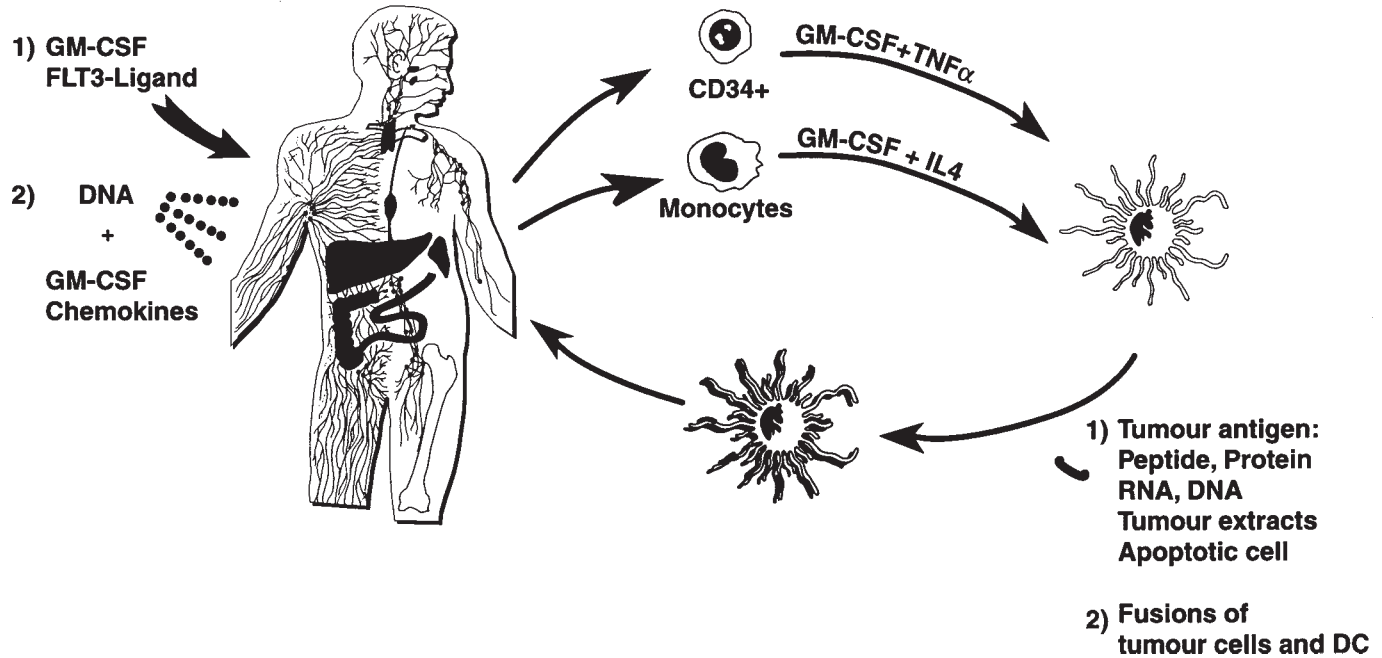
#### **4.1.2. TARGETING DCs IN VIVO**

Several regulatory factors are critical for the development of DCs in vivo. Among them, GM-CSF and FLT-3 ligand, particularly, have been shown to mobilize DC progenitors and enhance the number of mature DCs in vivo. In addition, FLT-3 ligand injections have been shown to induce tumor rejection, with the working hypothesis being that this effect is the result of an increase in DC number (70).



## IN VIVO

## EX VIVO



**Fig. 3.** Kick-starting tumor immunity with dendritic cells. Different strategies can be envisaged for dendritic cell tumor immunotherapy. (This figure is an adaptation of a figure published in *Hematologie* [1999] 5, 546–548).

Moreover, *in vivo* transfection of DCs with TAA cDNA is possible, using a Gene Gun. This method consists of a cutaneous bombardment with gold beads bearing the TAA cDNA. Although the yield of transfection *in vivo* is low, DCs transfected this way have been found to migrate to LNs draining the bombardment site (71). Therefore, one attractive strategy would be to increase the number of DCs at the target site by injecting GM-CSF, MIP-3 $\alpha$ , or FLT-3 ligand prior to Gene Gun bombardment.

## 4.2. Clinical Trials

The results of preclinical studies show much promise and provide a large array of DC-based strategies to induce tumor immunity. The results of the first DC-based clinical trials have prompted further questions regarding the selection of appropriate sources of Ags and routes of administration of DCs.

### 4.2.1. B-CELL NON-HODGKIN'S LYMPHOMA

B-cell lymphomas have the advantage of carrying a tumor-specific target on their cell surfaces, known as an idiotype. However, this is a weak Ag, and low-grade lymphomas remain incurable (72). DC-based vaccines have been developed at Stanford University Medical Center (73), where autologous DCs were purified from blood, pulsed (co-cultured) with the patient's idiotype protein, and reinfused intravenously. The culture medium contained autologous serum. Patients received four infusions of pulsed DCs, each followed by subcutaneous injection of soluble idiotype protein 2 wk later.

This trial included four patients with previously treated follicular lymphoma. No significant side effects were observed after injections. All the patients generated specific anti-idiotypic lymphocytes, but none produced anti-idiotypic antibodies. In one patient, a complete clinical remission was achieved. In another patient, whose disease was detectable only by molecular analysis of blood and BM, resolution was also complete. In a third patient, a minor response was observed, with regression of some peripheral LNs.

### 4.2.2. MELANOMA

Many clinical trials are ongoing using DC-based vaccines for stage IV melanoma patients. Recently, the first results of a trial led by the University of Zurich Medical School were reported (74). In this trial, DCs were generated from peripheral blood monocytes cultured in the presence of GM-CSF and IL-4 for 7 d. The culture medium contained FCS. Then, DCs were pulsed with either a cocktail of peptides known to be recognized by cytotoxic T-cells (MAGE-1 and MAGE-3 for human leukocyte antigen [HLA]-A1; tyrosinase, melan-A/Mart-1 and gp100 for HLA-A2) or tumor extracts (when patients lacked expression of HLA-A1 or HLA-A2). To increase the chances of encounter between pulsed DCs and specific T-cells *in vivo*, the investigators decided to inject DCs directly into an uninvolved inguinal LN.

This trial involved 16 patients who received weekly injections for 1 mo, followed by one injection 2 wk later and every month thereafter. The treatment was well tolerated, except for occasional fever and swelling of the injected LN for 1–2 d. No sign of autoimmunity was observed. Clinical responses were observed in five patients: Two presented a complete response with no trace of the disease after more than 15 mo, three had a partial response with regression of metastasis in various organs, and one displayed a minor response. In contrast to other DC-based trials for stage IV melanoma, this group obtained striking results. The chief differences are the presence of nonhuman proteins (i.e., FCS) in the growth culture medium and the direct delivery into LNs. This strategy may trigger inflammation of the injected LN, perhaps enhancing the immune response.

#### 4.2.3. PROSTATE CANCER

A phase II trial led by the Pacific Northwest Cancer Foundation in Seattle (75,76) involved 33 patients suffering from advanced, hormone-resistant prostate cancer. DCs were generated from peripheral blood monocytes with GM-CSF and IL-4 in the presence of autologous plasma. Patients received six intravenous infusions, at six weekly intervals, of DCs pulsed with PSM-1 and PSM-2 peptides. Hypotension and allergic reactions were noted in some cases after the infusions. Nine partial responders were identified, as defined by >50% reduction of prostate-specific antigen level in the blood.

## 5. TUMOR CELL VACCINES IN CNS TUMOR MODELS

Several active immunization protocols using tumor cells genetically engineered to secrete cytokines have been reported (77,78) to abrogate tumorigenicity, cure tumor-bearing animals, and induce long-lasting immunity to subsequent tumor challenge in extracranial tumor models. A number of groups have confirmed these findings in different brain tumor (BT) models. Experimental studies have demonstrated that subcutaneous vaccination of tumor cells, genetically engineered to produce *GM-CSF*, *IL-3*, or *IL-6*, stimulates a potent and persistent antitumor immune response against intracranial tumors and increases the survival of tumor-bearing mice (79).

Unfortunately, in practice, a number of barriers exist to the treatment of human BTs using genetically modified autologous tumor cell vaccines. For instance, these vaccines require the considerable task of *ex vivo* purification, culture, expansion, and transfection of tumor specimens, which is a difficult undertaking, even for tumors outside the CNS (80,81). In addition, it has been shown that immunizing nonhuman primates and guinea pigs with human BT tissue can induce experimental allergic encephalomyelitis (EAE) that is lethal (82). Vaccination with unfractionated tumor-derived Ags, such as those possibly contained in an autologous tumor cell vaccine derived from the CNS and modi-

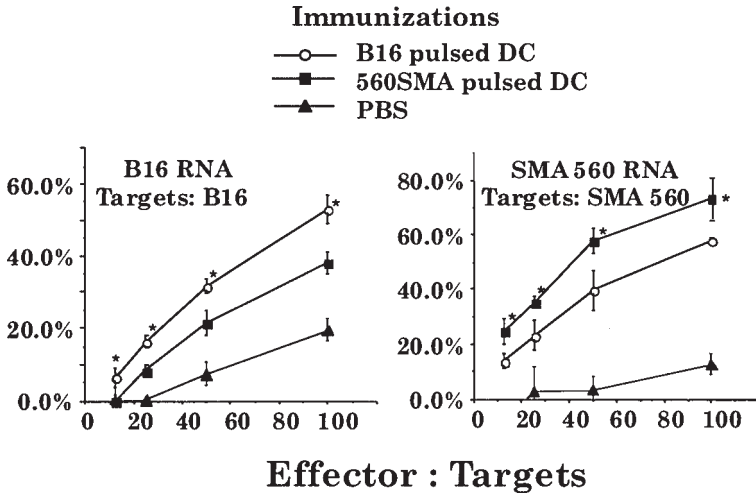
fied genetically, may lead to potentially disastrous consequences. This potential risk of autoimmune encephalitis may limit the use of whole-tumor-cell vaccines to a minority of patients (i.e., to those from which highly purified tumor specimens can be guaranteed).

