2003

MECHANISMS UNDERLYING PROTECTION AGAINST RT-2 GLIOMAGENESIS IN RAT BRAIN UTILIZING PRIMARY AND SECONDARY VACCINATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Dedication

This dissertation is dedicated to my parents, Genevieve and Richard Lister, Sr., who have given me so much support and encouragement during my years in graduate school. I would also like to dedicate this work to my sister, Susan, and brothers, Richard, David and Brian, who have cheered me on.

Acknowledgements

I would like to acknowledge a number of individuals who played a significant role in making this Ph.D. a reality. First and foremost, I would like to thank my Mother and Father for their tremendous love, patience, support and prayers over these past few years. Without them, this dissertation would not have been achieved. Secondly, I would like to thank my sister and friend, Susan, for her emotional support and a Vermont cabin to escape to. Thanks also go to my brothers, Richard, David, and Brian. My siblings' patience and sense of humor has kept me focused, upbeat and believing in myself. Thanks to my sister-in-law, Karen Taylor, for being there to help me with computer programs and formatting my dissertation. Thanks Karen for your friendship as well.

In addition, thanks go to the faculty members that have been influential in my becoming a Ph.D. candidate:

Dr. Randall Merchant, my advisor, receives a most heart-felt "Thank You" for his commitment, time and effort in getting me through all the hoops required of a Ph.D. candidate. I trust this long-term relationship will continue over the years.

Dr. Caroline Jackson, my mentor, has followed my graduate career from my enrollment at MCV. Her support has been instrumental in my developing a strong interest in research; as well as teaching. As a future mentor, I hope I can live up to her standards of not only her vast teaching skills but understanding and compassion as well.

Dr. Martin Graf, a mentor and friend. As a faculty member in Dr. Merchant's lab, Dr. Graf has provided invaluable assistance to me in the completion of my final experiments required for my dissertation. His insight and assistance has been invaluable. I look forward to having a long-term relationship both as researchers and friends.

My Committee Members: Dr. Kathleen McCoy, Dr. Linda Phillips, Dr. Milton Sholley, Dr. William Broaddus and Dr. Helen Fillmore. I thank them for supporting my research project and providing their time and support in completion of the required experiments and writing of this dissertation.

My friends, Dr. Lindy Hatch and Cricket Call. Lindy, you introduced me to MCV's Department of Anatomy and Neurobiology and faculty while I was at Montgomery College in MD. Thank you for both financial and emotional support.

Finally, I want to give a very special "Thank You" to Dr. Frances M. Christian. She has been a healer and support network for me. I could not have accomplished this goal without her inspiration, insight and invaluable time. She helped me build confidence in myself, that this undertaking was going to happen and be completed. Life has taken on a new outlook-- both exciting and challenging. She has my immense gratitude and love.

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List of Abbreviations

1°	primary
2°	secondary
α	alpha
β	beta
δ	delta
γ	gamma
Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
AINR	activation-induced nonresponsiveness
APC	antigen presenting cell
BBB	blood brain barrier
CD25R	IL-2 receptor
CD62L	L-selectin
CM	complete medium
CNS	central nervous system
CTL	cytotoxic T lymphocytes

DAB	diaminobenzidine
DC	dendritic cell
DMEM	Dulbecco's Modified Eagles Medium
DN	double negative
DP	double positive
eT	effector T cells
FBS	fetal bovine serum
FCS	fetal calf serum
GBM	glioblastoma multiforme
GM-CSF	granulocyte macrophage-colony stimulating factor
H&E	hematoxylin and eosin
HFP(s)	hind footpad(s)
IC	intracranial
IFN-α	interferon alpha
IFN-γ	interferon gamma
IL	interleukin
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-2R	high affinity IL-2 receptor complex

IL-2Rα	interleukin-2 receptor alpha
IL-2R-β	interleukin-2 receptor beta
IL-2R-γ	interleukin-2 receptor gamma
LAK	lymphokine activated killer
LN(s)	lymph node(s)
MFI	mean fluorescence intensity
MHC I	major histocompatibility complex I
MHC II	major histocompatibility complex II
MSC	myeloid suppressor cell
NK	natural killer
NN	nape of the neck
PBS	phosphate buffered saline
PGE_2	prostoglandin E_2
PMN	polymorphological neutrophil
RBC(s)	red blood cells
SP	single positive
ss	sterile saline
TCR	T cell receptor
$TGF\text{-}\beta_2$	transforming growth factor-$beta_2$
Th-1	T helper-1

Th-2	T helper-2
TIL	tumor-infiltrating T lymphocytes
TIMC	tumor-infiltrating mononuclear cells
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor-alpha
TNF-β	tumor necrosis factor-beta
u	micron
ul	microliter
VCAM	vascular cell adhesion molecule-1

Abstract

MECHANISMS UNDERLYING PROTECTION AGAINST RT-2 GLIOMAGENESIS IN RAT BRAIN UTILIZING PRIMARY AND SECONDARY VACCINATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2003

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Primary brain tumor affects some 18,000 adults in the United States each year (Silverberg *et al.*, 1990; Merchant *et al.*, 1997) and over 30% are high-grade anaplastic astrocytoma or glioblastoma multiforme (GBM) (Parney *et al.*, 1997). According to Kruse *et al.*, 1989, the treatment of patients with recurrent or persistent high-grade gliomas represents a major therapeutic challenge. The use of conventional therapy consisting of surgery, followed by radiation therapy and chemotherapy for gliomas, has been relatively ineffective (Jaecle *et al.*, 1994) despite the fact that these therapies are cytoreductive in nature (Black, 1991). Most malignancies will recur locally but may also

reappear at a different site within the brain. A brain tumor, once established, usually continues to outstrip the inhibitory action of any immunobiological defense mechanisms against it. Thus, malignant intracranial (IC) brain tumors represent a lethal neoplastic disease in which treatment has failed to extend the lifespan of afflicted individuals, with a GBM having a median survival rate of less than one year (Harsh *et al.*, 1987).This has prompted a search for other potentially useful methods to better understand the biology of brain tumors as well as better ways to treat them. The studies outlined herein, addressed the mechanisms behind the protection against tumor development provided by the various cells of the innate and cellular immune response in a rat brain tumor model. Investigations consisted of: 1) primary (1^{o}) vaccination involving a phenotypic examination and functional analysis of the cells of the innate immune response and the cells of the adaptive immune response infiltrating an RT-2 glioma and the expression of the CD25 receptor; 2) 1^{o} and secondary (2^{o}) vaccination that involved a follow-up on survival as well as the phenotyping of cells of the leukocytic immune response; 3) rechallenge of long-lived rats, from Experiment 2, in the contralateral (left) hemisphere; and 4) acquisition of memory T lymphocytes from vaccinated rats and the use of lymphocyte depletion studies to determine which cells were necessary to provide a protective vaccination against development of tumor. Thus, this study illustrated a potential therapeutic strategy to develop treatments for GBM patients as well as providing protection against development of tumor by the use of vaccines.

General Introduction

MECHANISMS UNDERLYING PROTECTION AGAINST RT-2 GLIOMAGENESIS IN RAT BRAIN UTILIZING PRIMARY AND SECONDARY VACCINATION

Glioma: The Classical Period

According to Burns (1800); gliomas with their macroscopical resemblance to normal brain tissue were known long before Virchow (Scherer, 1940). The English referred to gliomas as "medullary sarcoma", as "encephaloide" by the French, and as "fungus medullare" by German authors (Scherer, 1940). This period was considered one of macroscopical research. Virchow (1863/1865) created the name "glioma" and opened the period of combined macroscopic and microscopic study of these neoplasms (Scherer, 1940). Not only did he distinguish two main groups of brain tumors, glioma and sarcoma, he characterized gliomas as follows: 1) "gliomas are often enormous tumors of brain-like appearance with slow clinical evolution"; 2) " never showing a clear limitation towards the normal brain tissue, but resembling more a hypertrophy of the involved parts than real neoplasms"; 3) "microscopically they are formed by a proliferation of glia cells and sometimes of glia fibers - in the latter, the tumor is hard and in the former soft" (Scherer, 1940). These tumors correspond to what is now called "glioblastoma". Scherer, 1940 showed that clinical and pathological differences between the two main groups of brain

tumors – which dominate all modern glioma classification – had been perfectly characterized in 1865. For the next fifty years, Virchow's work determined the methods of glioma research. It was Strobe's (1895) work that provided the first really detailed microscopical description of gliomas. According to Scherer (1940), Virchow had described the essential differences of macroscopic behavior and mode of growth between "glioma" and "sarcoma". He points out that not only was Strobe able to confirm these differences, he was also the first to use new "specific" methods for the staining of the histological structures. According to Scherer, (1940), Strobe's description in all essential points were confirmed by Hennberg in 1897 and Storch in 1899.

High-grade oligodendroglioma, anaplastic astrocytoma and glioblastoma multiforme (GBM) affect approximately 18,000 Americans each year with 30% of these tumors being GBM, the most common primary central nervous system (CNS) tumor (Parney *et al.*, 1997). GBM is a rapidly progressive high-grade tumor of the supportive tissues of the brain. The use of conventional therapy consisting primarily of surgery, followed by radiation therapy, and chemotherapy for gliomas has been relatively ineffective (Kruse, 1994 and Merchant, 1997). A therapeutic strategy that would induce an immune response that targets tumor cells specifically may ultimately be a means of achieving their complete elimination. One of the most striking features of malignant glioma is the state of cellular immunosuppression observed in most patients studied over a period of twenty years (Roszman *et al.*,1991).Wellar and Fontana (1995) attributed the

immunodeficient state of glioma patients to several different factors: 1) immune paralysis resulting from the release of immunosuppressive factors secreted by the glioma cells; 2) treatment of glioma patients with immune compromising drugs like steroids and hydantoins; 3) the lack of specific tumor antigens; 4) the failure of glioma cells to present antigen; and 5) a lack of professional antigen presenting cells (APCs) in the brain.

The reported incidence of brain tumor is on the rise, although, early detection by Magnetic Resonance Imaging (MRI) and other advanced clinical diagnostic procedures may account for the apparent increase. Imaging technologies now make it possible to detect very small tumors before they cause clinical symptoms. Worldwide, the incidence of all forms of primary brain tumors ranges from 4.2 to 12.8 per 100,000 (Brewis *et al.*, 1966 and Liebowitz and Atler,1969). Baily (1948) and Zulch and Borck (1965) reported that medulloblastoma, cellular astrocytoma, ependymoma, pineal region tumor, teratoma and craniopharyngioma all showed peak incidence in childhood and young adults (less than 20 years of age). Tumors of older adults frequently involve the cerebral hemispheres and include astrocytoma, oligodendroglioma, meningioma, pituitary adenoma, neurinoma of the cerebellopontine angle and astrocytoma of the cerebellum, with GBM and metastatic tumors most commonly found in people between 50 and 60 years of age (Bruner, 1994).

Malignant gliomas have been able to evade aggressive conventional forms of therapy; such as, surgery, radiation and chemotherapy, despite the increase in the

understanding of the biology of tumors. Most surgically removed malignancies will recur locally, but about 5% of the time they will recur at a different site within the brain. A tumor, once established, usually continues to outstrip the inhibitory action of any immuno-biological defense mechanisms against it.

Reasons for this weak response by the host are: 1) the central immune system of those who develop tumor may be impaired so that an effective immune response can not be mounted against the tumor; 2) changes in the surface proteins of the tumor cells may block the immune response with peripheral sensitization of lymphocytes; 3) secretion of immunosuppressive factors, such as transforming growth factor-β (TGF–β), which decreases the ability of the host's immuno-surveillance system to recognize and attack the tumor; 4) the striking heterogeneity that characterizes GBM, in that there is no known tumor-specific antigen or receptor that is common (Debinski *et al.*, 1999) and 5) extreme dissemination of glioma cells within the brain due to passive perivascular cell placement mediated by the cerebro-spinal fluid (CSF) and an active cell migration (Pedersen *et al.*, 1995).

The significance and completion of this study was that despite the great hope of immunotherapy, cancer cells are masters of deceit and disguise that can readily alter themselves to evade immunologic recognition and attack. Because the race is between immune control and escape, the best strategies to combat cancer will need to attack it on several fronts. Opportunities that have been explored include constructing vaccines that

combine a variety of antigens (polyvalent vaccines); testing how well antibody and vaccine-based approaches work together; and combining nonspecific and specific immunotherapies and other cancer therapies. Many promising opportunities wait to be studied. Perhaps these therapies will yield cures – the universal objective of cancer researchers. Developing therapies that can change the nature of cancer from a progressive disease to one that may be controlled throughout life may be a more achievable and realistic aim.

Adaptive Versus Innate Immune Response

The immune system has evolved into two parts: one that is responsible for an immediately responding, non-specific response called the "innate immune response" and one that depends specifically on the immune challenge and requires plasticity and memory called the "adaptive or acquired immune system" (Becher *et al.*, 2000). Leukocytes are responsible for both adaptive and innate immunity. In 1997, Medzhitov and Janeway made it clear that these two systems are not separate, but are functionally intertwined and the actions of one has a profound effect on the other.

The innate immune system allows recognition of foreign material or matter via germ-line encoded pattern recognition via specific antigen-receptors generated by somatic recombination. Adaptive immune responses depend upon lymphocytes. B and T lymphocytes are the cellular members of the adaptive immune system. These are generated in primary lymphoid tissues- bone marrow and thymus. They populate the

secondary lymphoid structures- lymph nodes and spleen. These lymphocytes provide life-long immunity that can follow exposure to disease or vaccination.

Granulocytes are the first cells that migrate into tissues in response to invading pathogens. Granulocytes are a diverse collection of cells that include eosinophils, mast cells, basophils and polymorphonuclear (PMN) leukocytes- neutrophils. Their principal role in inflammatory and immune responses has long been thought to be the phagocytosis and killing of bacteria, via the generation of reactive oxygen intermediates and the release of lytic enzymes stored in granules (Cassatella, 1995).These cells are referred to as granulocytes because they contain prominent cytoplasmic granules. Non-granular cells of the innate immune response are monocytes and macrophages. Together, the adaptive and innate immune systems provide an effective defense system.

Primary and Secondary Organs:

The cellular members of the adaptive immune system are B and T lymphocytes. These cells are generated in bone marrow and thymus and recirculate in spleen and lymph node. They survey other organs through the blood and lymphatic systems in search of pathogens and/or foreign substances. Since the immune privilege of the CNS is not complete, Wekerle *et al.* (1987), suggested there is a certain degree of ongoing traffic of immune cells and molecules through the blood-brain barrier (BBB), and transport to regional lymph nodes. Due to this immune surveillance, trafficking in the neuro-immune network is initiated in the CNS.

Bone Marrow: Primary Lymphoid Tissue

The bone marrow is soft tissue found within the bones of the skeleton of the body and is the site of production of new blood cells or hematopoiesis. The various cell types it contains are adipocytes, stromal cells, and blood forming cells (hematopoietic tissue). The bone marrow is the location of stem cells of all blood elements, including the precursors of lymphoid cells. These stem cells- multipotent hematolymphomyeloid stem cells and their progeny - are organized into groups within fatty tissue. The various cell lines found in the bone marrow are red blood cells/erythroblasts, macrophages/monoblasts, platelets/megakaryocytes, polymorphonuclear leukocytes/myeloblasts, and lymphocytes/lymphoblasts.

The bone marrow is not the site of reaction with, or response to Ag. Marrow lymphocytes circulate from the marrow to other lymphoid organs and differentiate into lymphocytes capable of immune function. Cells originating in the bone marrow populate the thymus where they may differentiate into T cells, whereas B lymphocytes differentiate in the secondary lymphoid tissue.

Thymus: Primary Lymphoid Organ

Thymic structural heterogeneity derives from its embryological origins – its development requires significant input from all three germ layers. The epithelium is derived from the 3rd and/or 4th pharyngeal pouch endoderm. Phenotypic studies have shown that the first epithelium to develop is cortical, followed by small medullary islets

which expand in synchrony with the appearance of mature T cells, and subsequently the capsule toward the end of embryogenesis or even neonatal period (Boyd *et al.*, 1993).

The thymus is encapsulated by a thick outer layer of connective tissue and a thin connective tissue layer directly overlying the cortex. The subcapsular sinus separates these layers. Fibroblasts and type 1 collagen-rich trabeculae penetrate the cortex and terminate at the cortico-medullary junction (Boyd *et al.*, 1993). These trabeculae lobulate the thymus and provide a structural link with the inner medulla. Trabeculae contain fibroblasts, adipocytes, eosinophils, neutrophils, plasma cells, mast cells, macrophages and granulocytes and are well vascularized and innervated. Lining the subcapsule and trabeculae is a basement membrane supporting a flattened epithelium. This creates a barrier between the external and internal thymic environments. Boyd (1993) describes the internal base of the trabeculae as having perivasculature spaces which constitute the blood-thymus barrier.

