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Tumor Immunity Following Adenovirus Mediated Herpes Simplex Thymidine Kinase Gene Transfer to Experimental Rat Gliomas

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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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With respect and thanks,

Maulik R. Shah

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List of Abbreviations

ATCC	American Type Culture Collection
AV-LacZ	Recombinant Adenovirus Expressing The
AV-TK	Recombinant Adenovirus Expressing The Herpes Simplex Thymidine Kinase Gene Under Control Of The Rous Sarcoma Virus Promoter
BBB	Blood Brain Barrier
BCG	Bacillus-Calumet-Guerin Strain Of Mycobacterium
ß-gal	b-Galactosidase Gene Under Control Of The Rous Sarcoma Virus Promoter
BRL	Bethesda Research Laboratories
BSA	Bovine Serum Albumin
C. Parvum	Corynebacterium Parvum
CD	Cluster Designation
CGTB	Clinical Gene Therapy Branch
CNS	Central Nervous System
Con A	Concavalin A
CTL	Cytotoxic T-Lymphocytes
DMEM-10% FCS	Dulbecco's Modified Eagle Medium Supplemented With 10% Fetal Calf Serum
DMEM-2% FCS	Dulbecco's Modified Eagle Medium Supplemented with 2% Fetal Calf Serum
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence Activated Cell Sorter
FDG	Fluorescein-Di-B-Galactoside
GCV	Ganciclovir (Syntex Corporation)
GFAP	Glial Fibrillary Acidic Protein
HBSS	Hanks Balanced Salt Solution
HSVTK	Herpes Simplex Thymidine Kinase Gene
IFN	Interferon
IL	Interleukin
Ip	Intraperitoneal
LacZ	B-Galactosidase Gene
MCV	Medical College Of Virginia
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MOI	Multiplicity Of Infection
NCHGR	National Center For Human Genome Research (Bethesda, MD)
NIH	National Institutes Of Health (Bethesda, MD)

NK	Natural Killer Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
Pfu	Particle Forming Units
PHA	Phytohemagglutinin
Taa	Tumor Associated Antigen
TGF	Tumor Growth Factor
TNF	Tumor Necrosis Factor

Abstract

TUMOR IMMUNITY FOLLOWING ADENOVIRUS MEDIATED HERPES SIMPLEX THYMIDINE KINASE GENE TRANSFER TO EXPERIMENTAL RAT GLIOMAS

By Maulik Raj Shah, Ph.D.

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

Virginia Commonwealth University, 1997.

Major Director: Dr. Jack L. Haar, Director of Graduate Studies; Professor of Anatomy.

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Previous studies have determined adenovirus mediated herpes simplex thymidine kinase gene transfer (AV-TK) to be effective for the treatment of experimental gliomas. In this study we report three distinct phenomenon. First, animals with complete regression of subcutaneous tumors upon intratumoral injections of AV-TK with concomitant Ganciclovir® (GCV) administration developed tumor immunity. These animals had the ability to reject a subsequent inoculum of lethal doses of tumor cells. This tumor immunity was long standing and protective as far as 6 months

from the time of initial tumor ablation. Of interest, adoptive transfer of splenocytes from AV-TK treated-tumor ablated animals to naïve animals conferred resistance to tumor formation upon injection of lethal doses of tumor cells. This data strongly indicated the mechanism of tumor immunity was cell mediated. Further analysis of the anti-tumor immune response implicated CD8αß cytotoxic T-lymphocytes as the effector cell. Animals with complete tumor regression survived over 300 days and showed no signs of tumor relapse. Therefore, treatment of solid unifocal tumors with AV-TK and GCV may be able to prevent tumor relapse through the generation of an anti-tumor immune response.

Secondly, we determined that AV-TK and GCV treatment efficacy was dependent on tumor antigenicity. Two different subcutaneous tumor models were utilized; the weakly immunogenic 9L and the strongly immunogenic RT2. For the same dose of intratumorally injected AV-TK, a greater percentage of RT2 tumor were eliminated as compared to 9L. Final survival efficacy was dependant on the tumor type and the initial tumor size.