## 6. DCs AND INDUCTION OF ANTITUMOR IMMUNITY AGAINST CNS TUMORS

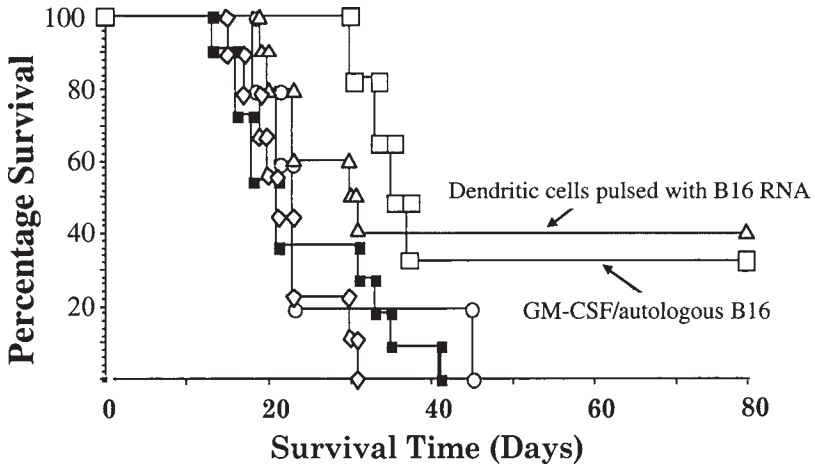
As described in the previous section, studies have shown that immunizing mice with DCs pulsed with specific Ags engenders protective tumor immunity in the treated animals (57,60,83,84). More recently, effective antitumor immunity in mice has been induced using DCs pulsed with unfractionated tumor-derived Ags in the form of peptides (59,85), cell sonicates (86), and mRNA (87). The advantages of vaccinating with total tumor-derived material are that the identity of the tumor Ag(s) need not be known and that the presence of multiple tumor Ags reduces the risk of Ag-negative escape mutants. The potential benefit of using total tumor Ags in the form of mRNA is that RNA can be amplified from a small number of tumor cells; hence, DC vaccine treatment may be extended to patients with BTs from which only a small, possibly microscopic, biopsy can be taken for diagnosis.

Furthermore, techniques could be developed in order to improve the quality of the material used for Ags. These could include methods for the enrichment of bona fide tumor cells from patient specimens by *ex vivo* purification, combined with the use of RNA subtractive hybridization techniques. Such techniques may reduce the concentration of self-Ags, while increasing the representation of tumor-specific Ags or TAAs in the vaccine preparation. This would be of crucial importance for vaccinations with CNS tumor-derived Ags, because increased Ag selectivity may diminish the risk of severe autoimmune complications and improve the likelihood of antitumor responses in the brain.

In order to establish the basis for a safe and more universally available vaccine therapy for CNS cancers, a few groups have explored the use of tumor Ag-loaded DCs for BTs. Initial studies in C57BL/6 mice compared the efficacy of DC-based vaccines (pulsed with either tumor extract or tumor-derived RNA) with that of cytokine gene-modified tumor vaccines for the treatment of tumors within the CNS (88). Using the B16/F10 murine melanoma (B16) as a model for CNS metastasis, it was shown that vaccination with BM-derived DCs, pulsed with either B16 cell extract or B16 total RNA, can induce specific CTL responses against B16 tumor cells (Fig. 4). Both types of DC-based vaccines were able to protect animals from CNS tumors and led to prolonged survival in mice with pre-established tumors. DC-based vaccines were at least as effective, if not more so, compared to vaccines with GM-CSF gene-modified B16 tumor cells (Fig. 5). Furthermore, DC-based vaccination was associated with a dra-



**Fig. 4.** Enhanced induction of CTL by dendritic cell vaccines. Induction of specific lytic activity against tumor cells by immunization with DCs pulsed with tumor-derived total RNA. Triplicate C57/BL6 mice were immunized three times with B16 tumour RNA or SMA560 RNA or PBS. 7 d later, splenocytes were isolated and restimulated for 5 d with irradiated tumor cells. Cytotoxic activity was then measured using the targets indicated in each panel.



**Fig. 5.** Dendritic cells (DCs) pulsed with tumor cell RNA are effective vaccines. Vaccination with DCs pulsed with tumor RNA protects against CNS challenge with B16 tumor and is equipotent to vaccination with GM-CSF-producing B16 cells. Vaccination of C57/BL 6 mice was performed a total of three times before intracranial tumor challenge with B16 cells. Mice were evaluated until death. Median survival, range, number of animals, and significance compared to PBS-immunized animals based on log-rank analysis for each group are as follows: PBS, 21, 13–42,  $n = 10$ , closed squares; DC pulsed with 560SMA RNA, 21, 15–31,  $n = 9$ ,  $P = 0.40$ , open diamonds; DC pulsed with B16 RNA, 31, 19–80,  $n = 10$ ,  $P = 0.0001$ , open triangles; GM-CSF modified B16 cells, 36, 30–80,  $n = 6$ ,  $P = 0.022$ , open squares.

matic enhancement of inflammatory infiltrate and large areas of hemorrhage and necrosis within the tumor.

More recently, two groups have reported the use of DC-based vaccines for the treatment of CNS tumors in animals. Okada et al. (89) developed a BT model using a C3 sarcoma cell line expressing the tumor-specific, MHC-I-restricted peptide epitope, E7 (49–57). Syngeneic C57BL/6 mice receiving intravenous injections of DCs pulsed with E7 peptide were effectively protected against a subsequent intracerebral challenge with C3 tumor cells. This systemic immunization strategy was effective in a therapeutic model. In vivo depletion of CD8<sup>+</sup> cells, but not CD4<sup>+</sup> or asialo-GM1<sup>+</sup> cells, abrogated the efficacy of E7-peptide-pulsed DC therapy of established tumors, indicating a pivotal role of specific CD8<sup>+</sup> T-cell responses in mediating the antitumor effect (89). Liau et al. (90) have demonstrated that peptide-pulsed DC therapy, using unfractionated acid-eluted tumor Ags, led to prolonged survival in rats with established 9L intracranial tumors. Associated with this therapy was a significant increase in peri- and intratumoral infiltration of both CD4<sup>+</sup> and CD8<sup>+</sup> cells within the brain. In addition, cytotoxicity assays demonstrated specific CTL activity against 9L tumor cells (90).

These reports have demonstrated that vaccines based on DCs pulsed with tumor extracts, RNA, acid-eluted peptides, or synthetic peptides are all active against tumors within the CNS. These studies establish a sound basis for further preclinical and clinical studies of DC-based vaccines for the treatment of BTs. Given the real concerns that vaccination with unfractionated Ags from the CNS may lead to potentially disastrous consequences in human patients (such as autoimmune encephalitis), research efforts must now also focus on the safety aspects of these immune-based treatments for BTs.

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# 16

## Death Ligand/Death Receptor-Mediated Apoptosis for Treatment of Brain Tumors

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*Wilfried Roth, MD and Michael Weller, MD*

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### 1. INTRODUCTION

The poor prognosis for patients with malignant gliomas calls for novel therapeutic approaches in this field of oncology. At present, involved-field radiotherapy is the single most effective treatment. Most of the currently available chemotherapy strategies fail because of the resistance of glioma cells to cytotoxic agents. Mutations of tumor suppressor genes (such as *Rb* or *p53*) or enhanced expression of oncogenes (such as *bcl-2* or *bcl-X*), both common features of malignant gliomas, may inhibit the efficient killing of tumor cells by cytotoxic drugs or radiotherapy. Because activation of apoptotic cell death cascades is now considered to be a powerful instrument to rapidly and specifically induce the death of target cells, many researchers are seeking ways to utilize the apoptotic signaling pathway for therapeutic purposes.

During the past few years, the phenomenon of cells committing a form of suicide (e.g., apoptosis) gradually became a focus of interest, both in basic biology and in experimental medicine. Triggering cell death by employing apoptotic pathways has specifically gained importance in the development of novel therapies for malignancies. Apoptosis is morphologically characterized by membrane blebbing, cellular shrinkage, and condensation of chromatin. The execution of apoptotic cell death may exhibit rapid kinetics, because cells can be degraded

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within a few minutes. In general, the biological function of apoptosis consists of the capability of an organism to dispose of needless or even dangerous cells, without causing damage to the surrounding tissue. Needless or dangerous cells may include autoaggressive immune cells, virally infected cells, tumor cells, or obsolete cells during embryogenesis. Thus, apoptotic cell death is essential for development, immune homeostasis, and tumor prevention. It has also been proposed (1) that premature apoptosis can result in neurodegenerative diseases, such as Alzheimer's disease or Parkinson's disease; deficient apoptosis may cause autoimmune diseases or malignant tumors.