The subcapsular/subtrabecular region is one of the most important in the thymus. It initiates thymopoiesis and produces thymic hormones (Kendall, 1991). Determinates specific for this region have been identified and the region labeled by Thy.1 in humans (Boyd, 1993).

The thymic cortex can be differentiated from the medulla because the lymphocytes are much more closely packed in the cortex. There are no afferent lymphatics in the thymus. The cortex is an area of active proliferation, with turnover of

cells every 3 or 4 days (Miller, 1993). According to Boyd (1993) and Miller (1993), 1% of the lymphocytes produced leave the thymus via blood vessels; the other 99% are destroyed locally via apoptosis.

In the cortex, many thymocytes are located within vacuolar membranes of large epithelial cells known as thymic nurse cells. These lymphoepithelial cell complexes provide a close association between intact, actively dividing thymocytes and large cytoplasmic vacuoles of the epithelial nurse cells. It is within the thymic nurse cells, which are epithelial reticular cells, that thymocytes may learn to identify self from nonself. Prothymocytes arriving from the bone marrow develop receptors for major histocompatibility complex (MHC) markers. At the time of positive selection, the prothymocyte expresses both the CD4 and CD8 co-receptor molecules. At the end of the selection process, mature thymocytes ready for export to the periphery express only one of these two receptors. CD8 thymocytes have receptor for Class I MHC molecules. T cells that express CD8 are programmed to become cytotoxic effector cells. CD4 thymocytes have receptors that recognize peptides bound to self MHC Class II molecules. $CD4^+$ T cells are programmed to become cytokine-secreting cells. Thus, positive selection also determines the cell-surface phenotype and functional potential of the mature T cell, selecting the co-receptor that it requires for efficient Ag recognition and programming it for appropriate functional differentiation upon activation of the immune response.

High affinity self-reactive thymocytes, that might give rise to autoimmune disease, are removed by negative selection at the junction of the medulla after reaction with thymic dendritic cells which are rich in MHC molecules. The surviving cells are then ready for release into the circulation and for further maturation in the peripheral lymphoid organs.

The medullary epithelium, represented by Hassal's corpusles, express different phenotypes as differentiation from medullary epitheloid reticular cells to endocrine epithelium where mature Hassal's corpuscles occur. Thymocytes also show differentiation-related phenotypic changes from cortex to medulla as defined by phenotypic markers identified by monoclonal antibodies. The subcapsular cortex contains the least mature thymocytes. In the deep cortex more differentiated thymocytes are seen. Maturation to even more mature thymocytes occurs in the medulla. Further differentiation occurs after the cells leave the thymus and reach peripheral lymphoid organs.

Lymph Nodes: Secondary Lymphoid Organs

Lymph nodes (LNs) are small organs occurring in series along the course of lymphatic vessels. Their parenchyma consists of a highly organized accumulation of lymphoid tissue. Cells of this tissue recognize antigenic materials in the lymph that percolates through the node. This induces a specific immune response. LNs are rich in macrophages, which clear the lymph of undesirable cells, invading microorganisms, and

other particulate matter.

LNs are located in areas of lymphatic drainage in the body and serve as filters for tissue fluid in lymphatic vessels. Classically, the organ is divided into the inner zone, the medulla, surrounded by the outer zone, the cortex. In the classic concept of the LN architecture, the LN comprises a superficial cortex containing lymphoid follicles alternating with interfollicular regions, a deep cortex or paracortex and a medulla (van den Cord *et al.*,1985). There are three kinds of spaces in each of these regions: 1) an intralymphatic space; 2) an intravascular space; 3) and an extravascular space or interstitium. Both the vascular endothelium and lymphatic endothelium are specialized in different regions of the node (Fossum and Ford, 1985).

They point out that the intercalation of LNs in the lymph stream serves a dual purpose in the defense of the body. First, phagocytes residing within the LNs ingest invading organisms arriving via the lymph and thus reduce the load of organisms reaching the blood stream. Secondly, according to Gowans and Knight (1964), LNs are ideally located as control posts for the immune system. This is because lymph draining wide areas is funnelled through their narrow confines. This allows the surveillance function of the immune system to be concentrated in small LN volumes. This results in patrolling lymphocytes continuously entering LNs from the blood stream by crossing the wall of the specialized postcappillary venules (Gowans and Knight, 1964), to leave again within a few hours in efferent lymph if they fail to meet an Ag they recognize. Studies by

Herman *et al.*, (1972), Cahill *et al.* (1976), Hay and Hobbs (1977), and Drayson *et al.*, (1981) found that after local antigenic stimulation, specific lymphocytes are concentrated in the regional LNs. This is due to a combination of an enhanced rate of lymphocyte traffic into the nodes associated with increased blood flow and selective retention of Ag-specific lymphocytes within the nodes. The recruitment of specific lymphocytes from a large recirculating pool serves to amplify the immune response. The chances of a successful encounter between Ag and specific lymphocytes are, according to Unanue *et al.* (1984), further increased by the strategic position of APCs in the middle of lymphocyte traffic areas within LNs.

APCs function primarily by displaying Ag in highly immunogenic forms on their surfaces. Ag, according to Mandel *et al.*, (1984), can also be retained for prolonged periods on the surface of follicular dendritic cells within the nodes, whereby repeated stimulation of B lymphocytes occurs in the absence of renewed external challenge. This generates immunological memory in B cells over an extended period of time. In summary, LNs are the sites of confluence of lymph-borne Ag and blood-borne lymphocytes.

Spleen: Secondary Lymphoid Organ

Like the LN, the human spleen is a highly compartmentalized organ. Each compartment has its own structure, cell population, and functions. The lymphoid follicles and the surrounding lymphoid tissue is called white pulp and the sinusoidal area, which

usually contains large numbers of red blood cells, is called the red pulp. A zone of densely packed lymphocytes surrounding the central arteriole contains T cells, while B cells are found surrounding the germinal center. The mantel surrounding germinal centers is composed mainly of B cells, but also T cells believed to be pushed aside from the B-cell zone by formation of the germinal center. In humans, there are two main functions: production of immunological responses against blood-borne Ags and removal of particulate matter and aged or defective blood cells, particularly erythrocytes, from the circulation (van Krieken and Velde, 1988).

Cells of the Amnastic Immune Response:

T Lymphocytes

T lymphocytes are not able to recognize free Ag but rather their recognition of Ags involves an interaction of the T cell receptor (TCR) with fragments of Ag bound to either class I or class II major histocompatibility complex (MHC) molecules on the surface of APC. Cells that are capable of processing Ags and displaying them on their surfaces are referred to as antigen presenting cells (APC). As defined by Fabry and Hart, (1993), all of the events that take place between uptake of Ag and T cell recognition are called "Ag presentation". This Ag presentation is dependent on specific and non-specific signals. Ag-specific T cells are activated through their clonally restricted T cell receptor (TCR) complex. Class I restricted T cells show almost exclusively $CD8^{+}$

cytolytic/suppressor phenotypes, whereas Class II-restricted cells are $CD4^+$ T-helper cells (Fabry *et al.*, 1993). The TCR is a disulfide-linked heterodimer made up of two chains, either α and β chains on the αβ T cells or γ and δ chains on the γδ T cells (Allison *et al.*, 1987; Raulet *et al.*, 1989). The αβ chains function together as Ag receptors on mature αβ T cells. The precisely defined TCR repertoire is formed during thymic development using four different gene segments: the variable (V), diversity (D), joining (J), and constant (C) regions. These gene segments come together in random fashion and are expressed as a single protein molecule containing the α (VαJαCα) and β(VβDβJβCβ) chains (van Ewijk, 1991). Upon leaving the thymic environment, the individual mature cells have specificity. This specificity is determined by the variable regions of the αβ TCR α and β chains which form a combining site that has contact residues for both the MHC-bound peptide and the MHC molecule (Kappler *et al.*, 1987; von Boehmer *et al.*, 1989 and Fowlkes *et al.*, 1989).

Secondary signals are also generated as result of the TCR-Ag-MHC interaction and are transmitted through CD3. CD3 is a multimolecular complex expressed in a noncovalent association with the TCR on the surface of all mature T cells. The CD3 complex includes invariant chains γ, ξ, δ and Є (Clevers *et al.*, 1988). An example of a secondary signal is phosphoinositide-specific phospholipase C, which mediates breakdown of inositol phospholipids, in turn giving rise to two second messengers, inositol phosphates and diacylglycerol. According to Fabry *et al.*, (1993) the resulting

increase in intracellular Ca^{2+} concentration and translocation of protein kinase C (PKC) to the plasma membrane are thought to initiate the cascade of biochemical events leading to activation of T cell effector functions and clonal expansion. The consequences of T cell activation are the presentation of Ag and stimulation of the T cells. This results in lymphokine gene transcription, translation, and secretion. This in turn influences activation, proliferation, and differentiation of T cells, B cells, and a variety of other cell types in the microenvironment of the T cell response.

Several different APCs play a major role in T-cell activation. These include mononuclear phagocytes (monocytes, macrophages), B lymphocytes, and Langerhans-dendritic cells. These cells are referred to as "professional" APC. They generally express MHC Class II molecules constitutively, which can be further up-regulated with cytokines. They can also express several costimulatory molecules such as IFN-γ and Class II on B cells, and increased Class II on macrophages and dendritic cells.

T Lymphocytes of Both the Naive and the Memory Phenotype:

A fundamental feature of vertebrate immune systems is immunological memory (Mackay, 1991). The term "immunological memory" is used to describe the rapid and enhanced immune responses to specific pathogens that have been previously encountered. Memory is thought to be a feature among long-lived cells and this can be years later and produce a faster and larger response. These cells are also present at higher frequencies compared to their naïve precursors through clonal expansion (Mackay, 1991). A systemic immune protection is provided by these cells as they are distributed and

recirculate through the body. Mackay suggested that a major advance in understanding memory has been the characterization of numerous cell surface molecules that distinguish memory T cells from naive T cells.

T cell subsets were first identified by a CD45 monoclonal Ab (mAb) in 1981 (Sanders *et al.*, 1988). Moriority, (1985), Rose *et al.* (1985), and Tedder *et al.* (1985), several years later independently reported functional characterization of these subsets or their association with a particular disease (Sanders *et al.*, 1988). The CDw29 mAb 4B4 marker was identified by Morimoto and coworkers, (1985). The CDw29 mAb 4B4 showed increased binding to the same T cell subset that had decreased CD45 expression (Morimoto, *et al.*, 1985, Smith *et al.*, 1986, Sanders *et al.*, 1988; Budd *et al.*, 1987). Subsequently, additional mAbs were identified that distinguished T cell subsets sharing functional attributes of the CD45-high and CD w29-high subsets. Two flow cytometry was used to give a direct comparison of markers. These data showed that these differed in expression of CD45 and CDw29, but also that of five additional surface markers (Sanders, 1988; Me, 1988; Makgoba, 1988 and Sharrow, *et al.*, 1988). These markers are the most useful for enumerating or sorting T cell subsets.

It was thought that these subsets represented different lineages of T cells, e.g. $CD4^+$ and $CD8^+$. Tedder and coworkers, (1988) instead proposed that they consisted of cells at different maturational stages. According to Saunders *et al.*, (1988) this theory is now supported by the results from many laboratories that have shown that the peripheral blood T cells of the CD45R- high subset convert to the CDw29-high subset, but

conversion in the opposite direction has not been reported. Thus, T cell expression of many surface molecules changes with T cell activation. These investigators singled out six aspects of functional differences between subsets: 1) differentiation into memory cells is accompanied by biochemical changes which make them more sensitive to CD2 and CD3 receptor-mediated activation and may result in enhanced responsiveness to low concentrations of foreign Ag; 2) activation of memory cells leads to secretion of large amounts of IFN-γ and Interleukin-3 (IL-3), while activation of naive cells results in little or no secretion. In contrast, naive and memory cells secrete comparable amounts of IL-2 in response to PHA stimulation; 3) memory cells differ from naïve ones in their use of accessory molecules CD4 and CD8. It is suggested that this may reflect higher affinity-specific receptors, enhanced signal transduction via CD3 or other biochemical changes affecting the function of accessory molecules in memory cells; 4) naive cells proliferate preferentially in response to certain immunologic stimuli such as phytohemmaglobin (PHA) and to autologous non-T cells. These cells are preferentially responsive to particular conditions of CD2 and CD3-mediated stimulation, naive cells can be activated preferentially when other signals are delivered; 5) the ability to mediate suppression is not unique to naïve cells because memory cells can also induce the suppression of Ag-specific B-cell responses; and 6) memory cells are slightly larger than naive cells. This cell size increases with activation and after memory cells have undergone prior activation. In summary, immunological memory's main feature is that it is specific, long-

term and systemic. An immune response in any one site will lead to a memory-type response upon challenge in another tissue. This is achieved through the dissemination and recirculation of memory cells throughout the body.

Westermann (1997) stated that in humans, approximately $5x10^{11}$ lymphocytes leave the blood each day, migrate into the tissues and return into the blood in their continuous search for specific Ag. T cells recirculate through the body by one of two basic pathways: 1) the vast majority recirculate by crossing high endothelial venules (HEV), and 2) after about18-20 hours residency in a LN, and leave through the efferent lymphatic duct (MacKay, 1991). T lymphocytes may also leave the blood in peripheral vascular beds, particularly within inflammatory lesions. Local LNs receive these draining cells via the afferent lymphatic vessels. $CD4^+$ T cells of both the naive and the memory phenotype enter LNs and Peyer's Patches via HEV. However, within the tissue their migratory behavior differs. About 10% of these lymphocytes enter LN and Peyer's Patches (PP) via HEV and leave via the efferent lymphatic vessel. This leaves over 90% of the lymphocytes migrating through these organs (Fossum and Ford, 1985). Due to the specialized environment of LN and PP, concentrated Ag is directly situated in the path of recirculating lymphocytes. This optimizes the chance of specific T cells finding their cognate Ag (Fossum and Ford, 1985).

Mackay, (1991), mentioned a study in which sheep were used to investigate these two compartments of recirculating cells. The study revealed that there is a striking bias in

the class of T cells that take these two migration pathways. These suggested migration pathways are: 1) T cells in peripheral afferent lymph, draining tissue such as the skin, were entirely of the memory type; and 2) T cells within efferent lymph; the vast majority of which originate from the blood via HEV, were overwhelmingly of the naive T-cell type. The study demonstrated that naïve T cells entered LNs by crossing HEV. Memory T cells entered tissues and drained to a regional LN through afferent lymphatics. The efferent lymph also was used by both naive and mT cells to leave the LNs and return to the blood. Once they left by the efferent lymphatics, they repeated their respective recirculation pathways.

Phagocytes in Gliomas:

Macrophages:

The monocyte-macrophage system is a network of specialized phagocytic cells widely scattered throughout the body. These cells include peripheral blood monocytes, dendritic cells, Kupffer cells of the liver, macrophages of LNs and spleen, fixed macrophages of other tissues, and CNS microglia (Lorusso and Rossi, 1997). Available evidence suggests that peripheral blood monocytes originate from bone marrow precursors and that tissue macrophages, including brain macrophages, are derived from circulating monocytes that migrate into tissues where they become resident (Kinney and Armstrong, 1997).

Bone marrow precursors therefore maintain the pool of peripheral blood monocytes and tissue macrophages. Fujita *et al.*, (1976) and Oehmichen and Huber, (1976), indicated that some of the CNS macrophages derive from resident microglia. del Rio-Hortega recognized microglia in 1932 by means of silver carbonate impregnation (del Rio-Hortega, 1919 and 1932). Microglia, originating from embryonic mesenchyme, distribute to the CNS white and gray matter where they become resident macrophages, and comprise between 5% and 20% of the total glial population (Lawson *et al.*, 1991).

As mentioned, monocytes originate from non-lymphoid precursors within bone marrow and circulate in the peripheral blood with a half-life of 1 to 3 days (Haynes and Denning, 1993). Monocytes leave the circulation by margination into the connective tissue. The maturation and differentiation of monocytes into macrophages is influenced by various processes including chemotactic factors (Mauri *et al*, 1987; Verghese and Snyderman, 1989). Lymphocytes (Balkwill, 1989), bacterial endotoxins (Levy and Cole, 1989), plasma proteins (Ross *et al.*, 1989) and cell surfaces are such examples (Berton and Gordon, 1983).

Macrophages bearing MHC Class II receptors and Interleukin-2 receptors (IL-2R) participate in the immune process as APCs. Other immune mechanisms include antibody-dependent cell-mediated cytotoxicity (ADCC) through the binding with Fc receptors of killer cells, and non-MHC specific restricted cytotoxicity (Tada and de Triboleta, 1993.)