In studying the importance of host immunity in tumor progression, we have determined that *in vivo*, the GCV mediated bystander effect was not sufficient to result in

tumor eradication without involvement of a host immune response. In athymic rats, 9L tumors failed to regress upon AV-TK and GCV treatment. In contrast, tumors of similar size were ablated upon treatment in immunocompetent animals.

Of related significance, adenovirus mediated gene transfer facilitated generation of tumor immunity. Animals with tumors ablated by AV-TK and GCV treatment developed an anti-tumor immune response which was protective against further tumor engraftment. In contrast, alternative treatments such as surgical excision of subcutaneous gliomas or tumor vaccination was not sufficient to protect against secondary tumor challenge. Injection of adenovirus altered the amount and phenotypes of tumor infiltrating lymphocytes from the NK phenotype towards tumor specific CD8+ CTL cells. This immunomodulatory property was potentially responsible for generation of the immune response.

Therefore, AV-TK was effective through two mechanisms. Transfer of the HSVTK gene confered GCV sensitivity resulting in substantial tumor regression and the adenovirus backbone served as an immune adjuvant to augment generation of host tumor immunity. This immunomodulatory property of adenovirus vectors is an added advantage to their use for cancer gene therapy.

A Rationale For This Study

The majority of malignant central nervous system (CNS) tumors are refractory to current modes of treatment including surgery, radiation, chemotherapy and biological response modifiers. The average survival of patients with glioblastoma multiforme, the most malignant form of glioma, post-operatively is approximately 12-13 months. The high morbidity and mortality associated with these CNS tumors has resulted in the development of alternative treatment modalities such as gene therapy.

Gene therapy offers potential as a treatment strategy for primary malignant CNS tumors. Clinical human trials utilizing herpes simplex virus thymidine kinase gene (HSVTK)transfer by a retrovirus vector into brain tumors have shown some efficacy. However, retrovirus mediated suicide gene transfer to focal neoplasms has multiple disadvantages including poor transduction efficiency, difficulty in virus concentration and *in vivo* virus inactivation. To overcome these limitations, we have created an alternate viral vector system, a recombinant adenovirus expressing the herpes simplex thymidine kinase

gene (AV-TK). This vector infected malignant glioma cells with much higher efficiency than reported for retrovirus mediated gene transfer. In a rat model, AV-TK treatment has resulted in regression and cure of subcutaneous and intracranial transplanted gliomas. However, factors governing treatment efficacy have yet to be determined. Studies in our laboratory indicated that animals treated with AV-TK may develop anti-tumor immunity. Interestingly, tumor eradication may be augmented and possibly dependent on the generation of this anti-tumor immune response.

Characterizing the immune mechanism governing anti-tumor immunity and determining the requirements for its generation will give insight into the potential for AV-TK as a treatment for unifocal systemic and CNS tumors.

Specific Aims

The main objective of this study is to characterize the anti-tumor immune response generated by the treatment of malignant gliomas with herpes simplex virus thymidine kinase gene transfer using an adenovirus vector and to evaluate the factors responsible for its generation as well as the immunomodulatory activity of AV-TK in mediating tumor eradication.

Our first goal is to characterize the immune mechanism responsible for long term tumor immunity in animals treated with AV-TK and GCV. The immune mechanisms investigated will include antibody and complement mediated cytolysis, antibody dependent cell mediated cytotoxicity, CD8+ MHC class I restricted cytotoxicity and NK cell cytotoxicity. Once the mechanism of tumor immunity is determined the immunophenotype of the effector mechanism will be characterized. Further characterization of the immune response will also require determining the ability of the immune effector mechanism to mediate tumor regression *in vivo*.