## 2. DEATH RECEPTORS AND THEIR LIGANDS

The natural induction of apoptosis often involves death ligands specifically interacting with their cognate death receptors (DRs), which are type I membrane proteins. They belong to the tumor necrosis factor receptor (TNFR) superfamily, which is characterized by two structural features: All family members share an extracellular cysteine-rich domain (2) and an intracellular death domain (3). The death domains consist of approx 80 amino acids. They are found in the signaling regions of the receptors and also in the recently identified proteins that transmit the death signal after having bound to the receptors, including Fas-associated protein with death domain (FADD), TNF receptor-associated death domain (TRADD), and receptor-interacting protein (RIP).

Since death domain-containing proteins can self-associate, ectopic expression of these proteins frequently leads to triggering of apoptotic cell death in the absence of external ligand. The physiological ligands of the DRs are type II transmembrane proteins and belong to the TNF superfamily. Although most of the ligands are synthesized as membrane-bound proteins, they also exist as soluble molecules. By forming homotrimers, the death ligands can activate their cognate receptors (4). Apoptosis-inducing receptor–ligand pairs are CD95–CD95L (5), TNFR–TNF (6), DR3–Apo3L, DR4–Apo2L, and DR5–Apo2L (7) (Table 1).

The process of apoptotic cell death triggered by death ligand–DR interaction is, in general, based on common mechanisms. Initially, binding of the trimerized ligands leads to oligomerization of the receptors and to a conformational alteration of the intracellular receptor tails. The altered structure of the receptor's death domain enables adapter proteins to bind the receptors via their death domains. Adapter proteins are characterized not only by a death domain, but also by a so-called death effector domain (DED), which allows caspases to join the complex of DR and adapter protein. Caspases are a family of cysteine proteases that are now considered the key executioners of apoptosis; however, the cleavage substrates that are essential to the death process have still not been clarified. All caspases are synthesized as inactive zymogens that can be activated by proteolytic cleavage. After further caspases have been cleaved, a sub-



**Table 1**  
**Death Ligands and Their Receptors**

<b>Ligands</b>			
TNF- $\alpha$	CD95 ligand = Fas ligand = Apo1 ligand	Apo2 ligand = TRAIL	Apo3 ligand = TWEAK
<b>Receptors</b>			
TNFR1	CD95 = Fas = Apo1	DR4 = TRAIL-R1	DR3 = WSL-1 = TRAMP = LARD
TNFR2	DcR3	DR5 = TRAIL-R2 DcR1 = TRAIL-R3 = TRID DcR2 = TRAIL-R4 = TRUNDD	

The table gives a survey of the death ligand/DR pairs and the respective abbreviations. CD95, DR4, and DR5 represent receptors with proapoptotic properties; DcR1 and DcR2 fail to transmit the death signal upon binding to Apo2L. DcR3 has been reported to act as soluble decoy receptor by neutralizing CD95L.

group of caspases, known as effector caspases, are activated. It is believed that the effector caspases also promote the activation of endonucleases, such as caspase-activated DNase (8). These endonucleases translocate to the nucleus, where they cause the degradation of DNA.

Here, the authors focus on the CD95L and Apo2L systems, in the belief that both systems have great potential for future application in the immunotherapy for malignant glioma. The employment of TNF signaling for treating malignant gliomas has little value in the management of these malignancies (9). The Apo3L–DR3 system has not been investigated in depth, but seems to be similar to the TNF system (10). Therefore, the TNF and Apo3 systems are not discussed in this chapter.

## **2.1. CD95 and CD95L**

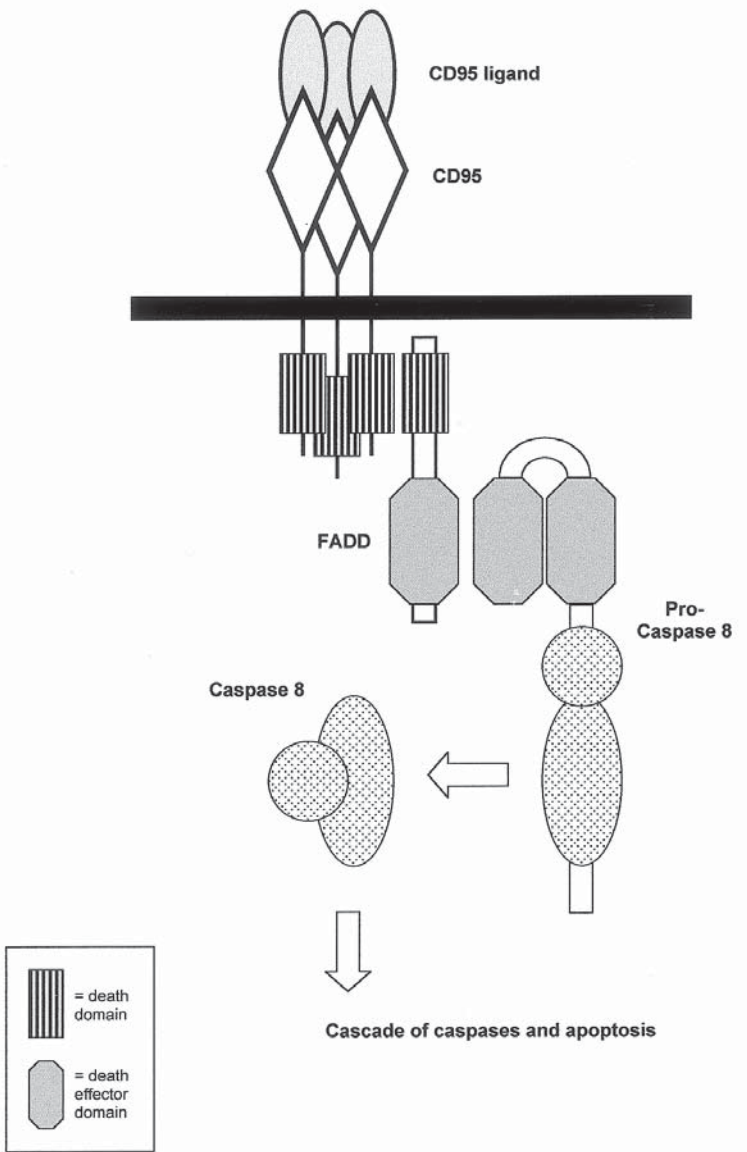
### **2.1.1. BIOLOGICAL PROPERTIES OF CD95 SIGNALING**

A decade ago, the CD95L–CD95 system began to be explored as a ligand–receptor pair that seemed to have solely one role, namely the killing of target cells (11,12). The ligand CD95L (= FasL = Apo1L) binds specifically to its cognate receptor, CD95 (= Fas = Apo1), to induce apoptotic cell death.

CD95-induced apoptosis fulfills several biological functions. *CD95* is expressed in numerous tissues, such as liver, thymus, and heart. On the other hand, expression of *CD95L* is rather limited physiologically (e.g., on activated T-cells). The CD95 system, together with the perforin system, is most important for T cell-mediated cytotoxicity. Thus, the CD95 system plays an outstanding part in the regulation of immune reactions, in maintaining "immunologically privileged" sites in the organism, and in the killing of virally infected cells and tumor cells (5,13).

Recently, the intracellular mechanisms of CD95-induced apoptosis have been investigated in much detail. After the binding of CD95L to CD95 and the thereby induced conformational change of the intracellular death domain, the adapter protein FADD binds, via interaction of the death domains, to CD95 (Fig. 1; 14). Through self-association of the DEDs, caspase-8 is coupled to the complex (15,16). The complex comprising of CD95, FADD, and caspase-8 is designated death-inducing signaling complex (DISC) (17). Since caspase-8 undergoes a change of tertiary structure caused by binding to FADD, the activation of caspase-8 then can occur by self-cleavage (18). The active subunits of caspase-8 induce the stepwise cleavage, which thereby activates several effector caspases (e.g., caspases-3, -4, -7, -9, and 10). However, the precise order of activation of these diverse caspases remains to be elucidated. Numerous substrates of caspases have already been identified, among them the DNA repair protein poly ADP-ribose polymerase (PARP), regulators of the cell cycle (e.g., Rb and MDM-2), several kinases, and diverse structural proteins (19). Despite extensive knowledge about target proteins of caspases, some crucial downstream steps on the way to the final completion of cell death remain obscure.

CD95-induced apoptosis is based on a signaling cascade that can be influenced by various other intracellular signaling factors. There is a multitude of metabolic pathways that can interact with the apoptotic death program. Each cell is provided with a complex regulatory system that allows various reactions to apoptotic stimuli. Whether a cell dies or stays alive depends on the balance of pro- and antiapoptotic forces. In this regard, the *Bcl-2* family is of particular importance (20). Proapoptotic family members include *Bax*, *Bad*, and *Bak*; *Bcl-2*, *Bcl-xL*, and *Mcl-1* belong to the antiapoptotic members of the family. *Bcl-2* was shown to inhibit CD95-induced apoptosis in glioma and other cell lines (21,22). Inhibitor of apoptosis proteins (IAPs) are another group of antiapoptotic proteins, composed of NAIP, c-IAP1, c-IAP2, X-IAP, and survivin (23–26). Most of these proteins can directly inhibit apoptosis by binding to caspases. A different mode of action is employed by FLICE-inhibitory protein (FLIP), which binds to the CD95–FADD complex, thereby inhibiting the recruitment of caspase-8 (27). Recently, a soluble decoy receptor, which binds to and inhibits the effects of CD95L, was identified (28). This antagonistic receptor, termed DcR3, was found to be predominantly expressed in malignant tissues, suggesting a novel immune-evasive strategy for CD95-positive tumors.