Ag presentation by macrophages is a process involving two signals. Signal 1 is

Ag-specific and originates from the binding of the APC receptor to the peptide-MHC complex. Signal 2 is Ag-nonspecific and is triggered by the B7 molecule exclusively expressed by APCs, which interacts with CD28 expressed on Th cells. Signal 1, in the absence of Signal 2, results in T cell inactivation or "T cell anergy". Macrophages may also be stimulated to release lysosomal enzymes following interaction with antigen-antibody complexes (Ferreri et al., 1986) and with lymphokines, e.g. IFN-γ, and to operate selective targeted-phagocytosis and opsonin-mediated phagocytosis (Berry and Butt, 1997).

Neutrophils:

Neutrophils, also known as polymorphonuclear (PMN) cells, are terminally differentiated, short lived cells, and incapable of proliferation. PMNs are the most common type of leukocyte in blood, but not in rats, and constitute 40-70% of the circulating nucleated cells (Wheater *et al.*, 1993). Histologically, the prominent features of the PMN cells are (1) their highly lobulated nucleus; when mature, there are usually five lobes connected by fine strands of nuclear material; (2) their high motility and phagocytic activity; and (3) their principal function in the acute inflammatory response to tissue injury where they ingest and destroy damaged tissue and invading micro-organisms, particularly bacteria (Wheater *et al.*, 1993).

The cytoplasm of neutrophils is lightly stippled with azurophilic granules which represent large lysosomes. These are often referred to as primary granules that appear

during neutrophil maturation. These granules contain characteristic acid hydrolases but also a number of bacteriocidal agents including myeloperoxidase.

To be cytotoxic after antigenic activation, neutrophils require stimulation by complement factors, leukotrienes, or other cytokines whereas lymphocytes become cytotoxic or antibody-producing after antigenic challenge. Experimentally, there are data that neutrophil infiltration into gliomas can occur in response to cytokines. Wright and Merchant (1997) showed a marked infiltration of leukocytes (mostly neutrophils) in the RT-2 rat glioma model after an IC injection of tumor necrosis factor-α (TNF-α).

Fossati *et al.*, (1999), reported on the results of an analysis of infiltration of neutrophils in human gliomas. Sections of tissue were stained for $CD15^+$ and myeloperoxidase$^+$ cells. Their analyses showed that over 70% of all gliomas analyzed had significant neutrophil infiltration and there was a marked and significant correlation between tumor grade and the extent of the neutrophil infiltration. Of the low grade tumors, only 40 to 50% had significant infiltration; however, in GBM over 85% of the samples analyzed had significant infiltration. The presence of neutrophils in areas of necrosis suggests that these infiltrating cells may play an important role in glioma pathology; i.e., they could be responding to necrotic lesions or could be promoting cell death and destruction.

A mounting body of evidence indicates that PMN survival can be extended following exposure to microenvironmental signals in infection and immunity. These

signals include lipopolysaccharide (LPS), IL-1β, TNF-α, interleukin- 6 (IL-6), IFN-γ, granulocyte-colony stimulating factor (G-CSF), and granulocyte macrophage-colony stimulating factor (GM-CSF) (Cassatella, 1995). Neutrophils should be considered not only as active and central elements of the inflammatory response, but also as cells that through cytokine secretion, may significantly influence the direction and evolution of the immune response.

Granulocytes:

Mast Cells:

Mast cells (MCs) occur in many organs and tissues, including the CNS of several species, including humans (Ibrahim, 1974). MCs are highly granulated connective tissue cells that store and produce a number of inflammatory mediators (Compton, 1952). Neumann, 1890, identified them over a century ago in brain infarcts and at the edge of multiple sclerosis. According to Hebda *et al.*,1993, tissue MCs are normally distributed widely throughout different organs such as the skin, upper and lower respiratory tract and gastrointestinal tract. In most issues, including the skin, MCs are generally concentrated around small blood vessels, lymphatics and nerves (Eady *et al.*, 1979). Specialized intercellular contact between mast cells and fibroblasts have also been documented. In addition to their cytoplasmic projections, mast cells have the expected normal compliment of cellular organelles (Greenberg and Burnstock, 1983). However, the most

characteristic morphological feature of these cells is the presence of numerous cytoplasmic granules which are individually enclosed in a bilayered membrane (Craig *et al.*, 1988).

Two types of granular cells are seen and are referred to as Type I cells and Type II cells (Ibrahim, 1974). Both types have been seen in the CNS of the mouse, rat, hamster and cat while the CNS of the guinea pig, rabbit, dog, monkey and man contained almost exclusively Type II cells (Ibrahim, 1974). Type I cells in the adult were generally oval or rounded with a central oval or rounded nuclei. Ibrahim, (1974), describes these cells as containing round and uniform granules, about 0.2-0.5 um in diameter which often masked nuclear detail. By electron microscopy the cells were seen embedded in an extension of the basement membrane system of the blood vessel and had pseudopod-like surface extensions (Ibrahim, 1974).

Type II cells of the rodents and the monkey, and some of those of the dog and adult human were regular oval or spindle-shaped (Ibrahim,1974). By electron microscopy, Ibrahim, 1974, has shown that these cells had many features in common with Type I cells, the main differences being the larger and variable granule size and the greater homogeneity and darkness of the granules, probably reflecting their greater lipid content.

Regarding distribution of these cells within the CNS parenchyma both types of cells were located around arterioles and venules but not around capillaries (Hebda et al.,

1993). No cells were normally seen at a distance from a blood vessel except in the leptomeninges, in the nerve fiber layer of the olfactory bulbs and in the choroid fissures and plexuses.

Type I cells are scanty in the mouse but more plentiful in the adult rat. MCs, according to Gruber *et al.*, 1995, are often found near blood vessels in the resting state and it is widely recognized that mast cells associate with newly forming micro-vasculature. Thus, early accumulation of masts cells locally at a tissue site appears to potentiate vessel growth.

Type II cells were numerically proportional to the richness of regional blood supply. Gray matter, because of its wealth in such vessels, contained more cells than did white matter (Ibrahim,1973). For the same reason the cerebral cortex contained more cells than most other grey matter areas.

Mast cells (MC) are involved in infammatory and hypersensitivity reactions (Galli *et al.*,1984) and occur in many peripheral tissues, in perivascular regions, in close apposition to innervating sensory or autonomic nerve fibers (Bienenstock *et al.*, 1987) and also within the peripheral nervous system and CNS (Johnson, D. and Krenger, W., 1992). As suggested by Leon *et al.*, 1994, neurons and immunological mediators such as neuropeptides or immunoglobulin E (IgE) can affect the state of mast cell activation. According to Ratzlaff, R. *et al.*, (1992) secretory products of activated MCs can stimulate or facilitate axon reflexes, thereby inducing positive feedback loops. Activated MCs also

secrete a wide array of pluripotent cytokines and other inflammatory mediators and therefore, may act as bidirectional carriers of information between the nervous and immune systems (Leon, A. *et al.*, 1994).

MCs are also classically associated with allergic response and are secretory cells found primarily in connective tissue such as the lungs and skin (Dimitriadou, 1990). These cells synthesize and secrete numerous powerful mediators such as : endorphins, histamine, kinins, leukotrienes, prostaglandins, serotonin, vasoactive intestinal peptide, proteolytic enzymes and phospholipases (Baloyannis, S. and Theoharides, T., 1992).

Bienenstock, J. *et al.*, (1987) and others report that a close relationship exists between the nervous system and the immune system. This could explain such pathological conditions as inflammatory processes, asthma, vascular headaches, certain tumors, and autoimmune diseases such as multiple sclerosis. All of these cases appear to be associated with an increase in the numbers of MC, which can be stimulated to secrete various neuropeptides and neurotransmitters such as acetylcholine (Fantozzi R. et al., 1978).

The close proximity between the cerebral MC and the cerebrovascular organs suggests that these cells could have important functions in controlling the integrity of the blood-brain barrier. Secretion of vasoactive molecules could make this barrier permeable to blood lymphocytes or pathogenic agents.

CD25/IL-2 Receptor:

An important requirement for proliferation of mitogen or Ag-stimulated lymphocytes is not only the production of IL-2, but also the interaction of this cytokine with its receptor. The IL-2R is formed on the cell membrane by the noncovalent interaction of two polypeptide chains (Greene, 1988; Wang *et al.*, 1987). The 55Kd protein (TAC Ag, CD25) represents one of these receptor proteins which associates with a 70Kd protein to form the functional high affinity IL-2R. The IL-2R subunit, IL-2Rα (p55, Tac antigen, CD25), is a protein of 251 amino acids (Minami *et al.*, 1992 and 1993). The gene for human IL-2Rα is located as a single copy on chromosome 10p/14-15 (Leonard *et al.*, 1984 and Kuziel and Greene, 1991). Post-translational processing of IL-2Rα involves glycosylation, sulfation and phosphorylation, resulting in its final MW of 55Kd (Hanisch and Quirion, 1996). Hanisch and Quirion also reported that IL-2Rα has a low affinity, whereas IL-2Rα in association with IL-Rβ, or in the trimeric complex αβγ, contributes to the high-affinity binding sites for IL-2 with Kd values in the range of 10^{-10} and 10^{-11} M. Expression of IL-2Rα in T cells, B cells and monocytes depends on induction by certain stimuli such as, Ag, IL-1, TNF β, mitogen or by IL-2 itself (Arima *et al.*,1992; Ballard *et al.*, 1989; Hatakeyama and Taniguchi, 1991).

As mentioned, the effects of IL-2 on the activation of its target cells are mediated through specific cell-surface receptors. The IL-2 receptor comprises at least three subunits encoded by different genes. The first component to be identified, IL-2Rα, is a 55

kDa protein that binds IL-2 with a Kd of about 10 nm and shares homology with the α-chain of the IL-15 receptor (Theze et al., 1996). The other identified subunits are IL-2Rβ and IL-2Rγ. IL-2Rβ is a 75 kDa protein containing a large intra-cytoplasmic domain of 286 amino acids. IL-2Rβ is shared with IL-15 whereas IL-2Rγ is a 64 kDa protein that also participates in the formation of the IL-4, IL-7, IL-9 and IL-15 receptors (Theze et al., 1996). This chain is also critical for signal transduction via JAK-3.

The cytokine, IL-2, has multiple immuno-regulatory functions and biological properties not only related to T cells. Hanisch *et al.*, (1996) provided the following evidence that suggested that IL-2 is also a modulator of neural and neuroendocrine function: 1) extremely potent effects of IL-2 on neuronal cells was discovered that included activities related to cell growth and survival, transmitter and hormone release, modulation of bioelectric activities; 2) IL-2 may be involved in the regulation of sleep and arousal, memory function, locomotion and modulation of the neuroendocrine axis; 3) the concept that IL-2 could act as a neuroregulatory cytokine has been supported by reports on the presence in rodent and human brain tissues of IL-2-like immunoreactivity, IL-2-like mRNA, IL-2 binding sites, IL-2 Rα and β chain mRNA immunoreactivity and 4) IL-2 and/or IL-2R molecules mainly localize to the frontal cortex, septum, striatum, hippocampal formation, hypothalamus, locus cereleus, cerebellum, the pituitary and fiber tracts, such as corpus callosum, where they are likely expressed by neuronal and glial cells.

Elliot *et al.* (1992) reported that T cells, obtained from patients harboring malignant gliomas, exhibited a profound decrease in mitogen-induced proliferation. This was presumed to be a consequence of a failure of these cells to secrete normal amounts of IL-2 and express the IL-2R. They and others (Fontana *et al.*, 1982; 1984) report that glioma cells are capable of diminishing lymphocyte function and may be responsible for the marked inhibition of immune function in these patients. The finding that glioma cells secret factor(s) that suppress the function of lymphocytes from normal subjects supports this hypothesis (Roszman *et al.*, 1987).

Blood Brain Barrier:

Normal:

The most notable morphological difference between the CNS and tissues of other systems is the existence of a barrier that separates the CNS tissue from the blood, thereby preserving the CNS protected environment. The blood-brain barrier (BBB) was once considered to be a single membrane. However, it actually consists of luminal plasma membranes, cytosol and abluminal membranes of the endothelial cells. Because of morphological difference, for many years it was believed that the brain was an "immuno-privileged" site, possessing few if any immune elements (Fossati *et al.*, 1999). Wahl *et al.*, (1988) described the morphological substrate of the BBB in cerebral vessels as a continuous layer of endothelial cells with tight junctions and no or minimal vesicular transport. He presented functional characteristics of the BBB as: a) low diffusional

permeability for compounds, b) low hydraulic conductivity, c) high reflection coefficient, and high electrical resistance. The consensus of a limited BBB was based on the following: 1) the cerebral capillary endothelial cells that constitute the BBB differ from those of the peripheral circulation in that they form extensive tight, impermeable junctions between adjoining cells; 2) endothelium is devoid of fenestrations and transcapillary channels and express a very low rate of pinocytotic activity; and 3) this continuous barrier means that, for all but a few compounds, passage into the brain from the circulation is determined by the solutes' lipid solubility (Greenwood, 1990). According to Greenwood (1990), glucose and the amino acids that are essential nutrients to meet the high metabolic demand of the brain are transported across the BBB in specific transport systems that facilitate their entry into the brain.

The existence of all of these parameters are indicative of low diffusional exchange of water-soluble solutes and inhibition of the penetration of several mediators by enzymatic degradation at the endothelial border (Wahl *et al.*, 1988). It is known that lipophilic compounds can easily cross the BBB by simple diffusion, while transcellular transport of hydrophilic solutes is made possible by facilitated diffusion and active transport. These mechanisms, described by Wahl *et al.*, (1988), permit a highly selective exchange between blood and brain and provide an optimally controlled homeostatic environment for the brain in a physiological state.

BBB Under Pathological Conditions:

In many diseases that affect the brain, the cerebral endothelium plays an active part in the disease process with the BBB becoming disrupted. This modification can cause a dramatic increase in vascular permeability. Consequences related to these disease processes include vasogenic brain edema and possible development of secondary brain damage (Greenwood, 1991).

Greenwood (1991) postulated that increases in BBB permeability were associated with neoplasia, ischemia, hypertension, dementia, epilepsy, infection, multiple sclerosis, experimental allergic encephalomyelitis and trauma. Evidence suggests that the cerebral endothelium is under direct influence from the astrocytes (Arthur *et al.*,1987). The unique characteristics of the BBB, therefore, are due to factors released by or present on astrocytes (Beck *et al.*,1984). Both Arthur *et al.*,(1987) and Beck *et al.*, (1984) considered that in certain pathological conditions it is possible that the influence of the astrocyte and its factors are lost. This gives rise to structural changes in the endothelium following ischemia. For example, both intrinsic and newly formed capillaries are often devoid of BBB characteristics and resemble endothelium from peripheral tissue. Evidence also suggests that in many diseases of the CNS, mediator substances from damaged or activated cells can induce barrier dysfunction. Mei Liu (1988) suggested that these vasoactive compounds may originate from a number of different sources - the blood, the cells of the CNS or from the endothelial cells themselves.

Blood-Borne Mediators:

Schurer *et al.*, 1989 have demonstrated that the blood-borne products of the kinin and complement system, bradykinin and complement C3a, can increase the permeability of the BBB in experimental animals. Similarly, platelets, basophils, macrophages and active T lymphocytes are cells of the blood, that according to Schurer *et al.*, (1989), have been shown to be a major source of vasoactive compounds. T lymphocytes release substances, such as histamine (Dux and Joo, 1982), serotonin (Olesen, 1985) and leukotrienes (Black and Hoff, 1985) that disrupt the BBB.

The current study was based on the following rationale. 1) human brain tumors progress rapidly and are almost invariably fatale despite combination therapy involving surgery, radiation and cyto-reductive drugs. 2) Little success has been achieved employing aggressive experimental adjuvant therapy against weakly immunogenic human tumors. 3) Activation of lymph node lymphocytes which circulate through the blood and enter tumors can be induced by immunization.

Chapter 2:

PRIMARY VACCINATION IN THE INTRACRANIAL RT-2 GLIOMA MODEL

Introduction

Current advances in cell biology, tumor immunology, and molecular biology has helped rekindle an interest in tumor vaccines as our understanding of Ag processing and presentation has evolved. Livingston (1993) and Schirrmacher (1995) base the development of vaccines on three premises: (1) the presence of qualitative and quantitative differences between tumor cells and normal cells; (2) the immune system, with its complex assortment of cells and cell products, provides specific and nonspecific protection against foreign substances; and (3) the immune system can be "taught" to recognize these differences and mediate tumor rejection by specific active immunization using these vaccines. Our observations support the hypothesis that a primary vaccine is able to provide some protection against RT-2 gliomas.