Also of interest are the factors governing adenovirus mediated generation of tumor immunity. The role of tumor antigenicity, host immunocompetence and the putative HSVTK bystander effect will be evaluated in addition to adenovirus mediated effects such as local inflammation.

Lastly, the advantage of AV-TK treatment over traditional methods for the treatment of focal tumors will be studied. This comparison will include gene therapy, tumor vaccination and surgical tumor removal. Evaluation will include tumor free survival and protection from further tumor challenges.

Literature Review

Primary tumors of the central nervous system (CNS) are the second most common neoplasms of children and in adults are more prevalent than systemic Hodgkin's disease[1]. Each year in the United States approximately 14,000 cases of primary CNS tumors are diagnosed[2]. To date, no curative treatment for most types of neuroectodermal CNS tumors exists. Current treatment consists of surgical removal of the neoplasm followed by radiation with or without chemotherapy[2]. However, there is significant morbidity associated with this treatment regimen and in very few cases can it be considered a success. For malignant gliomas, there is a poor long term prognosis with a mean survival time following treatment of approximately 12-18 months[3].

The most common CNS tumors are neoplasms of neuroectodermal origin which include the classifications of astrocytoma, oligodendroglioma, glioblastoma multiforme, ependymoma and medulloblastoma. Of these tumors, glioblastoma multiforme is the most malignant primary brain tumor compromising 50% of CNS tumors of children and 25% of intracranial tumors in adults[4]. Glioblastoma multiforme

represents the classic brain tumor in terms of pathology and tumor progression. Glioblastomas tend to be focal lesions which do not metastasize outside of the CNS[5]. Instead, these tumors slowly infiltrate the brain parenchyma leading to progressive CNS symptoms. Unlike systemic tumors, a much smaller tumor burden is necessary to be lethal[2]. It is estimated that a 100 gram CNS tumor is invariably fatal. In the case of glioblastoma multiforme treatment must be considered palliative for it is rarely curative.

Due to the ineffectiveness of current treatment modalities, many researchers attempting to improve patient survival rates have concentrated on alternative biological approaches such as gene therapy and immunotherapy rather than pharmacological methods of treatment.

Tumor Biology

The body of scientific literature encompassing the field of tumor biology is quite vast[6] (see review by Sherbet, 1987). Carcinogenesis is a multistep process involving genetic alterations within a cell[7]. The accumulation of genetic defects often leads to uncontrolled proliferation[8]. Advances in our understanding of the genetic basis of cancer allows for gene modification to be a viable strategy for cancer treatment. A variety of protocols exist for the management of cancer. However, most treatment regimens include surgery, radiation and chemotherapy or a combination to be effective. The lack of tumor specificity often limits the usefulness of these strategies resulting in significant side effects and morbidity1. Thus, researchers have explored alternative manipulations. Of these experimental protocols, methods to augment host immunity seem to be one of the most promising[9, 10, 11]. The role of immune responses in modifying tumor progression is well[12].

Immunodeficient or immunocompromised patients lacking the ability to mount a cellular immune response are

susceptible to tumors suggesting that immune effector cells function in the surveillance against neoplasms[13]. When the concept of immunosurveillance was first postulated[12], the exact immune mechanisms responsible for recognition of the tumor antigens was unknown. Many research groups have since shown that host responses against tumors are predominately by cellular mechanisms[14] (See Review by Baxevanis, 1994). Three immune cell classes have been described with anti-tumor activity. These classes are cytotoxic-T lymphocytes (CTL) characterized by antigen specificity[15], Natural Killer Cells (NK) which lack MHC restriction[16] and Lymphokine Activated Killer cells (LAK) derived by high dose cytokine stimulation *ex vivo*[17]. Although all of these different immune cell populations (CTL, NK, and LAK) have shown reactivity against tumors, the most predominant physiological mechanism involves CTL[14]. Generation of CTL responses can be divided into two phases. The first phase involves interaction of regulatory lymphocytes (usually expressing CD4 antigen; CD4+) with tumor cells often by the recognition of tumor associated antigens (TAA)[18]. The second phase involves regulatory lymphocyte activation of cytolytic T-lymphocytes[19].