**Fig. 1.** Apoptosis signaling by CD95 ligand-CD95 interaction.

### 2.1.2. CD95 SYSTEM AND MALIGNANT GLIOMAS

Normal as well as malignant central nervous system tissues exhibit remarkable expression patterns of *CD95* and *CD95L* (29). *CD95* is not expressed in normal brain tissue, with the exception of endothelial cells (30,31). In contrast,

the majority of long-term human glioma cell lines and fresh ex vivo glioma cells express CD95 (32–36). It has been shown that in glioma tissues in vivo, CD95 is expressed mostly in perinecrotic zones (35,37). The level of CD95 expression in gliomas has been shown to correlate with the grade of malignancy (33). CD95L was found to be regularly expressed on glioma cell lines, as well as in primary astrocytic brain tumors in vivo (34,38,39). In contrast with CD95, CD95L is diffusely expressed throughout the tumor tissue. Moreover, in most cases, co-expression of CD95 and CD95L, without evident signs of apoptosis, could be demonstrated (34).

Glioma cell lines that are highly susceptible to soluble CD95L or agonistic CD95 antibody (Ab), such as the cell line LN-18, have also been shown to co-express CD95 and CD95L. No obvious induction of apoptosis can be observed, even if these cell lines are grown to dense monolayers (81). One explanation of this finding may be the activity of antiapoptotic proteins, which protect the cells from the induction of suicidal cell death, but most of the investigated glioma cell lines and ex vivo glioma cells have been demonstrated to be susceptible to CD95-mediated cell death (32,36,40). A natural CD95L, produced in mouse neuroblastoma cells, induced more potent antiglioma effects than an agonistic CD95 Ab (36,40,41). This preparation of CD95L seems to acquire its cytotoxic potency from strong aggregate formation (42). Moreover, a *CD95* gene transfer into CD95-negative resistant glioma cells rendered them sensitive to CD95 agonists. It has been shown that a certain threshold level of CD95 expression is necessary for the induction of apoptotic cell death by CD95 Ab. However, above this threshold, CD95-mediated apoptosis did not correlate with *CD95* expression on glioma cells (43). These findings suggest that subcellular factors play an essential part in the execution of CD95-induced cell death of glioma cells (44). These intracellular modifiers of apoptosis may include *Bcl-2* (21). Other regulators of CD95-mediated apoptosis may also come into consideration, because several pro- and antiapoptotic members of the *Bcl-2* family have been detected in glioma cell lines and tissues (45–47). Thus, a CD95-based therapy for malignant gliomas would have to target not only the expression level of *CD95*, but also the intracellular components of the signaling cascade that determine vulnerability to the induction of cell death.

Several studies have been published that, in principle, show promising in vivo results of experimental CD95-targeting therapies for malignant tumors. As early as a decade ago, it was shown (11) that agonistic CD95 Ab induces regression of human B-cell tumor xenografts in *nu/nu* mice. Intraperitoneally administered soluble CD95L efficiently killed CD95-positive, intraperitoneal Yac-1 lymphoma cells (48). Arai et al. (49) demonstrated that adenoviral *CD95L* gene transfer, by local injection in immunodeficient mice, resulted not only in the regression of CD95-positive renal carcinoma xenografts, but also in the destruction of CD95-negative colon carcinoma tissues. CD95-positive carcinoma cells

died by apoptosis; the CD95-negative tumor was massively attacked by locally activated immune cells, which consisted mostly of neutrophils. Similar results were obtained by implantation of *CD95L*-transfected tumor cells into syngeneic or *nu/nu* mice (50). The local inflammatory response caused rejection of *CD95L*-expressing lymphoma, hepatoma, and melanoma cells.

To date, no in vivo study of a direct *CD95L* gene transfer for the treatment of malignant gliomas has been published. However, there are several promising reports on the in vivo treatment of rodent gliomas by viral transfer of other proapoptotic genes, some of which are part of the CD95-dependent killing cascade. Thus, malignant glioma cells can be effectively killed both in vitro and in vivo by gene transfer of *caspase-1* (51), *caspase-3* (52), *caspase-6* (52), or *FADD* (53).

A further important finding is the efficacy of the combined treatment of tumor cells with CD95L and cytotoxic drugs. Human malignant glioma cells are highly susceptible to synergistic induction of cell death by co-treatment with CD95L and various anticancer drugs (40,54–58). It has been shown that the combined application of CD95L and topoisomerase I or II inhibitors, vincristine or taxol, are effective in achieving prominent synergistic activation of apoptotic cell death in human glioma cells (40,54,56). The precise mechanisms underlying this synergistic induction of cell death are not completely understood, although the synergy of CD95L and taxol has been shown to involve inhibition of the protective effect of *Bcl-2* on CD95-mediated apoptosis. Because taxol phosphorylates, and thereby inactivates *Bcl-2*, CD95L-triggered apoptosis is facilitated (54).

Some cell types respond to cytotoxic drugs with upregulation of *CD95*, whereby the proapoptotic effects of CD95 agonists are enhanced (59,60). Moreover, it has been proposed that several cell types directly employ the *CD95* system to commit suicide in response to anticancer agents. Thus, the treatment of neuroblastoma, hepatoma, and lymphoma cells with anticancer agents was reported to result in autocrine or paracrine cell death mediated by direct CD95L–CD95 interactions (60–62). However, numerous cell types, including glioma cells, do not commit CD95-mediated suicide in response to chemotherapy with teniposide or doxorubicin (55,63–65). Hence, the mechanisms underlying the synergy of CD95 agonists and cytotoxic drugs seem to be both drug- and cell-type-specific. To date, no effort to treat human patients with CD95L has been reported, chiefly because of profound safety concerns, which are discussed in Section 3.2 (66).

In the last few years, a refined mechanism of immune evasion used by tumor cells has been revealed: the so-called CD95 counterattack (67). Until recently, the principal immune-evasive strategies employed by malignant glioma cells were thought to be the release of immunosuppressive factors, such as transforming growth factor  $\beta$  and prostaglandin E (68). However, regarding the CD95 counterattack hypothesis, glioma cells were found to be no exception from

tumors in general, in that they can kill attacking immune cells by utilizing the CD95 system. The CD95L expressed on glioma cell surfaces can trigger apoptosis through interaction with CD95 on the cytoplasmic membranes of T-cells (34,38,39).

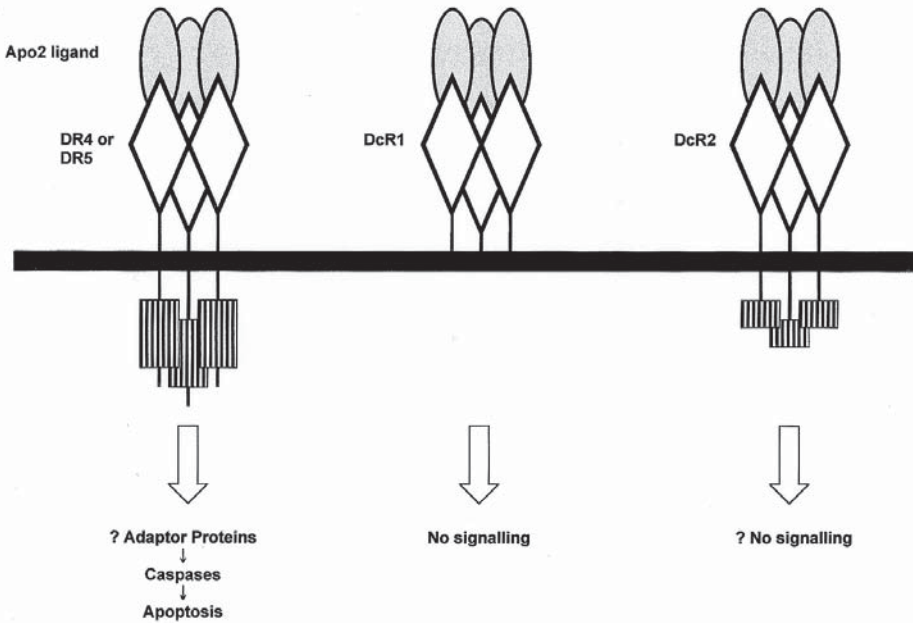
## 2.2. *Apo2L and Its Receptors*

### 2.2.1. BIOLOGICAL PROPERTIES OF APO2L SIGNALING

Another apoptosis-inducing system with a potential role in malignant gliomas has recently emerged, which seems to be different from the CD95 system, in that the ligand, Apo2L, binds to at least four distinct receptors. Apo2L, a type II protein, triggers apoptotic cell death when binding to one of the agonistic receptors, DR4 or DR5 (69–75). In contrast, cell death is inhibited by binding of the so-called decoy receptors, DcR1 (73,75–77) and DcR2 (78–80).