The first set of studies began by investigating the survival times of rats that were vaccinated in the hind footpads (HFPs) with $4x10^6$ irradiated but viable RT-2 cells. Seven days later, the animals received intracerebral (IC) injection of viable RT-2 glioma cells. Ten thousand RT-2 cells in 5 μl of phosphate buffered saline (PBS) were injected over a

thirty minute period. Subsequently, 30% of the primed animals survived the IC challenge. These findings suggested that a 1° vaccination of $4x10^6$ cells per HFP provided some protection, but there was a 70% failure rate in prolonging survival.

The second set of studies was designed to ask whether the amount of the inoculum ($4x10^6$ irradiated cells versus $8x10^6$ irradiated cells) affected survival. Data did show that this experiment resulted in significant differences in the rate of survival and/or length of survival from Experiment 1.

The RT-2 Glioma

The cell line, RT-2, was provided by Dr. G.Y. Gillespie and was derived from a glioma induced by the inoculation of the Rous sarcoma virus (Schmidt-Ruppin strain) into the brain of neonatal Fischer 344 rats. The RT-2 glioma cells are plastic-adherent and maintained in Dulbecco's Modification of Eagle's Media (DMEM) supplemented with 5% fetal calf serum (FCS). Their *in vitro* doubling time is 14.5 hours.

It has been suggested and shown that immunotherapeutic approaches for treatment of brain tumors can harness the control of a patient's immune system and direct it towards tumor (Kruse *et al.*, 1989; Merchant *et al.*, 1990). The RT-2 glioma model has been used extensively in our lab and others, is well characterized, and shares many features with human gliomas (Watts and Merchant, 1992). The rat RT-2 glioma provides a model for immunotherapy for brain tumors. A major factor of significant importance is that the use of syngeneic RT-2 tumor cells in the syngeneic Fisher 344 rats is the

experimental results provide a provision of a consistent genetic basis for interpretation of results.

A synopsis of these characteristics follows. Watts and Merchant (1992) showed that glioma-bearing rats experienced ataxia with increasing tumor burden. These rats also lost approximately 25% of their body weight over the course of a two week study period. Immunological status showed a progressive decline as well as behavioral impairments and decreased functional activity. At seven days post tumor inoculation, they showed that animals displayed slight contralateral paralysis and delayed response to painful stimuli. By Day 13 post RT-2 implantation, most rats exhibited diarrhea and a lack of grooming. Papilladema and periorbital hemorrhage due to increased intracranial pressure was also demonstrated. Without treatment, animals died between 14 and 17 days after IC implantation of 10,000 RT-2 cells.

Seven days after RT-2 glioma implantation, histological examination of brains revealed a small tumor with BBB permeability changes evidenced by extravasation of endogenous IgG in the adjacent cortex immediately around the tumor. Other changes that were demonstrated were: some of the vessels within the tumor displayed a little leakage of the protein tracer outside the vessel wall; less extravasation of IgG was seen at early stages in tumor growth due to limited neovascularization of the tumor; there was no central necrosis due to small tumor size; and) and there was limited hemorrhage within the tumor.

As the glioma grew, they showed that there was no increased BBB permeability throughout the entire tumor and adjacent brain. The glioma was greatly increased in size at day 10 and 13 post-tumor implantation and compression of the underlying white matter had caused ventricular collapse. Also noted was the shift of the tumor-bearing hemisphere past midline as well as distortion of the contralateral hemisphere. Briefly, a summation of their findings regarding the presence of IgG in the tumor are that IgG extravasation was observed as an intense, dark brown immuno-reactive product within the surrounding cortex and white matter and that some of the larger vessels within the tumor exhibited permeability to circulating IgG. However, because of the densely

organized tumor cells, the tracer was confined to the region immediately adjacent to the vessels. They also showed that limited extracellular space within the growing tumor prevented diffusion of IgG far into the tumor parenchyma; that the most intense staining was seen at the growing edge of tumor at Day 13; and that as the tumor enlarged, more edema and hemorrhage was seen within the edematous peritumoral tissue.

Primary Immunization

Since no conventional treatment for glioma is deemed curative at this time, tumor biology, host reactions (immunogenic manipulations) and gene therapy have become a primary focus of investigators in the neuroscience field. Approaches to cancer using immunotherapy can be placed under the following three categories: (1) nonspecific immunotherapy consisting of methods designed to stimulate, augment or boost the

immune capacities of the cancer patient nonspecifically by various means, using adjuvants such as bacillus Calmett-Gurin (BCG), *Corynebacterium parvum*, levamisole, and polynucleotides; (2) active or specific immunotherapy or vaccination, referring to the specific immunization of the patient with treated tumor cells, "tumor Ag" preparations, or cross-reacting viral or bacterial Ags in an attempt to specifically or selectively augment functional immune reactions; and (3) passive or adoptive immunotherapy involving the administration of immune cells, serum, or Ags from a specifically immunized host, in an effort to transfer specific immunity to the cancer patient. Since exposure to tumor Ags and cytokines *in vitro* causes $CD3^{+}CD8^{+}$ populations of cytotoxic T lymphocytes to develop anti-tumor activity, we wanted to generate therapeutic cytotoxic effector cells *in vivo* against the RT-2 glioma cell line. The following experiments provide a glimpse of the usefulness and promise that manipulation of the immune system holds.

Materials and Methods:

Animals:

Inbred female Fischer 344 rats weighing 120 to 140 grams and ranging in age from 4 to 6 months were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals are housed in a climate controlled, AAALAC approved vivarium and were provided free access to rat chow and water. All experimental animal procedures have been approved by the VCU/MCV Institutional Animal Care and Use Committee.

RT-2 Cell Line and Cell Culture:

The RT-2 glioma cell line was cultured in complete medium consisting of Dulbecco's Modified Eagles Medium (DMEM) (Gibco BRL, Grand Island NY) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco BRL) and non-essential amino acids. They were maintained as adherent monolayers in culture flasks, incubated at 37° C in an atmosphere of 5% CO_2 and 95% air and passed biweekly using 0.5% trypsin. RT-2 glioblastoma cell line is completely lethal when implanted IC into naïve syngeneic rats. Irradiation of RT-2 cells was performed using the Cesium irradiator for 10 minutes. These cells were maintained on ice in 50 ml tubes.

Mat-B III Cell Line and Cell Culture:

MAT-B III is a mammary adenocarcinoma cell line induced by dimethylbenzanthracene in a Fischer 344 rat (Segaloff, 1966).The Mat-B III tumor cell line was cultured in complete medium consisting of DMEM (Gibco BRL, Grand Island NY) supplemented with 10% FBS (Gibco BRL) and non-essential amino acids. They were maintained as adherent monolayers in culture flasks, incubated at 37° C in an atmosphere of 5% CO_2 and 95% air and passed biweekly using 0.5% trypsin. Mat-B III cell line was completely lethal when implanted IC in RT-2 vaccinated rats. Intracranial

Tumor Implantation:

Tumor cells for IC implantation were trypsinized and washed twice with PBS and a final suspension of 1×10^4 viable cells per 5 μl of PBS was made. Animals were

anesthetized by an intraperitoneal (IP)injection of ketamine·HCl (87 mg/kg) and xylazine (6.5 mg/kg). The scalp hair was shaved, wiped with betadine and an incision was made over the cranial midline. Animals were placed in a stereotactic apparatus and bregma was located and used as a reference point for injections. A hand-held Dremel drill was used to create a shallow depression 4 mm to the right of bregma and 1mm posterior to the coronal suture. Five μl of tumor cell suspension ($1x10^4$ cells) was injected into the parietal lobe of the brain at a depth if 3.5mm using a Hamilton syringe and a 26-guage needle secured to the arm of the stereotactic apparatus. The needle tract was sealed with bone wax and the incision closed with surgical staples.

Treatment #1:

To determine if a 1° immunization with irradiated RT-2 glioma cells would sensitize rats to reject an IC challenge of viable RT-2 cells; and, to determine if the immunization would be tumor specific, a study was conducted to determine the survival times of rats after receiving a 1° immunization in the right (R) and left (L) HFPs with irradiated RT-2 tumor cells. There were 3 groups of animals with 10 animals in each group as seen in Table 1. At Day 1, Group A (control group-1) received sterile phosphate buffered saline (PBS) at 100 μl per HFP. Group B (control group-2) and Group C (experimental group) received $4x10^6$ irradiated RT-2 cells per HFPs. After one week, $1x10^4$ viable RT-2 glioma cells were IC injected into the right cerebral hemispheres of Group A and Group C rats. Group B animals received $1x10^4$ viable MAT-B III cells IC.

The MAT-B III cells functioned as a control to show specificity of the vaccine to the RT-2 cell line. All animals were monitored for survival.

MAT-B III Cells IC

MAT-B III cells are from a mammary adenocarcinoma cell line induced by dimethyl-benzanthracene in a Fisher 344 rat (Segaloff, 1966). The Mat-B III cells functioned as a control to show specificity of the vaccine to the RT-2 cell line. (refer to Table 2.1). All animals were monitored for survival (see Figure 2.1).

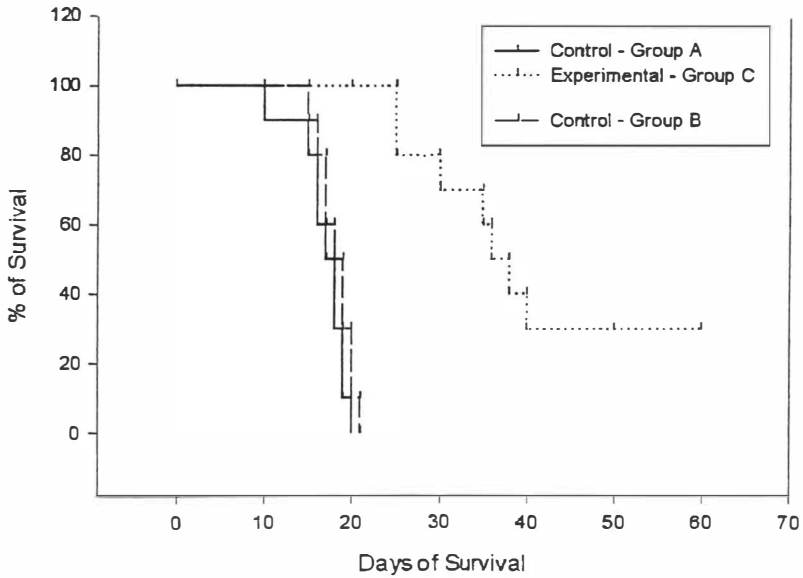
Table 2.1 Vaccination and IC Challenge Schedule

Day 1	Group A (control group)	n=10 – received PBS (100μl/HFP)
	Group B (control group)	n=10 – received irradiated RT-2 cells ($4x10^6$/HFP)
	Group C (experimental groups)	n=10 – received irradiated RT-2 cells ($4x10^6$/HFP)
Day 7	Group A	n=10 – IC injection- 10^4 viable RT-2 glioma cells
	Group B	n= 10 – IC injection- 10^4 viable MAT-B III tumor cells
	Group C	n=10 – IC injection – 10^4 viable RT-2 glioma cells

Results

As seen in Figure 1, the per cent survival in Control Groups A and B was 0%. A survival rate of 30% was attained in the experimental Group C and this extended beyond 60 days. The PBS control group (A) showed a standard survival of 16 days; the MAT-B III group (B) showed an average range of survival of 18 days; and the RT-2 model (C) demonstrated 100% survival up to 28 days and 30% survival beyond 60 days.

Figure 2.1 Survival data from I.C. RT-2 glioma implantation.
Kaplan-Meier survival plot in which 3 groups of animals were implanted with $1x10^4$ RT-2 glioma cells in the brain as previously described. All animals received a HFP vaccination of sterile PBS (0.1 ml) or $4x10^6$ irradiated RT-2 glioma cells. The results are representative plots of at least 3 independent studies.


120
100
80
60
40
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0
% of Survival
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40
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60
70
Days of Survival
Control - Group A
Experimental - Group C
Control - Group B

Treatment #2:

To determine if survival is influenced by the size of the inoculum. Experiment 2 consisted of three groups with eight animals per group as seen in Table 2. Day 1, Group A (control group) received 100 μl of PBS /HFP. Group B (experimental group-1) received $4x10^6$ irradiated RT-2 tumor cells in the NN. Group C (experimental group-2) received $4x10^6$ irradiated RT-2 tumor cells/HFP. At Day 7, IC injections of 10^4 RT-2 cells were given in the right cerebral hemisphere were given to animals in all three groups. Day 1- Group A (n= 8) received 100 μl PBS. Group B (n= 8) received $4x10^6$ irradiated RT-2 tumor cells Group C (n= 8) received $8x10^6$ irradiated RT-2 tumor cells Day 7- All 3 groups received IC injections of 10^4 RT-2 tumor cells in the R cerebral hemisphere.

Table 2.2 Represents the 1° vaccination schedule and IC challenge schedule.

Day	Group	No. of Animals	PBS or RT-2 Cells Received
1	A	8	received 100μl PBS (HFP)
	B	8	received $4x10^6$ irradiated RT-2 cells (NN)
	C	8	received $8x10^6$ irradiated RT-2 cells (HFP)
7	A B C	8	received IC injections of 10^4 viable RT-2 cells in the R cerebral hemisphere

Results:

As seen in Figure 2, rats in Group A and Group B were all dead by day 17 post IC

challenge with viable RT-2 cells. Experimental C showed extended survival with 30% of these animals in the experimental group surviving beyond 60 days. In this case, the amount of the inoculum did make a difference in extending survival of rats receiving a total of $8x10^6$ irradiated RT-2 tumor cells in the HFPs versus $4x10^6$ irradiated RT-2 tumor cells in the NN. The experiment should be repeated using these to sites for immunization but the amount of the inoculum should be the same.

Discussion:

The current forms of immunotherapy for human cancers can be broadly separated into two major categories, 1) cellular immunotherapy that is preventive and the subject of this dissertation, and 2) antibody-guided immunotoxin therapy. Any immune response eventually mounted against malignant gliomas *in vivo* in response to a 1° vaccination is clearly insufficient, as seen in Figure 1, for eradication and prevention of tumor cells and tumor development. Data reported here, using the highly immunogenic RT-2 glioma as a primary vaccination followed by IC challenge, resulted in a 30% survival of challenged rats. Rats challenged with MAT-B III showed a 100% death, which indicated that there was specificity of the vaccine.

These data also raise questions regarding the fate of lymphocytes generated in the primary immune response. Primary responses of T lymphocytes are initiated when Ag localizes in discrete micro-environments of the lymphoid tissues. These tissues are the peri-arteriolar lymphocyte sheaths of the spleen and paracortex of the LNs (Sprent,

1994). Naïve recirculating lymphocytes not only perfuse these areas, but recognition of Ag at these sites causes a selective sequestration of Ag-reactive T cells.

According to Sprent (1994), the MHC-bound antigenic peptides induce T cell trapping, such as displayed by interdigitating dendritic cells. Following trapping of T cells, there is a rapid proliferation of T cells that is assisted by the influence of cytokines. Cell expansion is a reflection of Ag concentration and affinity of the responding cells. The proliferating cells are capable of acquiring various effector functions and are then released into both vascular circulation and central lymphatic vessels after a period of several days (Sprent, 1994). These primary immune responses, however can lead to either rapid clearance of Ag, or to an immune response of brief duration followed by a quick loss of the bulk of the effector cells generated in this response (Springer, 1994). This suggests that in 70% of our rats, the primary immune response was an immune response of brief duration and disappearance of the bulk of effector cells. The 30% survival of rats in this experiment suggested that the actions of the APCs were such that the immune response resulted in the production of effector T cells and probably memory T cells. Direct evidence that proliferating T lymphocytes generated in a primary immune response may be rapidly eliminated has come from studies with super-Ags. Webb *et al.*, (1990), Kawabe and Oichi, (1991), and McDonald *et al.*, (1991) have shown that primary immune responses to super-Ags initially involve a strong Vβ-specific proliferation response in the spleen and LNs. This is followed by the entry of large numbers of blast

cells into the blood and lymph. The progressive disappearance of responding T blasts is associated with a disappearance of the APCs (Webb *et al.*, 1990).

The survival and proliferation of CTL during the effector phase of the immune response was probably critical for the elimination of tumor cells. Guiuntoli II et al., 2002, reported that the expansion and cytolytic function of tumor-reactive human CTL can be enhanced by CD4+ helper T lymphocytes through costimulatory signals that were mediated by cell surface molecules. Their work suggested that these costimulatory receptors on CD8+ CTL were capable of interacting with corresponding ligands on T-helper lymphocytes that resulted in enhanced proliferation and survival of the CTL during the effector phase of antitumor responses. These findings underline 1) the importance of Ag-specific T- helper lymphocytes for regulation and maintenance of CTL immunity; and 2) have implications for the design of therapeutic vaccines for cancer.

Chapter 3

PRIMARY IMMUNIZATION FOLLOWED BY SECONDARY IMMUNIZATION

Introduction

A milestone in tumor immunology was the formulation of the cancer immuno-surveillance theory by Burnett in the 1960s. This theory postulated that cell-mediated immunity plays an important role in the control of cancer. The specific immune response can be divided into the humoral immune response - mediated by B lymphocytes and their Ag recognition product immunoglobulin and the cell-mediated immune response, which involves T lymphocytes. T lymphocytes as a whole are a diverse group of cells that can be broken down into the following subsets: Th cells, cytotoxic T (cT) cells and suppressor T cells (sT). Zimmerman *et al.*, (1996) subdivided T cells into naive, effector and memory subsets. Naive T cells are resting cells that have not encountered Ag after release from the thymus; whereas, effector T cells are activated cells. The presence of and recognition of Ag by effector T cells, enables them to perform specialized functions with high efficiency and without further differentiation. In Chapter 2, we found that a 30% survival was attained with a 1^{o} vaccination only. Hence in this chapter, we investigated survival of rats after receiving a 1^{o} vaccination in the NN followed 14 days later by a 2^{o} vaccination of irradiated RT-2 glioma cells in HFPs. We theorized that the mechanism of

protection against the development of tumor in the animal glioma models, post 1^{o} and 2^{o} vaccination with irradiated glioma cells, is an *in vivo* stimulation of effector and cytotoxic memory $CD8^{+}$ T lymphocytes.

Materials and Methods

The materials for Chapter 3 are the same as described in Chapter 2.

Treatment #3

We wanted to determine if a 1^{o} inoculation of irradiated RT-2 glioma cells in the NN, followed 14 days later by a 2^{o} inoculation of irradiated RT-2 cells in the HFP would provide protection against development of glioma and prolong survival. The experiment had two groups of 10 animals per group as seen in Table 3.1. Group A (n=10) was a control group that received a 1^{o} vaccination of 100 μl of PBS per NN. At Day 14, this group received a 2^{o} vaccination of 100 μl of PBS per HFP. An IC challenge of 10^{4} RT-2 viable glioma cells was done on day 21.Group B (n=10) was an experimental group that received on day 1 a 1^{o} vaccination of $4x10^{6}$ irradiated RT-2 glioma cells in the NN. On day 14, this experimental group received a 2^{o} challenge of $4x10^{6}$ RT-2 cells per HFP. At day 21, all 10 rats received an IC challenge of 10^{4} viable RT-2 glioma cells.

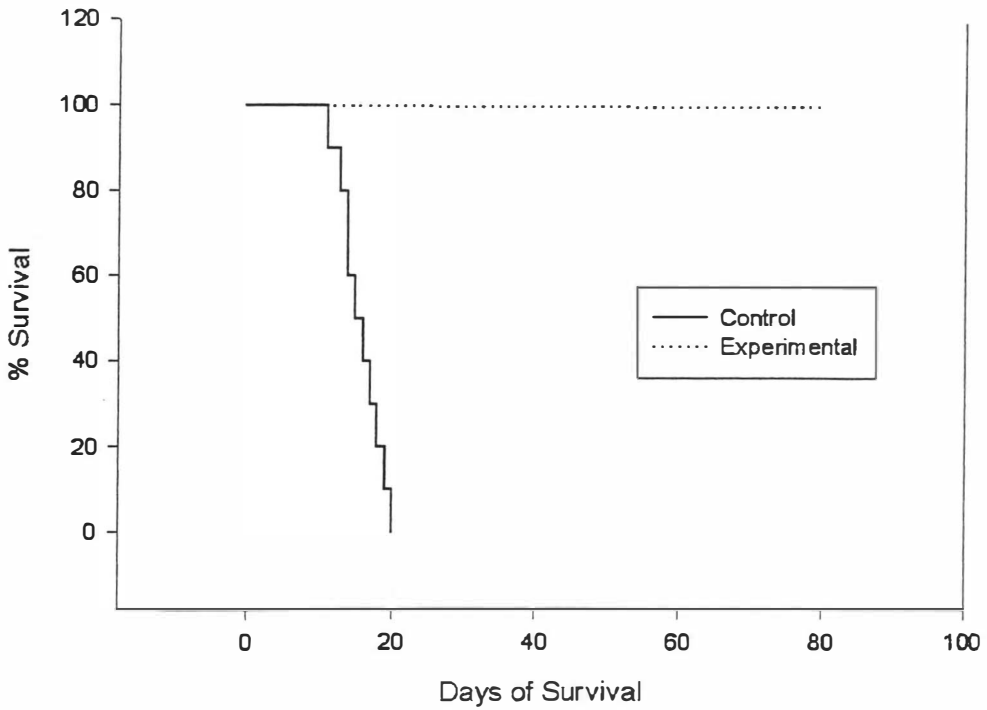
Table 3.1 Represents Control Group A and Experimental Group B.

Group	Day of Treatment	Animals	Treatment
A	1	N=10	control group 1° vaccination- received 100 ul of PBS in the NN;
	14	N=10	control group - 2° vaccination received 100ul PBS in the HFPs
	21	N=10	IC challenge in R cerebral hemisphere with 10^4 viable RT-2 cells
B	1	N=10	experimental group; 1° vaccination - $4x10^6$ irradiated RT-2 cells in the NN
	14	N=10	experimental group; 2° vaccination – $4x10^6$ irradiated RT-2 cells per HFPs
	21	N=10	IC challenge in R hemisphere – 10^4 viable RT-2 cells

Results

As seen in Figure 3.1, rats in Group A (n=10) were all dead from tumor by day 17. Rats in Group B (n=10) attained 100% survival and showed no signs of tumor at the site of IC injection. These rats survived beyond 180 days.

Figure 3.1. A 1° and 2° Vaccination with Irradiated RT-2 Glioma Cells.
Groups of animals received a 1° vaccination with either sterile PBS or $4x10^6$ irradiated RT-2 glioma cells in the NN on Day 1. A 2° vaccination was administered on Day 7 of either PBS or $4x10^6$ irradiated RT-2 cells per HFP. A Kaplein-Meier survival plot, which has combined the results of three independent studies, is shown. The survival of the RT-2 vaccinated group shows significant survival and protection in comparison with the non-vaccinated group. $P< 0.0001$.


120
100
80
60
40
20
0
% Survival
0
20
40
60
80
100
Days of Survival
Control
Experimental

Discussion

Vaccination had an important role in reducing the mortality that is caused by RT-2 tumor. The ultimate goal of the vaccine was to develop long-lived immunological protection, whereby the first encounter with RT-2 glioma is remembered, which leads to enhanced memory responses that either completely prevent re-infection or greatly reduce the severity of the disease.

The memory T cell compartment consists of both $CD4^+$ and $CD8^+$ T cells that can acquire effector functions to kill infected cells and secrete inflammatory cytokines. Effector $CD4^+$ T cells help in B-cell responses and enhance $CD8^+$ T cell development, through the activation of APCs or secretion of cytokines such as IL-2, IL-4 and IL-5.

The context in which the T cell recognizes Ag, the abundance of Ag and the duration of Ag exposure are important parameters that can affect the speed and nature of the T-cell response (Kaech *et al.*, 2002). The tenets of T cell immunologic protection is well established in that the anamnestic response that is mediated by memory $CD4^+$ and $CD8^+$ T cells is more rapid and aggressive than the primary response.

Kaech *et al.*, (2002), reveal the physiological basis for the heightened recall responses of memory T cells: "(1) as a consequence of clonal expansion during the primary infection, experiments in mice have shown that there can be a substantial increase (1000 fold) in precursor frequency of Ag-specific T cells in immune animals

compared with naïve animals; (2) as naïve T cells differentiate into memory T cells, their gene-expression profile is reprogrammed by changes in chromatin structure and the profile of active transcription factors. For example, the genes that encode IFN-γ and cytotoxic molecules, such as perforin and granzyme B, are not expressed in naïve $CD8^+$ T cells, but are constitutively expressed in effector and memory $CD8^+$ T cells. Elevated levels of the messenger RNA transcripts endow memory $CD8^+$ T cells with the capacity to produce larger quantities of these proteins more rapidly than naïve T cells. (3) Memory $CD8^+$ cells express a different pattern of surface proteins that are involved in cell adhesion and chemotaxis from naive T cells, which allow memory T cells to extravasate into non-lymphoid tissues and mucosal sites. This enables memory T cells to survey peripheral tissues. Recently, memory $CD8^+$ T cells that reside in these peripheral tissues have been termed 'effector' memory T cells, whereas those that are found in lymphoid organs are termed 'central' memory T cells; (4) memory T-cell populations are maintained for a long time due to homeostatic cell proliferation which occurs at a slow, yet steady pace. The rate of this homeostatic cell division must equal the rate of cell death, because the number of $CD8^+$ T cells remains relatively constant over time." We can conclude from this, that when the secondary immune response in this experiment came into contact with the given Ag, it differed from the response produced after the first antigenic encounter as seen in Figure 2. The capacity to produce a secondary immune response or anamnestic response persisted long after the first encounter or contact with

the Ag. Thus it is the increased number of Ag-specific T cells, and their faster responses, anatomical location, and longevity that explain how memory T cells controlled long-term protective immunity in this vaccination treatment.

Treatment # 4:

We wanted to determine if a heightened immune response occurs at the site of IC challenge in immunized rats that received a 2° vaccination. Contrast and comparison of the prophylactic immune response to an endogenous immune response was performed at days 3, 5, and 7 post IC injection using three color flow cytometry and histology. Day 5 showed significant changes in the presence of lymphocytes in the vaccinated versus the control animals as seen in the tables of FACs analysis and histograms that follow.

Methods: FACS Analysis of Infiltrating Leukocytes

RT-2 tumor tissue was excised and forced through a 70 μm nylon cell strainer (Fischer Scientific) using the butt of a sterile 3 cc syringe plunger. Cell suspensions were washed three times with cold 5% FBS-PBS. Viable leukocytes were enumerated using a hemacytometer and trypan blue exclusion and then were treated with a battery of fluorescently conjugated mAbs for FACs analysis. $1x10^6$ cells were stained in V-bottom, 96-well microplates (Costar Brand, Fischer Scientific) in a volume of 50 μl of 5% FBS-PBS. Cells were suspended in a cocktail containing three monoclonal antibodies (mAbs)coupled to FITC, PE or biotin and incubated in the dark for 30 minutes on ice.

The mAbs were isotype controls, CD3ε, CD4, CD8β, His 48 and CD45 (Pharmingen, San Diego, CA). After the primary Ab incubation, the cells were washed twice and incubated in streptavidin-PerCP (Bectin Dickenson Immunocytochemistry Systems, San Jose, CA) using a 1:250 dilution for 30 minutes on ice. Finally, the cells were washed twice in a volume of 50μl of 5% FBS-PBS, fixed in 1% paraformaldehyde and stored at 4° C until analysis was done. Three color flow cytometry analysis was performed using a Coulter Epics XL-MCL flow cytometer.

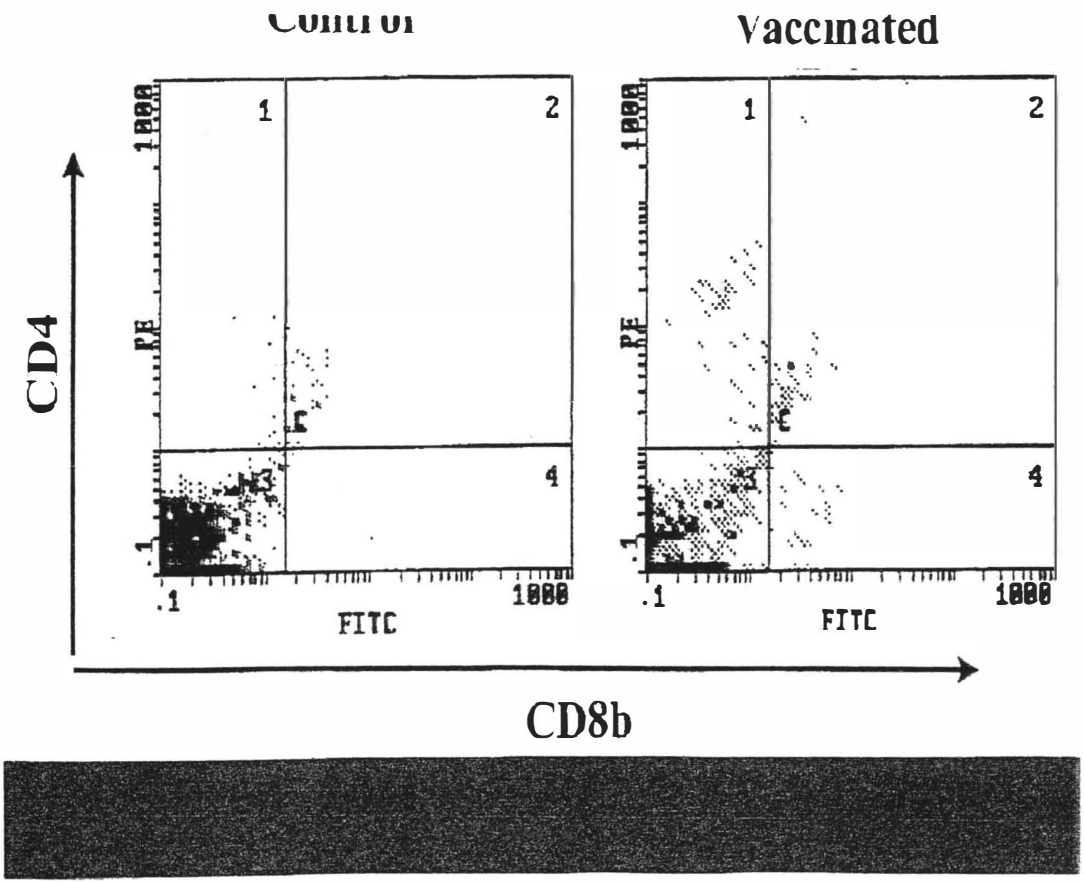
Table 3.2: FACs Analysis of Cell Populations of Vaccinated Animals at Day 5 Post IC Challenge. Day 5 Vaccinated – CD3/4/8.

Region ID	%	Count
C1	2.49	21
C2	2.84	24
C3	93.1	787
C4	1.54	13

Table 3.3: FACs Analysis of Cell Populations of Non-Vaccinated Animals at Day 5 Post IC Challenge. Day 5 Control – CD3/4/8.

Region ID	%	Count
C1	1.05	13
C2	2.59	32
C3	95.9	1185
C4	0.49	6

Figure 3.2 Normal FACS staining for Tables 3.2 and 3.3 of T lymphocytes at Day 5 post IC challenge with viable RT-2 glioma cells. Lymphocytes were isolated from the brain for CD3Є, CD4 αβ T lymphocytes and CD8 αβ T lymphocytes in experimental animals (vaccinated) and control animals (non-vaccinated) animals.


Control
Vaccinated
1
2
4
3
1000
.1
PE
FITC
CD4
CD8b

Preparation and Staining of Brain Tissue From Day 1 and Day 5 Tumors

Fixation of tissues was done by immersion with glutaraldehyde. In general, the tissue was fixed in 3% glutaraldehyde on a 0.1 M sodium phosphate buffer at 4° C and at a pH of 7.2-7.4 overnight. Tissue was then washed in 0.1 M sodium phosphate buffer with 2 changes, 5-10 minutes each. Tissue was postfixed with 2% osmium tetroxide in the 0.1 M buffer for 1 hour. This was followed by washing for 5 minutes with the 0.1 M sodium phosphate buffer. Dehydration with a cold ethanol series followed. Vials were placed on a shaker to aid in dehydration. Ethanol series consisted of : 1) 50% ethanol for 5 minutes at 4° C; 2) 70% ethanol for 5 minutes at 4° C, 3) 80% ethanol for 10 minutes at 4° C; 4) 90% ethanol for 10 minutes at 4° C; 5) 100% ethanol for 15 minutes each, three changes at room temperature; and 6) propylene oxide for 20 minutes each, three changes, at room temperature. This step was followed by infiltration of the tissue with a half-and – half mixture of propylene oxide and Epon resin for 8 hours overnight. Samples were placed on a shaker to facilitate infiltration. The previous infiltrate was replaced with pure Epon resin and infiltration was done on a shaker for 8 hours. Embedding tissue in beem capsules and addition of resin followed. Beem capsules were placed in the oven at 60° C for 2-3 days. Thick section were cut and slides stained with hematoxylin and eosin (H&E) or toluidine blue by Ms. Judy Williamson of the Department of Anatomy and Neurobiology.