The biological function of the Apo2L system has not been clarified. Apo2L, like CD95L, induces caspase-dependent apoptosis. However, it has also been reported that nuclear factor- $\kappa$ B, which is a transcription factor with mitogenic signaling properties, can be activated by Apo2L in some cell types (75). Therefore, other physiological functions of Apo2L, besides merely induction of apoptosis, may be pondered. Unlike CD95L, Apo2L is expressed in many tissues, and its expression pattern to a great extent coincides with the expression of the proapoptotic receptors, DR4 and DR5 (69). Since, under physiological circumstances, co-expression of ligand and receptor does not lead to apoptotic cell death, powerful antiapoptotic mechanisms must be in existence. On the one hand, it is believed that antagonistic decoy receptors, among them DcR1 and DcR2, protect DR4- and DR5-positive cells from Apo2L-induced apoptosis. Thus, the balance of proapoptotic and antiapoptotic receptors could be decisive for the susceptibility of cells to Apo2L. On the other hand, a role for intracellular antiapoptotic proteins can be anticipated, whereby Apo2L-triggered cell death is blocked in tissue that is abundant in the agonistic receptors, DR4 and DR5. Further investigations will have to unravel the meaning and importance of these remarkable expression patterns, and the precise mechanisms by which potentially susceptible cells are protected from apoptosis.

Forced expression of the death receptors, DR4 and DR5, both of which belong to the class of type I membrane proteins, results in the induction of apoptotic cell death (72,73,75). DR4 (= TNF-related apoptosis-inducing ligand [TRAIL]-R1), as well as DR5 (= TRAIL-R2), harbor a death domain (Fig. 2; 72,73). The two decoy receptors, DcR1 (= TRAIL-R3) and DcR2 (= TRAIL-R4), antagonize Apo2L-induced apoptosis by binding the ligand without transmitting the death signal (82). DcR1 is a membrane-bound protein similar to DR4, but lacks a cytoplasmic tail and thus a signaling domain. Forced ectopic expression of DcR1 renders cells resistant to Apo2L-induced cell death



**Fig. 2.** Apo2L-triggered signaling. Induction of apoptosis by DR4 and DR5 and modulation of signaling by the decoy receptors, DcR1 and DcR2.

(73,75,83). DcR2 is similar to DcR1, but harbors a truncated cytoplasmic death domain (78,79). Because no proapoptotic signaling is possible through this truncated sequence, DcR2 is also acting as an antagonistic receptor. Apo2L-mediated apoptosis depends on the activity of caspases, because several caspase inhibitors can abrogate DR-initiated cell death signaling (71,72,75). Apo2L, like CD95L, can evoke activation of caspase-8; however, it has been suggested that caspase-10 may also be involved in DR4/DR5-mediated cell death (72,73,84) and caspase-3 is activated by Apo2L-induced signaling (84). One unanswered question is how the death signal initiated by ligand–receptor binding is transmitted to the caspases. It is still unclear whether Apo2L signaling is dependent on the adapter protein, FADD, or not. Some authors (74,85,86) have reported inhibitory effects on the induction of apoptosis by dominant-negative FADD; other authors (71,72,87) have obtained results suggesting that Apo2L-triggered cell death is completely independent of FADD. Moreover, there are similar contradictory findings regarding whether other adapter proteins, such as TRADD, RIP, or TNF receptor-associated factor (TRAF2), can bind to DR4 and DR5.

### 2.2.2. APO2L SYSTEM AND MALIGNANT GLIOMAS

Considering that Apo2L and DR4/DR5 are co-expressed in many tissues, the existence of efficacious apoptosis-protective mechanisms has been postulated.



These may be comprised of decoy receptors and, perhaps more vital, intracellular modifiers of apoptosis. The importance of the latter is supported by the finding that the expression of agonistic vs antagonistic receptors often does not correlate with susceptibility to Apo2L-induced cell death (79,84,88,89). It is an intriguing finding that many tumor cell lines are susceptible to Apo2L-mediated apoptosis, but untransformed cells are usually resistant (69,70). *DcR1* has been shown to be abundantly expressed in normal tissue, but only to a limited extent on malignant cells (73). Therefore, an Apo2L-based therapeutic approach could be specific for malignant tissue. Actually, 8/12 investigated malignant glioma cell lines proved to be positive for *DR4*, and 11/12 positive for *DR5*. Compared to this, only 4/12 glioma cell lines have been found to be *DcR1*-positive, and 2/12 *DcR2*-positive (89). In most cases, the glioma cell lines were susceptible to exogenous Apo2L-induced cell death (10/12), irrespective of the coincidentally expressed Apo2L (89,90). However, the expression pattern of the four receptors did not correspond to sensitivity to Apo2L-initiated apoptosis (89). This finding is in agreement with reports about absent correlations obtained with other malignant tumor cell lines (79,84). Thus, Apo2L-receptor interactions represent a complex regulatory system, probably including still unknown agonistic and antagonistic receptors or intracellular factors that control the different pathways of apoptosis.

For possible Apo2L-based therapy for malignant gliomas, it is imperative to consider the expression of ligand and receptors in normal brain tissue. It has been shown by immunohistochemistry that virtually all investigated malignant gliomas (23/23) expressed Apo2L (90), but Apo2L was not found in normal brain parenchyma (69). Given the fact that Apo2L is expressed on most human tissues, the expression pattern in the brain is unique. Moreover, in brain tissue, mRNA of *DR5*, *DcR1*, and *DcR2* could not be found (73,75,78). To the authors' knowledge, expression of *DR4* in the brain has not yet been investigated. There are few reports dealing with therapeutic strategies to utilize the Apo2L system against malignant tumor cells. The majority of tumor cell lines have proven to be susceptible to Apo2L, including glioma cells (89), lymphoma cells (91), melanoma cells (84,92), and myeloma cells (93).

Recently, for the first time, the powerful antitumoral effects of Apo2L have been demonstrated *in vivo*. The authors showed that the systemic administration of human Apo2L can suppress the growth of human mammary, colon, and lung adenocarcinoma xenografts in mice (100,101). Importantly, no systemic toxicity was observed. Moreover, we have recently observed that the local administration of soluble Apo2L exerts prominent antitumoral activity against intracranially growing human glioma xenografts in athymic mice (102). Two intratumoral injections of Apo2L resulted in long-term survival of treated mice and in the eradication of the glioma xenografts. Importantly, we did not detect any Apo2L-mediated local toxicity in the brain of treated mice.

### 3. CLINICAL CONSIDERATIONS

#### 3.1. Resistance

A major obstacle to the established chemotherapy for malignant gliomas is the resistance of glioma cells to many cytotoxic agents. The most important advantage of a DR-based therapy would be the direct activation of caspases, leading to rapid and definite activation of the tumor cell's death program. The strategy of employing the intrinsic apoptotic program of tumor cells could overcome many of the known resistance mechanisms. Mutations of tumor suppressor genes, such as *p53* or *Rb*, frequently confer the resistance of malignant gliomas to cytotoxic treatments. However, DR-triggered apoptosis could circumvent these antiapoptotic mechanisms, resulting in enhanced anti-tumor activity. Another impediment to several treatment strategies is the local, as well as systemic, immune suppression caused by the production of transforming growth factor  $\beta$  or similar cytokines by malignant gliomas. These mechanisms may hamper active cellular immunotherapy approaches, but fail to inhibit the apoptotic cascade, once activated by death ligands.

However, there are also some obstacles for a CD95L- or Apo2L-based therapy for malignant gliomas. The complete absence of CD95 or DR4/DR5 on the surface of tumor cells would result in resistance to proapoptotic ligands. Nevertheless, it has been shown that a *CD95* gene transfer in *CD95*-negative, apoptosis-resistant glioma cells can render them susceptible to CD95 agonists, suggesting intact signaling pathways in many tumor cells, even when the receptors are missing (21). Moreover, a low level of DRs could be increased by certain cytokines, as the expression of *CD95* was demonstrated to be increased after pretreatment with TNF- $\alpha$  and interferon  $\gamma$  (IFN- $\gamma$ ) (32). However, the expression level of DRs on tumor cells may not be most decisive for the efficacy of such a therapeutic approach. Because several glioma cell lines are susceptible to death ligand-induced apoptosis only if protein synthesis is simultaneously inhibited, the existence of cytoprotective proteins must be proposed. Therefore, a successful DR-based therapy may also have to neutralize the intracellular antiapoptotic factors. This could be accomplished by combined treatment with drugs that either affect RNA or protein synthesis in general, or interact with known inhibitors of CD95L- or Apo2L-induced apoptosis specifically. Further investigations will have to identify and evaluate the eligible candidates for this kind of combination therapy.

Some of the substances found to act synergistically together with CD95L (40) may exert their potentiating effects by blocking antiapoptotic proteins. The inactivation of *Bcl-2* by taxol was found to be associated with synergistic induction of apoptosis by *CD95L* and taxol (54). The topoisomerase I inhibitor, topotecan, sensitizes glioma cells to CD95-mediated apoptosis, by inhibiting RNA synthesis (56). Immune response modifiers can also enhance DR-mediated

cell death, as shown with IFN- $\gamma$ , IFN- $\alpha$ , and TNF- $\alpha$  (32,94). In vivo confirmation of the synergy of death ligands and common cytostatic agents would permit the application of lower doses of cytotoxic agents without loss of anticancer effectiveness. Moreover, it is conceivable that cells that are resistant to one death ligand may be sensitive to another ligand. Melanoma and myeloma cells have been shown to be resistant to CD95-mediated apoptosis, but susceptible to Apo2L-induced cell death (92,93).

Because it has been demonstrated that dexamethasone inhibits both CD95-mediated and cytotoxic drug-induced glioma cell death (95,96), the frequent use of high steroid doses to manage brain edema may be a potential problem for chemotherapies, and especially for death ligand-based therapies. Thus, it would be appropriate to avoid a simultaneous application of steroids and proapoptotic agents, as well as to reduce steroid doses to the absolutely essential minimum.