Results:

Based on our results in Treatment # 4, we decided to look at the histology of the area of brain parenchyma surrounding the needle track after IC injection of RT-2 tumor at days 1 and 5. We believe that 24 hours post IC injection an immune response is mounted against the tumor cells. Day 1 and day 5 thick sections were stained with toluidine blue or H&E. One animal per time-point was harvested for histology. Day 1 histology demonstrated significant numbers of red blood cells (RBCs) in the brain parenchyma at the site of the needle tract which was expected. At the same time, significant numbers of neutrophils were seen, not only in the brain tissue ,but within the vasculature. This acute inflammatory response at a site of injection would be expected due to the damage done to the brain parenchyma by the needle alone.

Day 5 histology demonstrated the presence of monocytes in the blood vessels (Figure 3.3), rolling along the luminal walls of blood vessels, tethering to the blood vessel wall and diapedesing through the endothelial cells into the brain parenchyma. Other cells of the immune system seen in this tissue were lymphocytes and mast cells. Mast cells were both granulated (Figure 3.4) and degranulated (Figure 3.5). Within the glioma cells of the tumor, many mitotic figures were seen in different stages of mitosis (Figure 3.6). Necrotic area within the tumor bed is seen Figure 3.7. Macrophages in the brain parenchyma are also detected filled with phagocytosed hemosiderin (Figure 3.8).

Figure 3.3 Histogram of Monocytes in Day 5 RT-2 Tumor
The histology demonstrates the presence of monocytes in the lumen of a blood vessel. Tissue was stained with Toluidine Blue; magnification 20X. Rolling monocytes are indicated by a thick arrow; and tethered monocytes by a narrow arrow.

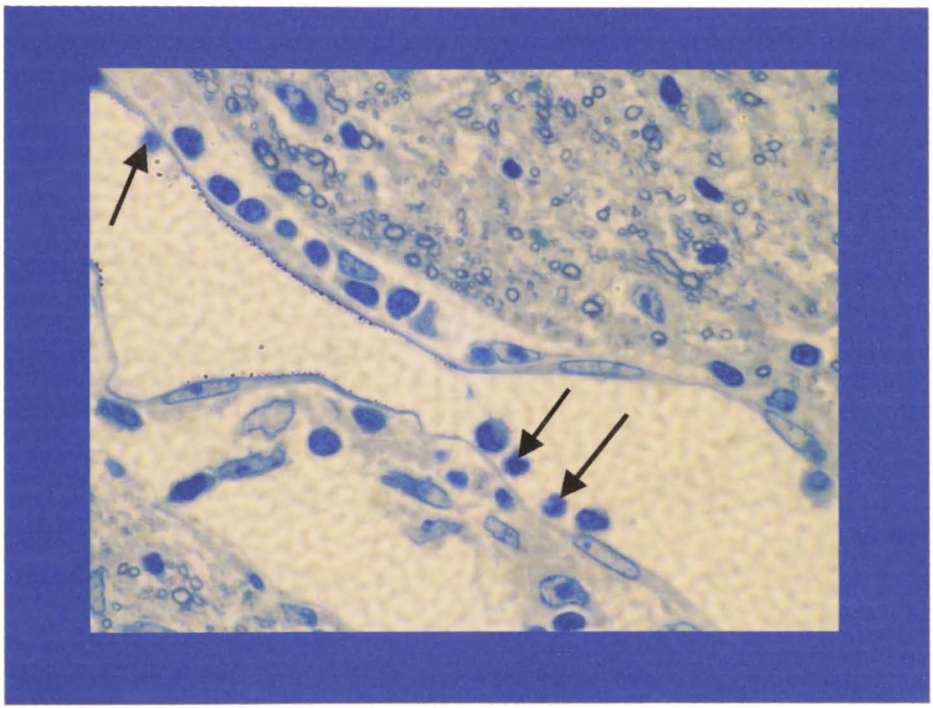


Figure 3.4 Histogram of Granulated Mast Cells
The histology demonstrates the presence of granulated mast cells located outside the lumen of a blood vessel. Tissue was stained with toluidine blue and magnification was 20X. Mast cells are indicated by arrows.

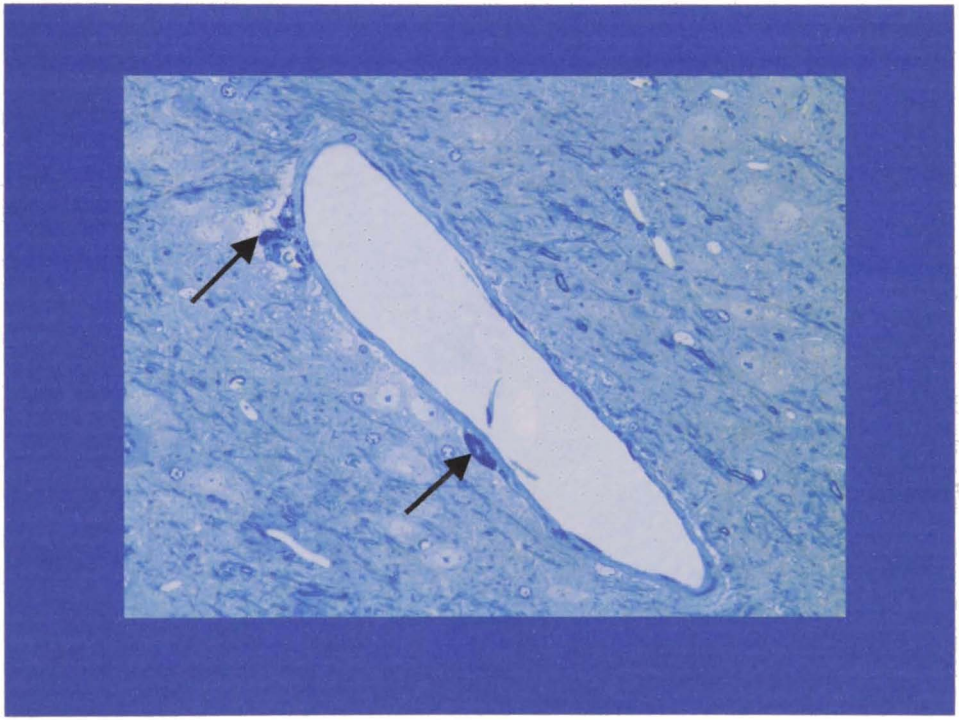


Figure 3.5 Degranulating Mast Cells in Neural Parenchyma
The histology demonstrates the presence of degranulating mast cells located in brain parenchyma. Tissue was stained with H&E; magnification 100X. Degranulated mast cells are marked by arrows.

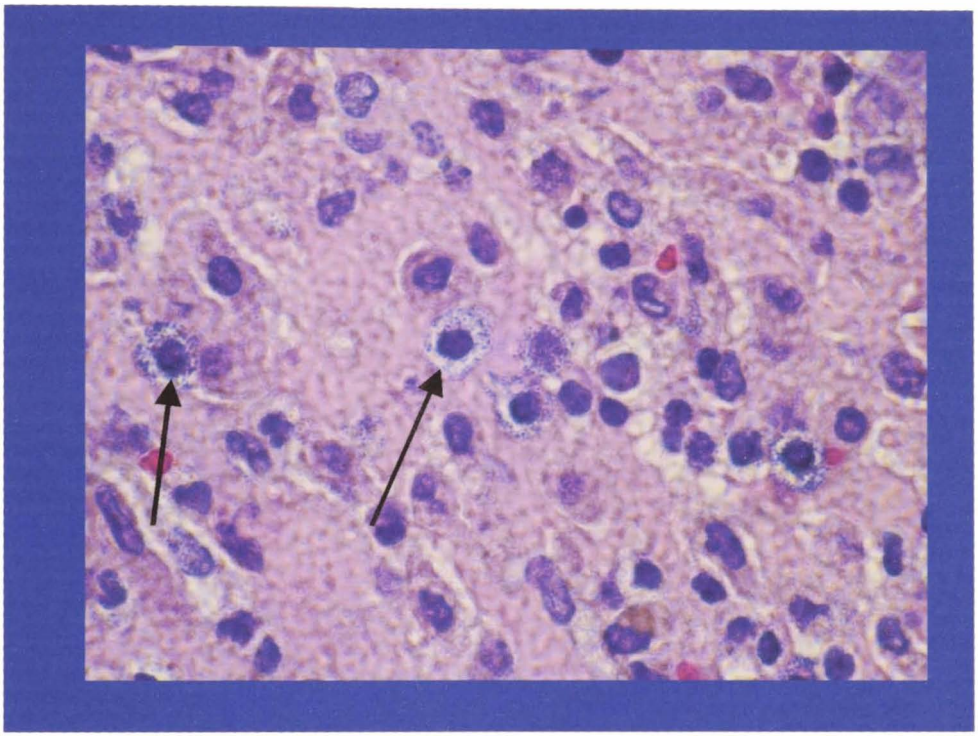


Figure 3.6 Mitotic Figures in Tumor Tissue
Mitotic figures are numerous in tumor tissue. The arrow marks a mitotic figure present in this histogram. The tissue was stained with toluidine blue; magnification is 100X.

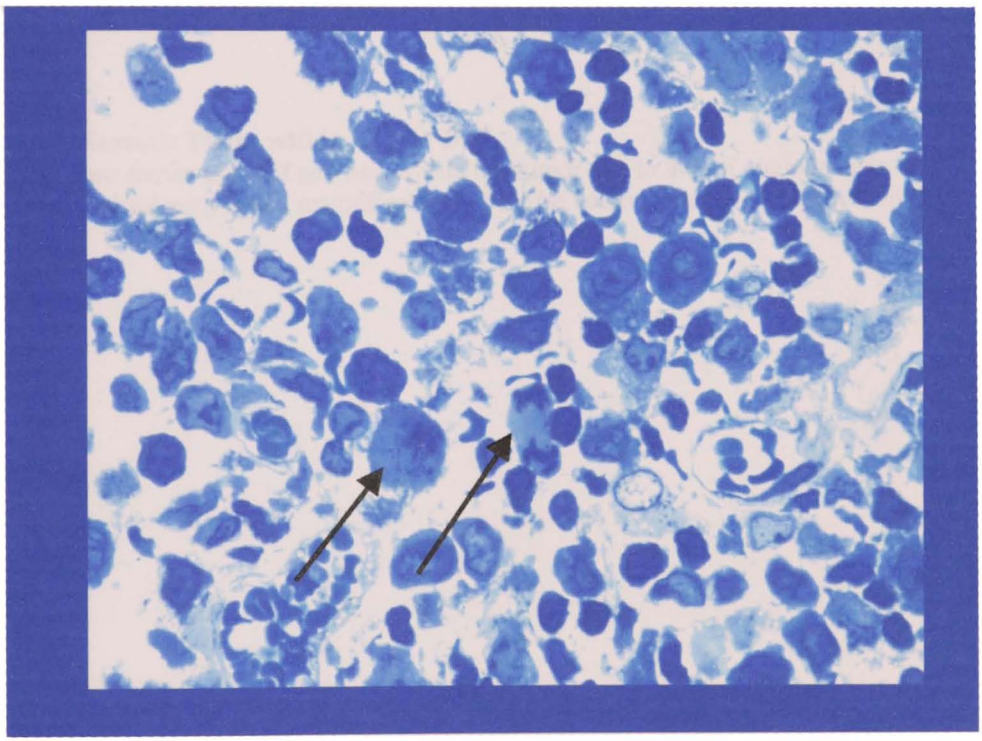


Figure 3.7 Necrotic Tissue within Tumor Bed
This histogram shows areas of necrosis within the tumor due to loss of angiogenesis. Tissue was stained with H&E; magnification 10X. The necrotic area is demarcated by asterisks.

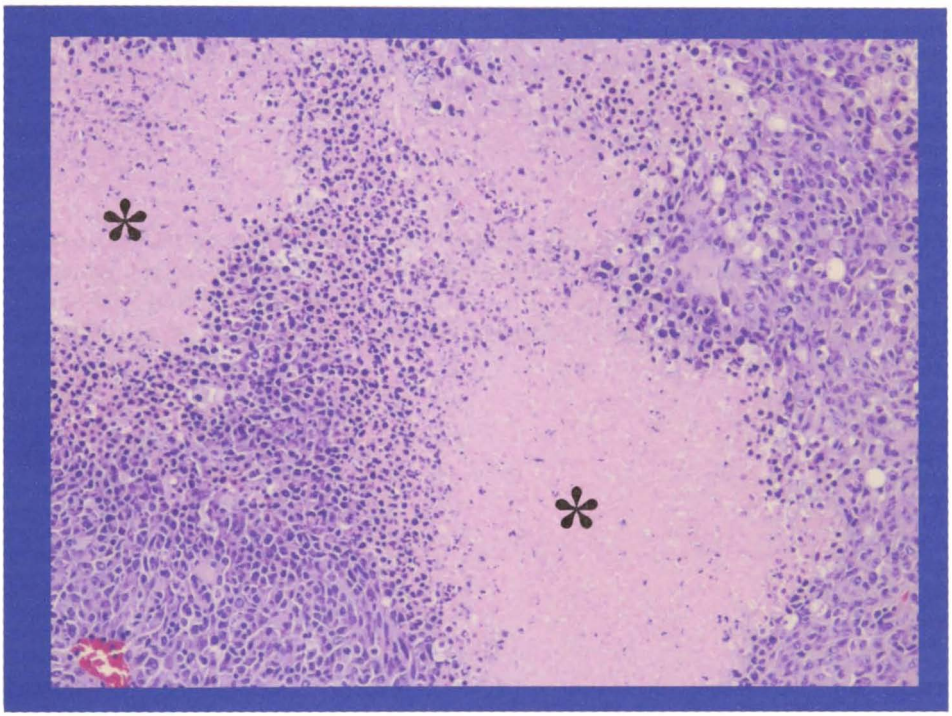
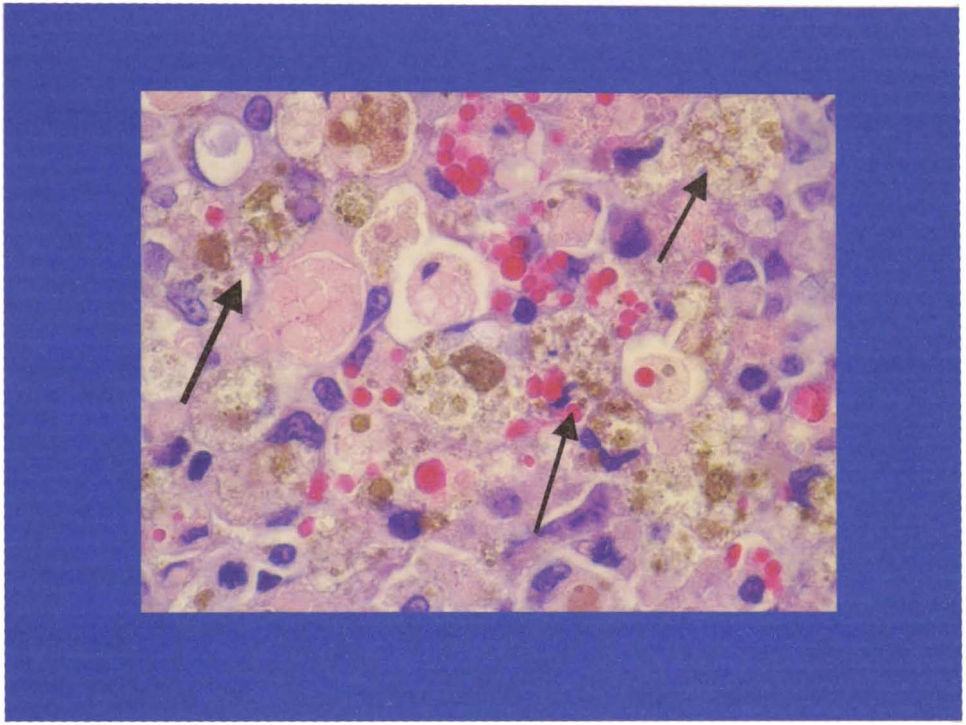


Figure 3.8 Hemosiderin-filled Macrophages. This is a histogram of macrophages containing phagocytosed red blood cells and the by product - hemosiderin. Tissue was stained with H&E. Magnification is 100X. Arrows are used to point out specific macrophages, but the hemosiderin-filled macrophages primarily fill the field.



Discussion:

There has been tremendous interest in the potential for immunotherapeutic manipulation of human brain tumors, and numerous studies have been performed to determine whether human brain tumors express Ags recognized by the immune system in the host of origin. As a result of those studies, a general consensus has been reached that human brain tumors are not antigenic/immunogenic. The difficulty with human studies is that there are too many unknows: 1) it is not known whether human gliomas are antigenic; 2) whether the assays that were employed were appropriate for detection of antitumor responses; and 3) whether tumors progressing in the brain would induce detectable systemic immune responses even if the tumors were antigenic. The rationale for the current study was that some of those issues needed to be addressed under conditions where the number of unknowns was reduced. Thus, our studies were conducted to determine whether a relatively well defined experimental glioma that was 100% lethal and known to induce detectable specific cytotoxic responses, would induce peripheral immune response when challenged in the brain. This work provided further support to the approach of developing methods of immunoprophylaxis of weakly immunogenic lines and human brain tumors.

Chapter 4

MEMORY LYMPHOCYTES FACILITATE SURVIVAL

Introduction

Immunological memory manifests itself as an accelerated and elevated response to a second stimulation by the same or closely related Ag (Mullbacher, 1994). Establishment of long term memory underscores all protective vaccination strategies. One of the characteristics of an immune response is the acquisition of memory which focuses on establishment of long-term memory in the absence of constant antigenic challenge. We used rats that survived tumor challenge in Chapter 3. These data are well represented in our experimental model using a 1° and 2° vaccination in RT-2 glioma challenged rats that have survived beyond 180 days post first IC challenge and were then rechallenged in the left contralateral hemisphere. We hypothesize that these methods were able to generate long-term Ag-specific cytotoxic T lymphocytes. These T cells were long-lived lymphocytes involved in the experimental rats surviving beyond 60 days after IC challenge and this was attained without Ag repriming. These observations suggested that T cell memory is long-lived in the absence of RT-2 tumor Ag.

Materials and Methods

Animals and the RT-2 cells were prepared as mentioned in Chapter 2.

Intracranial Tumor Implantation

Tumor cells for IC implantation were trypsinized and washed twice with sterile PBS. Viable cells were identified by trypan blue exclusion, enumerated on a hemacytometer, and a final suspension of 1×10^4 viable cells per 5 μl of PBS was made. Animals were anesthetized by an intraperitoneal injection of ketamine·HCl (87 mg/kg) and xylazine (6.5 mg/kg). The scalp hair was shaved, wiped with betadine and an incision was made over the cranial midline. Animals were placed in a stereotactic apparatus, bregma was located and used as a reference point for injections. A hand-held Dremel drill was used to create a shallow depression 4 mm to the left of the sagittal suture and 1 mm posterior to the coronal suture. Five μl of the tumor cell suspension (1×10^4 cells) was injected into the parietal lobe of the brain at a depth of 3.5 mm using a Hamilton syringe and a 26-gauge needle secured to the arm of the stereotactic apparatus. The needle tract was sealed with bone wax and the incision closed with surgical staples.

Methods of Determining Immunological Memory

Treatment #5:

To determine if immunological memory (long term memory) had been established in long term survivors. Group B animals from Table 4.1, that survived beyond 180 days after IC challenge of viable RT-2 glioma cells, were rechallenged in the contralateral left

hemisphere with RT-2 tumor cells. Group A (control group) (n=10) were unvaccinated animals that received RT-2 tumor cells in the left cerebral hemisphere.

Table 4.1 Long Term Memory in Experimental Animals

Group	# of Animals	Treatment	Survival
A	n=10 (control)	IC challenge 10^4 RT-2 cells	0%
B	n=10 (experimental)	IC rechallenge 10^4 RT-2 cells	100%

Results:

The control Group A, showed 0% survival. All of Group A (control rats) died by Day 21 post implantation. The control rats had received sterile PBS as a vehicle instead of RT-2 glioma cells. Results of rechallenge in the left hemisphere showed 100% survival for Group B experimental animals from Table 3.1. These animals survived beyond 60 days.

Discussion:

Peripheral T lymphocytes can be classified into two groups: naïve and memory T cells. The focus of this study was to examine the duration of T cell memory in long-lived rats. The ability of a T cell to recognize a specific peptide epitope in the context of MHC molecules by APCs from tumor cells is provided by its T cell receptor. The interaction between T cell receptor and the Ag-MHC triggers proliferation and clonal expansion of specific T cells. Some of the progeny of the Ag-responsive T cells develop into Ag-specific memory T cells. This subpopulation of T cells is maintained within the host and provides immune surveillance. In the event of reactivation by subsequent re-exposure to

the tumor, specific memory T cells become activated and clonally expand with greater magnitude than during the initial response. It is generally accepted that specific immune T cell memory persists after an encounter with an Ag and may help to protect the immune host against subsequent exposure to that pathogen.

The experiments performed in this chapter suggested that specific immunity was induced by 1° and 2° vaccinations with viable irradiated RT-2 glioma cells in HFPs and the NN. The first IC challenge was in the right cerebral hemisphere. The experimental rats that survived were rechallenged 180 days beyond the first challenge in the left cerebral hemisphere. Control rats died between days 16 and 18 after IC challenge with tumor cells. 100% survival was seen in the left cerebral hemisphere challenged rats. We have reported on a series of experiments that suggested that immunological memory in rats that received multiple vaccinations with the immunogenic RT-2 glioma cell line could be developed and persist. In the first experiments, rats vaccinated by subcutaneous injections of RT-2 cells were challenged in the right cerebral hemisphere. The immunological response was specific and these animals remained protected after a rechallenge with RT-2 tumor cells in the left cerebral hemisphere. This work provides further support to the approach of developing methods of immunoprophylaxis to treat weakly immunogenic lines and human brain tumors.

Chapter 5:

MEMORY LYMPHOCYTES PROTECT AGAINST THE DEVELOPMENT OF RT-2 GBMS

Introduction

Active, specific immunotherapy with anti-cancer vaccines offers the possibility of stimulating the intrinsic immune response of a patient to his or her tumor, with the development of long-term immunologic memory through helper and cytotoxic T cells. This vaccine may assist prophalactically in the protection of tumor development and rejection of existing tumor cells. It may also help in surveillance against their re-emergence. A draw back to some experimental studies may be seen in the fact that vaccination-based immune therapy for cancer must be shown to work in the presence of a pre-existing neoplasm. In contrast to this type of immune treatment, we want to address the use of classical vaccination strategies which are preventive.

These experiments examined the use of various leukocytic suspensions, from the spleens of 1° and 2° vaccinated rats that had survived for more than 210 days, as vaccines. Seven days prior to harvesting the spleens, animals received a booster of 10^4 irradiated RT-2 glioma cells in the right hind flank.

Materials and Methods

Animals and the RT-2 cell line were treated as mentioned in Chapter 2.

Treatment #6:

To deplete various lymphocytic cell populations from spleen cells of rats that received a 1° and 2° vaccination at least 210 days prior to harvesting spleens. Splenocytes were selected for transfer because of convenience versus use of lymph nodes. Table 5.1 gives a breakdown of leukocytic makeup by % as determined by Dr. Martin Graf.

Table 5.1 Percent makeup of leukocytic cells in spleen

Leukocytic Cells	Percentage
B Lymphocytes	35%
$CD4^+$ T Lymphocytes	25%
$CD8^+$ T Lymphocytes	10%
Natural Killer Cells	10%
Monocytes	10%
Others	5%

The purpose was to examine and determine which effector and long-term memory cells were necessary for transfer to provide protection to naïve rats before IC challenge with viable RT-2 glioma cells. The spleen-donor rats (n=6) received a booster of 10^4 irradiated RT-2 glioma cells delivered in sterile PBS in the right hind flank to up-regulate the secondary immune response.

Seven days post hind flank injection, spleens (n=6) were harvested from the vaccinated rats. To obtain cells, spleens were aseptically minced and pushed through a

nylon mesh by use of the butt of a plunger from a sterile 3 ml syringe. The leukocytic population was pooled and consisted of the following groups as seen in Table 5.2. Group 1: T and B cells together as a passive transfer of a whole cell suspension; Group 2: $CD4^+$ T cell-depleted suspension; Group 3: $CD8^+$ T cell-depleted suspension; Group 4: pan T cell-depleted suspension; and Group 5: sterile PBS (control group). Purity of cells transferred was 90%. The number of cells transferred was $4x10^8$ per group. For future studies, we will alter the numbers of the various lymphocytes (increase or decrease) transferred accordingly.

Twenty four hours after receiving an IP injection of a particular cell suspension or PBS, experimental and control rats received IC injections of viable RT-2 cells ($1x10^4$) in the right cerebral hemisphere. The animals were observed for survival.

Results:

Group 1 = whole cell suspension = 2/3 rats survived. Group 2 = $CD4^+$ T cell-depleted suspension = 3/3 rats succumbed to tumor. Group 3 = $CD8^+$ T cell-depleted suspension = 2/3 rats survived. Group 4 = pan T cell-depleted cell suspension = 1/3 rats survived. Group 5 = sterile PBS control = 3/3 rats succumbed to tumor. See Table 5.2.

Table 5.2 Depletion Studies and Survival

Groups	Cell Suspension	N=3	Animal Survival	Deaths	Days Survived
1	whole cell suspension	3	2	1	30 Days
2	$CD4^+$ T cell-depleted	3	0	3	20 Days
3	$CD8^+$ T cell-depleted	3	2	1	30 Days
4	pan T cell depleted	3	1	2	30 Days
5	sterile PBS	3	0	3	17 Days

Discussion:

A central observation in immunology is the finding that upon a secondary contact with a given Ag, the immune response obtained differs from the response produced after the first antigenic encounter. The secondary response that produces effector/memory T lymphocytes persists long after the first encounter with Ag. This memory property of the immune system, together with two other characteristics, specificity and diversity, makes the immune system an attractive biological entity to investigate prophylactic treatment by use of vaccines to prevent development of or treatment of brain tumors. The morbidity and mortality caused by infectious diseases has been reduced by the important role of vaccination. The ultimate goal of vaccines is to develop long-lived immunological protection, whereby the first encounter with a pathogen is remembered. This leads to enhanced memory responses that either completely prevent re-infection or greatly reduce the severity of the disease. It has been well established that the anamnestic response that is mediated by memory $CD4^+$ and $CD8^+$ T cells is more rapid and aggressive than the primary immune response (Kaech, et al., 2002). This faster T-cell response can control secondary infections quickly and fully eliminate the pathogen. In our previous experiments based on mT cells, the emphasis was placed on $CD8^+$ cytotoxic lymphocytes for long-term survival.

However, in this last experiment that involved depletion studies of $CD4^+$ T cells

and $CD8^+$ T cells in cell suspensions created from harvested spleens of long-term survivors to RT-2 glioma challenge, CD8-depleted cell suspensions given to rats followed by IC injection of viable RT-2 tumors demonstrated long term survival. The suspension that was CD4-depleted was not able to protect against the development of RT-2 tumor and ultimate death. These rats did survive longer than naïve rats that received IC challenge only, but they finally succumbed to tumor. The results of this experiment suggested that the presence of transferred memory $CD4^+$ T cells played a significant immunological role required to provide protection against IC tumor challenge with viable RT-2 glioma cells. This is in contrast to the lack of survival seen in animals that received $CD8^+$ T cells and no $CD4^+$ T cells in the cell suspension.

The results of this experiment suggested that the presence of memory $CD4^+$ T lymphocytes were required for protection against a challenge of viable RT-2 glioma cells. The protective mechanism could possible have been that $CD4^+$ T cells were capable of production of the cytokines IL-2 and IL-4.

The production of these cytokines created a division of $CD4^+$ T cells into two separate entities. T lymphocytes are divided into Th-1 cells that are responsible for production of IL-2, INF-γ, and IL-12. Th-2 cells are responsible for the secretion of IL-4, IL-3, IL-5 and IL-6.These cytokines play a pivotal role in the communication network of cells. Pleiotropic functions associated with the IL-4 cytokine was first described to enhance DNA synthesis of purified resting mouse B cells (Topp *et al.*,1995). Topp *et al.*,

1995, suggested that the following cell lines were involved in the production of IL-4: $CD4^+$ T lymphocytes and to a lesser extent by mast cells and human tumor-associated natural killer cells. $CD4^+$ T cells are pivotal in the immune response in that they control the development and activation of B lymphocytes, $CD8^+$ T cells, macrophages, and other inflammatory cells (Bunce and Bell, 1997).

Functional properties of IL-4 include (1) activation of resting B cells, (2) enhancement of IgE production in activated B lymphocytes, (3) an increase in expression of the low-affinity receptor for IgE, (4) a co-stimulant for resting T cells, and (5) their activation of cytolytic $CD8^+$ T lymphocytes.

A number of studies have examined the ability of naïve and memory $CD4^+$ T cells to synthesize cytokines after activation. Similar observation on $CD8^+$ T cells have been performed but observations suggest that naïve and memory $CD8^+$ populations can synthesize cytokines, but to a lesser extent than the $CD4^+$ population (Akbar, 1991). According to Akbar, 1991, IFN-γ, IL-3 and IL-6 are preferentially synthesized by the $CD4^+$ memory T cell population rather than by the naïve cells.

These results strongly indicate that $CD4^+$ T cells were required for long term survival. These cells were a mixed bag. Therefore, three possibilities existed: 1) a humoral response promoted by $CD4^+$ T cells; 2) CTL response promoted by $CD4^+$ T cells; and 3) presence of cytotoxic $CD4^+$ T cells. Our primary focus was number two.

The data presented here can be interpreted in a number of ways, but a hypothesis

which incorporates our findings is the lack of induction of effective antitumor reactivity against parental RT-2 cells in a CD4-depleted cell suspension. This could be due to a paucity of memory helper cell function, as well as a consequence of suppressor factors expressed by tumors. The ability of these rodents to survive beyond 20 days, possibly represents an attempt by mCD8 $^{+}$ cytotoxicT cells to perform in a cytotoxic role. However, without $CD4^{+}$ effector/memory T cell help, the numbers of $mCD8^{+}$ cytotoxic T cells probably declined along with production of IL-2. This reduction in IL-2 could inhibit the activation of naïve T cells to become a large CD4 and CD8 effector population. This, along with tumor suppressor factors, had a potential significant effect allowing tumor to escape the cytolytic activities of $CD8^{+}$ lymphocytes.

The mechanism(s) by which $CD4^{+}$ T cells contribute to the initiation and persistence of $CD8^{+}$ T cell responses remain incompletely defined. Until recently, CD4-dependent help for CTL responses was thought to result primarily from the production of IL-2 to support activation of $CD8^{+}$ T cells (Wagner *et al.*, 1980; Keene, J. and Forman J., 1982). An additional mechanism for the provision of help in which $CD4^{+}$ T cells interact with APC in a CD40-dependent manner to "condition" the APC for effective activation of $CD8^{+}$ T cells has been recently demonstrated (Ridge, J. *et al.*,1998; Bennett, S. *et al.*, 1998; and Schoenberger, S. *et al.*, 1998).

In some cases, CD4 help is necessary to initiate CTL responses. However, $CD8^{+}$ T cells can produce their own IL-2 response to costimulation to drive activation in the

absence of $CD4^+$ T cells (Azumi, M. *et al.*, 1992; Harding, F. and Allison, J., 1993; and Deeths, M. and Mescher, M., 1997). A CTL response occurred in the absence of help but the response only persisted if CD4-dependent help was available. This was true in a number of models of virus infection, tumors, and autoimmunity (Tham, E. *et al.*, 2002). This suggested that different mechanisms might operate at differing stages of $CD8^+$ T cell activation, differentiation, clonal expansion, and survival.

Tham, E., *et al.*, (2002) demonstrated that if IL-2 was absent 3 days after the activation response in $CD8^+$ lymphocytes was initiated, the cells lost the ability to make further IL-2 in response to Ag and costimulatory ligands, and clonal expansion ceased. They described this non-responsiveness as resembling the "anergic" state originally described for $CD4^+$ T cell clones following TCR engagement in the absence of costimulation. In the $CD8^+$ CTL population, it was distinct, in that it occurred following full stimulation through both the TCR and costimulatory receptors. Tham, E. *et al.*, (2002) have termed this state of $CD8^+$ T cells "activation-induced nonresponsiveness" (AINR) to distinguish it from the classical anergy that occurs when T cells receive just one signal. Further examination resulted in the demonstration that the AINR state was reversed if continued proliferation was supported for a short time (1-2 days) by provision of help in the form of IL-2. After this, the $CD8^+$ T cells regained the ability to produce IL-2 and continued to respond to Ag in an autocrine-driven manner for prolonged times (Tham, E., *et al.*, 2002).

In conclusion, helper-independent initiation of CTL responses have been demonstrated in numerous models examining virus infection, tumors, and autoimmunity but these responses have often failed to persist in the absence of $CD4^+$ T cell help. Naïve T cells responded to Ag and costimulation by producing IL-2 to drive their initial clonal expansion, but within a few days the cells became AINR. They could still be signaled through the TCR to carry out effector functions but could no longer produce IL-2 to sustain expansion. However, the CTL could continue to proliferate if provided with IL-2. This suggested that the basis for the requirement for $CD4^+$ T helper cells is to sustain effective CTL responses in many instances.