### 3.2. Safety

After Ogasawara et al. (66) had reported that intravenously injected CD95 Ab caused fatal liver toxicity in mice within hours, CD95L has been considered too toxic for any therapeutic application. However, since then, many reports have been published, which suggest that locally administered CD95L is a powerful agent to achieve various desired effects without causing systemic toxicity. CD95 agonists were tolerated in several animal models without unwanted side effects. Soluble CD95L was applied intraperitoneally to treat lymphoma (48), agonistic CD95 Abs and CD95L-producing adenoviruses have been injected intra-articularly to treat arthritis (97,98), and CD95L was targeted to subcutaneously growing renal and colon tumor xenografts by adenoviral gene transfer (49).

Regarding a potential intracranial application of CD95L into the brain, it is important to consider that CD95 is not expressed in healthy brain parenchyma, with the exception of endothelial cells, which have proven to be resistant to CD95 agonists (99). Thus, the expression pattern of DRs and death ligands, as well as the separated nature of the central nervous system compartment, suggest that a locally applied proapoptotic ligand may be well-tolerated by the healthy surrounding brain tissue and by the organism as a whole. Preliminary results show neither systemic toxicity nor damage to normal brain tissue after intracranial injection of CD95L (Roth and Weller, unpublished results). However, given the possibility that death ligands, despite all precaution, may enter the systemic circulation, CD95L scavengers, such as soluble CD95, could be used to neutralize the ligands.

In particular, Apo2L could become a powerful therapeutic tool against malignant gliomas for two reasons: First, Apo2L may have the ability to selectively kill tumor cells in vivo (100,101). Second, Apo2L holds promise to be a well-tolerated drug without systemic toxicity. Even high doses of intravenously

applied Apo2L were well tolerated by nonhuman primates (101). Importantly, local administration of soluble Apo2L exerts prominent antitumoral activity against intracranially growing human glioma xenografts in athymic mice without causing neurotoxicity (102). Future studies will show which mode of administration of Apo2L and which combination with other anticancer agents may turn out to be most promising for an effective treatment regimen for human malignant glioma.

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## Combining Radiation Therapy with Immunotherapy for Treatment of Brain Tumors

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*William H. McBride, DSc*

### *CONTENTS*

INTRODUCTION  
RADIOIMMUNOTHERAPY  
RT COMBINED WITH CELLULAR IT  
REFERENCES

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### 1. INTRODUCTION

Radiation therapy (RT) is a common component of the treatment of most intracerebral malignancies. In gliomas, it is used mostly to achieve local/regional control. In most cases, this is still a major challenge. Despite improvements in surgery, RT, and chemotherapy, patients often succumb to persistent local tumor growth or recurrence. Improvements in local control and survival rates may come from new technical advances in the delivery of RT (1–3). Also, as knowledge increases about the signal transduction pathways that determine the intrinsic radio-resistance of cancer, gene therapy approaches are being developed that should increase the probability of local control. However, rational appraisal of the impact of these, and other, newly introduced treatment options suggests that, although the patterns of failure may change, any improvement in survival is likely to be limited to selected patients and to be modest.

The rapid growth and/or significant size of many gliomas at the time of detection suggest that surgery and RT are likely to remain front-line therapies for some time to come. However, these approaches do not address the problem of dealing with cancers that spread diffusely outside the radiation field. Most patients with glioblastoma multiforme (GBM) fail within 2 cm of the irradiated area (1). New strategies are needed to deal with diffuse disease. These will prob-

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ably need to be combined with established therapies. Adjuvant chemotherapy has shown only very limited promise of efficacy against diffuse disease, and only in certain tumor types (4). Immunotherapy (IT) is conceptually appealing, because its potential specificity may cause specific cancer killing with minimal normal tissue damage, and because of its seek-and-destroy capabilities. It may be able to deal with diffuse cancer deposits, but it is still not clear what IT approach will prove best or how best to integrate IT into treatment plans. This chapter discusses radioimmunotherapy (RIT) and cellular IT combined with RT in the treatment of brain tumors (BTs).

## 2. RADIOIMMUNOTHERAPY

Most studies that have attempted to combine IT with RT have used radiolabeled monoclonal antibodies (MAbs). For solid tumors, in general, the therapeutic efficacy of this strategy has shown only limited success in selected patients, but various novel approaches are being explored that may improve the outcome.

RIT approaches for BTs have attached  $\beta$ -emitters, such as Iodine-125 ( $^{125}\text{I}$ ),  $^{131}\text{I}$ , or ytterium-90 ( $^{90}\text{Y}$ ) to MAbs. These deliver sparsely ionizing radiation that is relatively inefficient at killing cells.  $^{125}\text{I}$  also emits Auger electrons that have a higher cytotoxic efficiency; however because these have a short path length,  $^{125}\text{I}$  molecules must be internalized into the nucleus before they are effective. Isotopes that emit densely ionizing  $\alpha$  particles, such as astatine-211 ( $^{211}\text{At}$ ), have a higher relative biological effectiveness (5), but are difficult to produce and have a short path length that limits their killing effect to, at best, a radius of a few adjacent cells.

### 2.1. Radiobiological Considerations

Unlike external beam irradiation, RIT irradiation is delivered at a low-dose rate. The dose rate decreases further with time, because of physical decay of the radionuclide and biological clearance of labeled material, limiting the dose that is delivered. The general radiobiological considerations for external beam irradiation delivered at low-dose rate also apply to low-dose-rate RIT with  $\beta$ -emitters. To optimize the therapeutic efficacy of RIT, the Ab–radioisotope combinations should deliver the highest tumor dose with the lowest normal tissue dose. Low-dose rate allows repair of sublethal DNA damage at the same time that killing is taking place. This decreases overall lethality. However, it preferentially spares normal brain tissue (NBT), which has a greater capacity than tumor cells to repair DNA damage, resulting in a therapeutic benefit.

Comparison of external beam fractionated irradiation with RIT, in a range of syngeneic murine tumors and human tumors grown in immune-deficient mice, generally support these conclusions (6,7). Thus, treatment of human glioma xenografts with  $^{90}\text{Y}$ -radiolabeled Abs required administration of an approximately

threefold greater total dose, compared to high-dose-rate external beam treatments to achieve the same tumor regrowth delay (8). This was attributed mostly to the dose-rate effect. RIT resulted in a comparatively diminished tumor bed effect, as would be expected if RIT spares normal tissue surrounding the tumor. The sparing effect of low-dose-rate radiation would not extend to rapidly proliferating bone marrow progenitors, and systemic toxicity can be more dose-limiting than normal brain toxicity.

Because RIT delivers radiation at low-dose rate, it:

- will be more effective against tumors with poor DNA repair capacity.
- will be less effective against tumors that are proliferating so rapidly that the rate of cell growth outstrips the rate of cell kill.
- will be influenced by the presence of hypoxic cells to a lesser extent than high-dose-rate radiation. Hypoxic cells are well represented in many glial tumors and are relatively radioresistant (9–12).
- may cause tumor cells to accumulate preferentially in the radiosensitive G2 phase of the cell cycle.

Radiobiological modeling of the parameters that dictate cellular responses to low-dose-rate radiation (W. H. McBride and R. J. Suwinski, unpublished) suggest that, in most cases, the major factors determining outcome are the potential doubling time of the tumor and intrinsic cellular radioresistance.

## ***2.2. Biophysical Considerations***

A number of physical issues associated with the delivery of RIT limit the intratumoral dose and may outweigh the radiobiological considerations. They include:

- Pharmacokinetics of the administered Ab.
- Slow rate of accumulation of immunoglobulin when delivered by the vascular route.
- Short retention time in the tumor. Along with slow accumulation rate, this reduces the maximum dose and dose rate that can be achieved.
- Dose inhomogeneity. This will be dependent upon vascularity of the tumor, the extent of interstitial penetration of the Ab, and possibly the host cell content.
- Immunogenicity of the Ab. “Humanizing” the MAb can decrease this. The carboxy-terminal murine sequences can be replaced with human sequences, using recombinant DNA technology. Alternatively, the Ab can be made in mice genetically manipulated to produce human Abs.

Of these issues, heterogeneity in dose distribution resulting from the poor penetration of solid tumors by immunoglobulin, and a tendency of Abs to bind to intratumoral phagocytic cells through their Fc receptors, may be the most problematic, especially in the brain. This, in part, impacts the choice of radio-

nuclide. Longer-range  $\beta$  particles improve dose homogeneity, to an extent, although they may deposit more radiation in surrounding normal tissue. A number of approaches have been used to address the issue of poor Ab penetration of solid tumors. Fab' and Fab<sub>2</sub>' Ab fragments penetrate and distribute in tumors faster and more homogeneously than whole immunoglobulin. They are also more rapidly cleared from the circulation, minimizing systemic toxicity. However, because of the altered clearance rates, the intratumoral dose that can be achieved is generally decreased (13). One possible solution is to use Ab pretargeting. The Ab with an attached high-affinity receptor, such as streptavidin, is allowed to target and accumulate at the tumor site. Residual circulating Ab conjugate is cleared, and finally the radionuclide is delivered, attached to a small molecule, such as radiolabeled biotin (14,15). Penetration of glial tumors by Abs may also be enhanced by the use of drugs to open the blood–brain barrier (BBB). External beam irradiation can have a similar effect (16), although it may be less consistent and reliable in the central nervous system (17).