Chapter 6

GENERAL DISCUSSION AND LITERATURE REVIEW

The successful application of immunological methods to the diagnosis and control of clinical infectious disease during the 19th century had a resounding influence on the reach of medical aspirations (Hewitt, H., 1982). It fostered a hope that soon hardened into conviction that human malignant disease would eventually yield to similar approaches. In the 1800s, physicians noticed that tumors sometimes regressed in cancer patients who contracted bacterial infections. Therefore, the clinical support for the idea that the immune system might restrain the development of cancer emerged. William B. Coley, a surgeon at Memorial Hospital in New York City from 1892 to 1936, dedicated his life to creating therapies based on this observation (Old, L., 1998) These treatments are now considered immunotherapies because they aimed to attack disease with the body's own defenses that brought about complete tumor regressions in some individuals. Deliberate attempts were made by him to infect cancer patients with bacteria. He later developed a vaccine consisting of killed bacteria to prompt a tumor-killing response. However, because the results were unpredictable, they were not widely accepted.

During the 20th century, the search for oncogenic agents and exploration of immunological aspects of clinical cancer have persisted. In the early decades of the 20th

century, experimental cancer research was confined to the use of allo-grafted animal tumors. The high frequency of induced or spontaneous regression sustained the concept of tumor immunity. By the 1920's it had been disclosed that autochthonous animal tumors displayed no such phenomena, and the subject of clinical tumor immunity was considered closed (Leitch, A., 1920). In 1943 it was reopened; Gross had demonstrated that xenografts of a chemically induced tumor were immunogenic (Gross, L., 1943). However, the repeated demonstration during the 1950's, that animal tumors were of spontaneous origin, invariably failed to display the immunogenicity shown by nearly all chemically induced tumors was ignored (Foley, E., 1953; Prehn, R., 1957; and Revesz, L. 1960).

In 1965, Rous (Rous, P., 1965), a pioneer of viral oncogenesis, used the occasion of his Nobel address to warn against generalization of a concept of viral oncogenesis beyond the meager evidence supporting it. Cancer-causing viruses were not, he said, an "Open Sesame!" to the cancer problem (Hewitt, H., 1982). Proponents of a topic that was confidently flourishing previously came to write discouraging titles as "Back to the drawing board..." (Alexander, P., 1977), "The questionable immunogenicity of certain neoplasms" (Weiss, D., 1977) "...non-rejectability of spontaneous tumors... (Klein, G., and Klein, E., 1977); and "The case history of Mr. T.I. – Terminal patient or still curable?" (Nossal, G., 1980). Still , the link between immunity and cancer remained firmly fixed in the minds of many people. During the 1960s and 1970s there was wide

acceptance of the "immunosurveillance" model put forth by Lewis Thomas of New York University and MacFarlane Burnett of the Hall Institute in Melbourne, Australia (Old, L., 1998). This theory held that the immune system constantly seeks out and destroys emerging cancer cells. Tumors, it proposed, arise when this policing mechanism failed. Because accumulating evidence suggested that the immune system attacked only tumors caused by viral infections and this accounted for only a small number of tumors, the theory appeared flawed.

Coley's approach to cancer therapy was described as nonspecific: it strengthened the overall activity of the immune system instead of selectively arousing those elements most able to combat cancers. During the last century, scientists developed a range of other nonspecific immunotherapies – e.g. Bacillus Calmette-Gurin, or BCG as a vaccine to treat bladder cancer (Old, L., 1998). Although this vaccine illustrated the potential of non-specific immunotherapies, it acted locally, provoking inflammation only in the bladder. Most cancers become lethal because they spread and give rise to tumors at a distance. To eliminate these growths, immunotherapies had to be capable of seeking out incipient tumors in all parts of the body. To accomplish this, many research oncologists turned in the 1970's and 1980's to molecules the body produced in response to viral and bacterial infections, now called cytokines, to help orchestrate the defense response. The cytokines included such proteins as interferons, interleukins, and tumor necrosis factor. Extensive clinical testing of this nonspecific approach dampened enthusiasm because

relatively few patients appeared to benefit from cytokine therapy alone (Old, L., 1998).Cytokines have proven more valuable in combination with one another or with other treatments.

The discovery of antibodies at the end of the 19th century provided the means to search for such cancer-specific Ags and later opened the way for extensive studies of antibodies as potential immunotherapies for cancer (Old, L., 1998). Antibodies are a critical component of the immune system and circulate in the blood and bind to foreign Ags. Therefore, they are able to mark Ag-bound invaders for destruction by scavenger cells called macrophages, by other cells and by special blood protein components, collectively called complements.

In 1975, the search for Ags became easier. This was made possible by the discovery made by Cesar Milstein and Georges J. F. Kohler of the University of Cambridge (Old, L., 1998). These researchers demonstrated that Ab-producing cells could be made to survive indefinitely if they were fused with cancer cells. This technique enabled scientists to produce unlimited supplies of identical Abs, because any given Ab-producing cell produces only a single species of Ab. This method profoundly effected cancer immunology for several reasons. First, it provided a powerful new method to search for cancer antigens. Secondly, defined Abs could be produced in sufficient amounts to put Ab-based therapies to the test (Old, L., 1998).

Monoclonal antibodies have revealed a large array of Ags that exist on human

cancer cells. However, virtually all these Ags are also found on normal cells. These normal cells might be damaged by an Ab-based therapy. This overlap does not preclude their use as therapeutic targets for several reasons: the Ag in normal tissues may not be accessible to blood-borne antibodies; the cancer cells may express more Ag than normal cells do; and antibody-induced injury of normal cells may be reversible. In the Ab-based vaccines discussed, the injected Ab is derived from an animal. The treatment is considered passive immunotherapy: the immune molecules are given to patients, who do not produce them on their own. A vaccine, however, is deemed active immunotherapy because it rouses an immune response in the individual who needs protection. The technology of Ab production gave rise to high expectations as well as to premature and unrealistic assertions about Abs as "magic bullets" (Old, L., 1998).By recognizing specific Ags, it was hoped that monoclonal Abs would home in on cancer cells and trigger an immune attack that destroyed the target cell but ignored normal cells lacking the cancer Ags. Many expected that these bullets would be more deadly by loading them with toxic chemicals; the Abs would carry the toxins directly to the tumor, where the poisons would kill cancer cells. At first the technology was declared a failure. The reality of the situation was far more positive. The concept remained sound, and slow, steady progress has been made in developing Ab therapies. Today, Abs can target cancer cells and act on other cell types and molecules necessary for tumor growth. Antibodies can neutralize growth-factors chemicals needed by tumor cells and their blood supply and

thereby inhibit a tumor's expansion. Antibodies can also target the stroma, the connective tissue between tumor cells (Old, L.,1998). This and other Ags that mark tumor stroma or blood vessels have become attractive targets to researchers devising Ab-based therapies.

As previously mentioned, tumors of the CNS are poorly responsive to the three modalities of conventional anticancer therapy: surgery, radiation, and chemotherapy. However, since the demonstration of immunogenicity of chemically induced tumors in mice, it has been the goal of tumor immunologists to develop novel approaches to cancer therapy based on manipulating antitumor immunity (Prabir, K. *et al*., 1992). Some of the strategies presented by these authors include (a) active specific immunotherapy with autologous or allogeneic irradiated tumor cells or their extracts in the presence of adjuvants,(b) infusion of large doses of interleukin-2 (IL-2) with or without lymphokine-activated killer (LAK) cells, and (c) adoptive immunotherapy involving *in vitro* activation and amplification of tumor-infiltrating lymphocytes followed by their infusion into a tumor-bearing host. Clinical trials employing these approaches have been conducted. The use of immunotherapy as a clinical tool has been an area of enthusiasm and intense investigations.

Adoptive immunotherapy, whereby immunoreactive cells with antitumor activity were transferred into the tumor bed at the time of debulking, was an approach for potential treatment of high-grade gliomas. With the advent of recombinant DNA techniques, recombinant IL-2 (rIL-2) was available in sufficient quantity to allow the

generation of greatly expanded numbers of activated lymphoid cells for therapy (Kruse *et al.*, 1989).

Lillehei, K. *et al.*, 1987 began clinical trials that involved intratumoral implantation of both preparations of lymphocytes, together with rIL-2, in patients with recurrent, high grade gliomas. The lymphokine activated killer (LAK) cells and autologous-stimulated lymphocytes (ASL) were successfully generated from the PBL of brain tumor patients.

Kruse *et al.*, 1989 described two separately activated preparations of cells, derived from peripheral blood to use therapeutically against brain glioma. One preparation was widely known as lymphocyte activated killer cells. The other preparation was termed autologous-stimulated lymphocytes. These are lectin-responsive and combinant IL-2-responsive cells. Clinical researchers have reported the use of LAK cells against brain tumors (Jacobs, S. *et al.*, 1986 and Merchant *et al.*, 1988). Others have reported the similar use of ASL (Ingram, M. *et al.*, 1987; and Ingram *et al.*, 1987). Two separate studies examined the capacity of PBL obtained from glioma patients to respond to PHA and rIL-2 stimulation. Yoshida *et al.*, 1987, concluded that PBL from patients with malignant brain tumors produced a level of IL-2 lower than that of normal control subjects because of the decreased number of IL-2 producing T cells. PHA-activated PBL from both responded equally well to rIL-2. Elliot *et al.*, 1987, also found that rIL-2 did not improve the PHA responsiveness of PBL from patients who had not received

radiation, steroids, or phenytoin. Kruse *et al.*'s, 1989 data showed that there was a statistically significant difference in the short-term cytotoxicity against all targets of brain tumor patients' PBL stimulated with rIL-2 alone (LAK) versus those stimulated with PHA and rIL-2 (ASL). They have found that in long-term cytotoxicity assays this difference disappears. Kruse *et al.*, 1989, suggested that different types of activated cells were responsible for the target cell lysis, and that they perhaps killed in a mechanistically different fashion. The difference may have been due to the fact that LAK cells did not recycle as well as other activated T-cells and/or NK cells. Their work also suggested that the effector cells could produce stasis of glioma by producing lymphokines such as tumor necrosis factor, leukoregulin, interferon, and lymphotoxin (Merchant, R. *et al.*, 1986).

The results of this study by Kruse *et al.*, 1989, showed that the production of ASL was labor intensive, involving long-term culture and a greater chance of contamination. Kruse *et al.*, 1989, studied the LAK cells and ASL preparations and compared them to the starting PBL by growth and viability measurements, cytotoxicity, phenotype expression using specific monoclonal antibodies to characterize cell surface proteins, and morphology as seen by electron microscopy. They determined that the two preparations were distinct and that both were tumoricidal against known tumor cell lines. However, LAK cells and ASL derived from the peripheral blood of patients with intrinsic brain tumors exhibited a wide variation in their capacity for *in vitro* growth. This could be explained, in part, to the chemotherapy that patients received (Kruse *et al.*, 1989). The

modern vaccine story starts in the 1940s and 1950s with a fundamental discovery of tumor immunology. Scientists found that when chemicals or viruses induced tumor in mice, the tumor bore Ags that could immunize other mice of the same strain against transplants of the tumor. Subsequent studies showed that immune system cells known as T lymphocytes taken from immunized animals could transfer immunity against tumors in healthy animals of the same strain. Workers also devised techniques to show that the T cells from immunized mice could kill tumor cells grown in test tubes as well (Old, L., 1998). Antibodies elicited by the tumor cells generally failed to transfer immunity or kill tumor cells.

The next challenge was to isolate the tumor Ags recognized in this system so that they might be tested in a vaccine. Thierry Boon, 1993, and his colleagues at the Ludwig Institute for Cancer Research in Brussels developed a method to do just that (Old, L., 1998). The technique revealed two main categories of tumor Ags that evoked a T cell response in melanoma patients. According to Old, L., 1998, the first category of Ags called MAGE, BAGE, and GAGE that were produced by tumor cells but not by normal cells outside the testes. The second category of Ags, including tyrosinase and Melan A, were called differentiation Ags; they were made from by both melanoma cells and melanocytes, normal cells from which the tumor cell arises.

Another source of information about potential tumor Ags comes from the discoveries concerning genetic changes in cancer cells. Among the most attractive targets

for vaccines are abnormal proteins that are made when genetic mutations turn normal genes into cancer-promoting versions. A long list of cancer-related genes – known as oncogenes and tumor suppressor genes – are being compiled. Although much uncharted areas lie ahead, there are now more vaccine-based therapies than before and promise hope for the future.

The studies that comprised the formal research basis for this dissertation included the following projects: 1) examination of the ability of a 1^{o} vaccination to protect against RT-2 tumor and the demonstration of that specificity; 2) examination of the ability of a 1^{o} and 2^{o} vaccination paradigm to provide protection against an IC challenge of viable RT-2 glioma cells; 3) examination of memory T cells in vaccinated and aged rats and 4) examination of depletion studies to see if passive transfer of effector/memory cells was possible.

To address the questions put forth, a highly immunogenic glioma cell line (RT-2) was used. The RT-2 glioma cell line is uniformly lethal, whether implanted s.c. or IC. It provides an efficient animal model in which to explore the efficacy of lymphokine and cellular immunotherapy for brain tumors. The RT-2 glioma model has been used extensively in our lab and others, is well characterized, and shares many features with human gliomas.

We have described an experimental system in which vaccination with irradiated RT-2 glioma cells activated an immunological response against the tumor. This was

achieved in the 1° vaccination paradigm with 30% survival and in the 1° and 2° vaccination paradigm with 100% survival seen in animals with IC challenge in the right cerebral hemisphere with viable RT-2 tumor cells. These data have described an experimental system in which a population of resting, primed $CD4^+$ T lymphocytes and $CD8^+$ CTLs can be generated in vivo and migrate to and initiate an immune response to the tumor cells.

Immunological memory is described as the capacity of the immune system to respond faster and more efficiently against an Ag it has encountered in the past. T cell memory is believed to result from an increase in the frequency of Ag-specific T lymphocytes (Ahmed, R. and Gray, D., 1996; and McHeyzer-Williams, M. and Davis, M., 1995). Our results suggested that these populations of cells contributed to the 100% survival rate.

Specialized cells known as memory T cell and B cells, and long-lived effector B cells (plasma cells), which constitutively secrete high-affinity 'neutralizing' antibodies, are the basis of immunologic memory (Kaech, S., et al., 2002). The memory T cell compartment consists of both $CD4^+$ and $CD8^+$ T cells that acquire effector functions to kill infected cells and/or secrete inflammatory cytokines that inhibit the replication of pathogen. Effector $CD4^+$ T cells also help B-cell responses and enhance $CD8^+$ T cell development, through the activation of APCs or secretion of cytokines, such as IL-2, IL-4 and IL-15. Although there might be interesting parallels between T- and B-cell

development, this work focused primarily on the formation of effector and memory T cells.

In the first study, we found that a 1° vaccination of irradiated RT-2 cells in the HFPs or NN saw 30% survival of experimental animals. This was promising data that directed the next set of experiments. Again, in the second study a vaccination paradigm followed the first experiment. This one required a 1° and 2° vaccination of irradiated cells in the NN and HFPs. Complete survival was attained in the experimental animals and all control animals succumbed to tumor.

In the third set of experiments, we treated long-lived immunized rats with a secondary IC challenge in the opposite hemisphere 180 days post first IC challenge. This group of animals also resulted in 100% survival in the experimental rats and no survivors for the control group.

The fourth experiment was performed to answer the questions if we could passively transfer memory lymphocytes from spleen leukocytes from long-lived immunized and challenged rats. This was done in the form of depletion studies. The results were interesting in that the $CD8^+$ T cell depleted group survived longer than the $CD4^+$ T cell depleted group. Future studies should look at the naïve and memory populations using the CD45 mAbs markers that are available for humans, mice and rats. Then one could look at the relative numbers of naïve T cells versus memory T cells during a vaccination or vaccinations. The cytokines IL-2 and IL-4 involvement in $CD4^+$

Th-1 and Th-2 response versus $CD8^+$ CTL response should be looked at more closely to determine the roles of these two cytokines in memory cell production.

The role of $CD4^+$ cytotoxic T cells should also be studied to see if they have a potential role in prolonging survival in tumor-bearing rats or provide protection from development of tumor. Vaccination has had an important role in reducing the mortality and morbidity that is caused by infectious diseases. The ultimate goal of a vaccine is to develop long-lived immunological protection, whereby the first encounter with a pathogen is 'remembered', which leads to enhanced memory responses that either completely prevent reinfection or greatly reduce the severity of disease.

Many promising opportunities wait to be studied, and they give us reason to expect that powerful immunologic therapies will one day become a reality.

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