Direct instillation of radiolabeled Ab into surgically created resection cavities in BT patients has been explored in clinical trials as a means of circumventing the problem of penetration through the vascular route (18–24). In a recent 6-yr update, Riva et al. reported (25) the results of 105 patients with malignant gliomas injected with multiple courses of <sup>131</sup>I-labeled antitenascin MAbs by means of an indwelling catheter. Tumor burden was minimized by surgery, RT, and, in recurrent lesions, by a second operation. For 62 evaluable patients with high-grade malignant gliomas (58 glioblastomas and four anaplastic astrocytomas), 50% of whom had recurrent lesions, the treatment yielded a significant extension of median survival (23 mo) and of disease-free time to relapse (12 mo). There were nine partial responses, three complete responses, and 20 with no evidence of disease. The most important factor in obtaining beneficial outcomes was limited extension of the neoplasm at the time of therapy.

### 2.3. Targets for RIT of Gliomas

Several glioma-associated molecules have been identified by MAbs as potentially useful targets for RIT in preclinical and clinical trials, including the epidermal growth factor receptor variant (EGFRvIII) (26,27), neural cell adhesion molecule NCAM, chondroitin sulfate proteoglycan (27), tenascin (18,19), and gangliosides (28,29). Ideally, a target for RIT should be well represented on the cell surface, and not shed in a form that will block Ab access to the cell surface. It is best if the radiolabel is internalized into the nucleus. This is particularly important for <sup>125</sup>I-based RIT.

As mentioned in Section 2.1., the most important radiobiological parameters contributing to the failure to control tumor with RIT are the potential doubling time of the tumor and its intrinsic radiosensitivity. Therefore, MAbs that slow

tumor proliferation and/or cause radio-sensitization would have an advantage. Interference with certain signal transduction pathways has been shown to cause radio-sensitization (30,31), and there is some evidence that monoclonals to EGFR have this ability. Of the large number of oncogenes that have been identified as aberrant in gliomas, only the *erbB* oncogene (encoding EGFR) is amplified in an appreciable number (32). This, as well as overexpression of platelet-derived growth factor ligand and/or receptor, appears to give glioma cells a selective growth advantage. Aberrant expression of EGFR has been shown to correlate with a poor response to RT in patients with cancers of the uterine cervix (33) and head and neck (34,35). Radiation causes phosphorylation of EGFR on EGFR-overexpressing A431 cells, and can induce cellular proliferation through this pathway (36). On the other hand, Ab to EGFR, but not EGF itself, slows proliferation of A431 cells and radiosensitizes them, with resulting apoptosis (37). Preliminary data from patients with head and neck cancers receiving anti-EGFR, in combination with external beam radiation therapy are encouraging, and phase II clinical trials are underway.

The mechanism by which anti-EGFR MAbs radio-sensitize tumors has yet to be fully elucidated, but some intriguing suggestions have been made. Anti-EGFR, and not EGFR ligands, has been reported to trigger a specific early physical interaction between EGFR and DNA protein kinase (DNA-PKcs) or Ku70/80, both in vivo and in vitro (38). These molecules are responsible for repair of DNA double-stranded breaks following irradiation. The suggestion is that interference in EGFR signaling by the MAb impairs the DNA repair process. Apoptosis and DNA damage repair has recently (39) been linked by the finding that DNA-PKcs degradation can occur through activation of caspase-3, which is involved in apoptosis. The efficacy of RIT may therefore be enhanced by the use of MAbs that affect signal transduction pathways determining cell life or death after irradiation.

A phase II clinical trial of RIT with <sup>125</sup>I-labeled anti-EGFR has been reported in 25 patients with primary malignant astrocytomas. RIT was given intravenously or intra-arterially after surgical resection and definitive RT. Total cumulative doses ranged from 40 to 224 mCi. No significant life-threatening toxicities were observed. At 1 yr, 60% of the patients were still alive (40,41).

### 3. RT COMBINED WITH CELLULAR IT

The involvement of specific T-cells in autoimmune demyelinating disease has been known for some time, but only recently has it been recognized that the brain is not excluded from the effects of IT for cancer. Several studies have shown that immune T-cells can enter brain tissue and cause tumor rejection (42–46). These results, and new developments in anticancer vaccines, have reawakened interest in active specific IT and passive transfer of activated immune cells as therapeutic modalities against BTs.



Cellular IT for cancer is generally considered independent of surgery or RT. The various strategies that have been employed are reviewed elsewhere in this book. This section discusses only the advantages of a therapeutic approach that integrates cellular IT with frontline RT and some of the factors that may be important in developing such a strategy.

The recent dramatic increase in knowledge of the molecular requirements for the generation of tumor-specific rejection responses, and the ability to genetically manufacture more effective vaccines, are providing powerful, new, better-defined tools for the cellular IT of brain cancer. On the other hand, clinical responses are still relatively infrequent and inconsistent. It is still not clear how best to generate tumor-specific cytotoxic T-lymphocytes (CTLs) that can traffic to sites of tumor growth in sufficient numbers to cause rejection. The problem is aggravated if the tumor is rapidly proliferating. Such tumors can generate hundreds of millions of cells each day. Since the frequency of tumor-specific CTL in the body is unlikely to rise above  $1/10^4$ , and the number within a tumor is unlikely to exceed a million, each tumor-specific T-lymphocyte would need to kill hundreds of tumor cells a day to cause regression. Furthermore, this must be achieved in a microenvironment that is antagonistic to lymphocyte survival and function.

### ***3.1. Rationale for Combining Cellular IT with RT***

There are a number of factors that might diminish the effectiveness of cellular IT against BTs in addition to the limited number of CTLs that can be generated against tumor-associated antigens (TAA). Heterogeneity in the expression of TAA and their ability to be modulated may enable tumor cells to escape the immune response (IR). The tumor microenvironment is hostile to expression of lymphocyte function, both by virtue of being hypoxic and nutrient-deprived, and because BTs produce immunosuppressive factors, such as transforming growth factor  $\beta$  (TGF- $\beta$ ), prostaglandins, and interleukin-10 (IL-10) (47–50). IL-10 is produced by both microglia and astroglia and has been reported to increase glial cell proliferation and motility (51). Expression levels increase with the malignancy of gliomas. Defects in T-cell receptor signaling in lymphocytes from patients with gliomas have been reported (52), as well as a type-2 cytokine profile that is not generally considered as beneficial for the development of cellular immunity (53). The potential importance of immunosuppressive factors in the response to IT is suggested by the finding that antisense to TGF- $\beta$  had some efficacy in a rat glioma model (54).

Combining cellular IT with EBRT or brachytherapy would seem to offer a number of possible advantages:

1. Debulking of the tumor would reduce the burden to be eliminated by IT, as well as reducing the likelihood that antigen (Ag) loss variants will be selected.
2. Local RTs generally leave systemic IRs intact.

3. Irradiation should liberate considerable amounts of tumor Ags that could generate a tumor-specific IR.
4. The aftermath of RT may allow better access of immune effector cells and molecules into the tumor site. The BBB is temporarily affected (16), intratumoral interstitial pressure may drop, and adhesion molecules required for exit of immune cells from the circulation may be upregulated (55–57).
5. Reoxygenation after RT would promote a better environment for lymphocyte survival and function.
6. Radiation can generate a proinflammatory environment (55), which may counteract the immunosuppressive effects of factors such as TGF- $\beta$  (58).

Combining IT with RT has been attempted before for BTs (59,60). In clinical trials, RT has been combined with loco-regional administration of  $\alpha$ -interferon (IFN- $\alpha$ ) and IL-2, alone or in combination with lymphokine-activated (LAK) killer cells (61–64). Success has been modest. True integration of these modalities has yet to be achieved, and further studies are required before the potential advantages of the combination therapy can be properly exploited. Particular consideration must be given to the mechanisms of radiation-induced tumor cell death, the form of RT, the relative timing of the procedures, and toxicity to normal tissues, including the immune system.

### ***3.2. Radiation-induced Cell Death and Immunity***

Radiation kills cells by inducing rapid apoptosis, or by causing slow apoptotic or necrotic cell death during mitosis. Recent studies have made it clear that the nature and extent of tumor cell death impact the magnitude of the elicited anti-tumor IR. Apoptotic death is thought to present a benign stimulus to the immune system (65). Even under circumstances in which large numbers of cells are undergoing apoptosis, neither local inflammatory reactions nor systemic immunity are generally induced. In contrast, necrotic cell death can generate an inflammatory response. Under such circumstances, the rate of tumor cell death may be important. Thus, tumors expressing an immunogenic viral tumor Ag failed to generate a protective T-cell response if transplanted as small fragments, but did so if given as a single-cell suspension (66). The fragments were dealt with as though they were in an immunologically privileged site. This failure of the immune system to recognize tumor fragments was attributed to failure of Ag to reach the lymph nodes and other immune organs. Failure to respond to tumor may be further accentuated by the development of immunologic anergy. This is more readily induced by small numbers of tumor cells, which release few Ags, than by larger number of cells that are immunogenic (67).

The importance of the rate of tumor cell death, and presumably the concentration of tumor Ags, for the generation of immunity has also been seen in recent gene therapy studies. Although lethally irradiated B16 tumor cells were poorly immunogenic, equivalent tumor cells transfected with herpes simplex

virus-thymidine kinase (*HSV-TK*) and killed *in situ* by gancyclovir were found to elicit strong antitumor immunity (68). Similar results have been obtained using tumor cells transfected with *cytosine deaminase*, which were rapidly killed by administration of 5-fluorocytosine (69). The suggestion is that rapid and massive release of tumor cell debris may enhance the uptake and presentation of tumor Ags by tumor-associated antigen-presenting cells (APCs) (70–72). In these terms, ionizing radiation can be considered a silent killer. It kills tumor cells by apoptosis or slow mitotic death, which are processes that induce little immune recognition. Although it induces a proinflammatory cytokine profile (55), conventional RT is not, on its own, a powerful inflammatory stimulus.

A corollary is that increasing the rate or the manner of tumor cell death after irradiation may be beneficial in terms of the generation of immunity. This may be achieved by an appropriate choice of radiation treatment, or by addition of another adjuvant therapy, such as hyperthermia or photodynamic therapy, which induces more rapid cell death. Gene therapy approaches that increase tumor radiosensitivity are another alternative (30). For example, *HSV-TK* plus gancyclovir and *cytosine deaminase* plus 5-fluorocytosine, both of which are cytotoxic and increase tumor immunogenicity (68), also sensitize tumors to the effects of irradiation (73–77). One might expect irradiation to release even more immunogenic Ags in combined treatment situations. If the rate of cell death after RT can be increased, one might expect to improve local control, and, perhaps more importantly, translate radiation-induced cell death into systemic immunity that can impact diffuse disease.

### 3.3. Forms of RT for BTs and Their Impact on Immunity

RT for the treatment of BTs has been administered in several different ways. There is little evidence as to how the form of RT affects antitumor immunity, but some consideration should be paid to its potential impact on combination with cellular IT. The conventional fractionated RT regimen is delivered by external beam and aims to deliver doses of 50–60 Gy in 25–30 fractions over 5–6 wk. History has shown that individual fraction sizes in excess of 5 Gy are associated with a risk of increased toxicity. In spite of this, radiation is also given stereotactically to BTs as a single dose of up to 30 Gy, or in a small number of fractions (1–3). This is achieved by limiting the field size. There is an inverse relationship between the volume of the brain that is irradiated and the dose that can be tolerated.

Interstitial implantation of radioactive sources (brachytherapy) is another option. Permanent and temporary, low- and high-activity implants have been used (78–80). Again, the treatment volume for brachytherapy is normally limited to 5–6 mm lesions in one hemisphere. Intensive focal RT, using brachytherapy or stereotactic delivery, has been shown to offer an improvement in median sur-

vival in selected patients. These are those with small volume disease or disease in a site that can tolerate radiation necrosis.

Most recently, three-dimensional inverse treatment planning and mini-multileaf collimators have been developed for the treatment of BTs. These allow a substantial increase in dose to sites of greatest tumor density, while delivering more precise and limited doses to sites containing more normal tissue (81–83). These advances promise to improve local control and alter the patterns of failure after RT, but failure outside the field because of diffuse disease will remain a problem.

These different forms of RT utilize different dose rates and doses. They are given over considerably different time periods, from minutes to months. It may be expected that the rate of tumor cell death will be different, as will the effects on the tumor microenvironment, tumor vasculature, and other normal tissues, including intratumoral host cells. Cell death will be most rapid if high-dose-rate, large radiation doses are delivered over a short time period. Such would be the case with high-dose-rate, temporary brachytherapy or EBRT delivered stereotactically.

Delivery of RT over an abbreviated time period will compromise the differential ability between the NBT and tumor to repair DNA damage. This may be obviated to an extent by limiting the amount of normal tissue in the field. On the other hand, it offers several potential advantages when planning adjunctive cellular IT.

First, radiation delivered in a conventional or an extended split-dose schedule can fail because of accelerated tumor cell repopulation, which occurs in tumors probably after a lag period following surgery, chemotherapy, RT, and, most likely, after IT (84). In essence, the initial cell loss leads to an increased rate of tumor proliferation and a larger growth fraction. Therapy administered at this time must deal with a mass that is proliferating much more rapidly than was the primary tumor, and, consequently, the therapy is less effective (85,86). The rate of proliferation of tumor deposits outside the field may also accelerate on removal of the primary tumor (87). The implication is that therapy should be given in the shortest possible time consistent with minimizing normal tissue damage. Radiation delivery that is delivered in a short time period would seem to offer the best opportunity for interfacing with adjunctive IT regimens with minimal delay.

A second potential advantage is that the BBB is affected most by high-dose irradiation with approx 20–30 Gy (16). Penetration of the tumor by immune cells or molecules may take advantage of this in the postirradiation period, before the barrier recovers. Doses higher than the conventional 2 Gy also promote a more proinflammatory environment (55), which may promote immunity. In addition, immune cells within the irradiation field are subject to the cytotoxic effects of radiation. Relative radio-sensitivities in this regard, are:

nonactivated lymphocytes > activated lymphocytes > newly immigrated monocytes > differentiated macrophages and microglia. In combining RT and cellular IT, immigration of immune cells into the tumor site after RT will be needed. Intratumoral lymphocytes decrease rapidly after tumor irradiation, but recover within a week (88). If the time period of irradiation is long, as in conventional RT, infiltrating cells will be repeatedly killed. High-dose radiation given at a high dose rate will kill fewer immune cells overall.

### ***3.4. Enhancing the IR to Ags Released After RT***

Approaches used to generate cellular immunity to BT Ags are dealt with elsewhere in this volume. In general, human BT-associated Ags are not well characterized, but potential targets are present, such as Ags of the MAGE family, tenascin, and EGFR (18,19,26,89,90). Ideally, for cellular IT of cancer, one would want to generate tumor-specific, cell-mediated CD8<sup>+</sup> CTL responses. CTLs recognize and respond to peptide moieties presented in association with major histocompatibility complex class I (MHC-I) determinants on the surface of APCs. Additional cell surface molecules (known collectively as co-stimulatory signals), which are present on professional APCs such as dendritic cells (DCs), are necessary to trigger the activation and proliferation of CTLs. In the absence of co-stimulation, CTLs that engage MHC-I and peptide complexes either die or enter a state of anergy in which they remain unresponsive to subsequent stimulation. Because most tumor cells lack the required co-stimulatory signals, they are normally poor at activating CTL responses.

CTLs can be generated *in vivo* or *ex vivo*. *Ex vivo* expansion of T-cells, followed by adoptive IT, has shown promise in preclinical murine BT models (43,91) and has been tested in a phase I clinical trial (92). Several strategies are being employed to generate *in vivo* antitumor responses. One approach is to use DCs, which are powerful APCs that can be readily cultured from progenitor cells in granulocyte-macrophage colony-stimulating factor (GM-CSF) plus IL-4. In animal models, injecting DCs pulsed with tumor extracts or total tumor RNA (42), with synthetic peptide Ag (44), or with acid-eluted tumor Ags (45), has generated CTL responses that could protect against and treat tumors implanted in the central nervous system. Another approach that has been shown to induce immunity against intracranial tumors uses cytokines or cytokine gene-transduced tumor cells as vaccines (61,93–96). Generally, in such studies, the cytokines are used to induce expansion of either T-cells or APCs, such as DCs. IL-12, which preferentially expands type 1 (Th1) T-cells that are involved in cell-mediated IRs, has been the subject of considerable interest (97–99). GM-CSF and IL-3, which appear to target APCs, have also shown some efficacy (100).

An important issue with respect to vaccination for BTs, which impacts consideration of how best to combine cellular IT with RT, is the route of administration. In one study, Thompson et al. (60) reported on antitumor IRs elicited in

C57Bl/6 mice by melanoma cells genetically engineered to produce either GM-CSF or IL-2. If the *GM-CSF*-transduced cellular vaccine was given as a subcutaneous injection, but not as an intracranial injection, it protected against intracranial tumor challenge. In contrast, direct intracranial injection of tumor cells secreting IL-2 was protective, but flank vaccination was not. Combination therapy, with the GM-CSF-producing vaccine administered subcutaneously and the IL-2 vaccine given in the brain, achieved a synergistic response. If cytokines are to be used to enhance IRs, more information is required about their requirements, in terms of the Ag that is presented, the availability of immune cells, and the type of responses that are generated in different sites.

It is evident from preclinical studies that IRs generated at sites distant to the brain can affect BT growth. However, direct instillation of agents into the brain may be required to provide an appropriate "danger signal" (101) to generate optimal tumor recognition. The precise mechanisms involved in immune recognition of BT cells remain to be determined. However, the process appears to require direct physical contact between the APCs and tumor cells/Ags (102). It appears to be potentiated by the presence of high Ag concentrations or of processed/degraded cell debris (103,104). The same factors are probably rate-limiting for the generation of antitumor CTL responses against BTs following RT.

Few studies have attempted to combine local IT strategies with RT for BTs. However, local intratumoral IL-3 (88) and IL-2 (105) expression has been shown, in non-BT models, to potentiate the response to RT and to enhance the generation of systemic immunity. The alterations in the tumor microenvironment, which occur in these situations, are complex and involve changes in host cell content, tumor hypoxia, and possible neovascularization. Further studies are required to explore the generation of local IRs prior to RT, since this approach in conjunction with systemic vaccination and/or adoptive IT may be most effective at increasing control of both local and diffuse disease. Approaches using APCs may be particularly appealing, because these cells are functionally radioresistant. In the final analysis, these local strategies may be limited by the tolerance of NBT. Cytokines in particular, such as IFN- $\alpha$  (106) or IL-2, may be potent radiosensitizers and could result in severe NBT damage when used in combination with RT.

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