Glioma Immunobiology

In previous years, the central nervous system was considered an immune privileged site due to a lack of lymphatic drainage and the protective nature of the blood-brain barrier (BBB)[20]. In 1977 however, Albright et al.[21] reported rejection of tumor allografts in the CNS. Other similar studies have resulted in a greater understanding of acquired immune responses to tumors of the CNS[22, 23]

The role of the immune system in glioma progression stems from the observation that patient survival can be correlated to the degree of tumor infiltration by lymphocytes[24]. Another important observation was that peripheral blood lymphocytes have the ability to cross the BBB and migrate into an intracranial tumor[25]. In many cases, glial tumor cells have been shown to express antigens which can be localized by antibody staining[26]. These studies question the axiom of the brain as an immune-privileged organ. Clearly there is a selective interaction of the host immune system with CNS tissue.

Glioma Associated Antigens

Recently there has been a great interest in glioma specific antigens that has been fueled by the development of a variety of monoclonal antibodies reactive against glial tumors[26]. These antigens have not proven to be useful in therapeutic regimens, however, due to their lack of tumor specificity[27]. Antibody staining has localized many of these antigens to normal as well as malignant brain tissue[28].

The majority of these glioma associated markers identify intracellular intermediate filament proteins[26]. This class of proteins is associated with cellular differentiation and is expressed through development[29]. Tumorigenesis usually involves cellular reversion to a less differentiated state in which these filament proteins are produced[29]. The first of these tumor-associated antigen proteins discovered was glial fibrillary acidic protein (GFAP), an indicator of astrocyte lineage[30]. Other glioma associated antigens in this family include the neurofilament proteins nestin and vimentin[31]. Nestin expression is also detected in neurons at the earliest stage of their differentiation but disappears by the sixth week of development[32].

The next largest group of glioma associated antigens

are cell surface antigens. Many malignant gliomas overexpress growth factor receptors including platelet derived growth factor receptor (PDGF)[33] and epidermal derived growth factor receptor (EGFR)[34]. The role of these antigens in glioma progression is discussed in a review by Kurpad et al.[26]. In summary, expression of these growth factor receptors makes cells susceptible to uncontrolled growth upon stimulation with the receptor ligand which is secreted as a normal physiologic paracrine factor.

In animal models, gliomas have been shown to express antigens against which cellular immune responses can be generated[35, 36]. Cellular immune responses against brain tumors in these models have been antigen specific. However, clinically only a single trial utilizing human glioma cell immunization was moderately successful[37]. The lack of a naturally occurring cellular immune response during glioma progression has been attributed to poor antigen presentation as a consequence of glioma induced immunocompromise[38].

Glioma Induced Immunocompromise

Peripheral blood lymphocytes from patients with glioma have a decreased capacity to proliferate in response to the mitogens Phytohemagglutinin (PHA)[39] or Concavalin A (Con A)[40] and also show reduced IL-2 production upon stimulation[41].

Furthermore, there is considerable evidence that Tumor Growth Factor-Beta (TGF-ß) is secreted by malignant glial tumors[42]. This glycoprotein inhibits proliferation and cytotoxic activity of LAK cells[43]. Much evidence exists pointing to TGF-ß as the primary immunosuppressive agent associated with gliomas[42]. Other potential immunomodulators resulting in decreased anti-tumor T-cell activity are Interleukin 1[44], Tumor-Necrosis Factor-alpha[45], and Interferon-gamma[46]. In other cases, the general lack of professional antigen presenting cells within the CNS has been associated with the development of T-cell anergy[47].

Glioma Specific Immunity

Gliomas have been shown to be highly antigenic in animal models but weakly immunogenic unless effector cells are secondarily stimulated with IL-2[48]. The favored explanation is that tumor bearing animals may prime lymphocytes into cytolytic precursors but without cytotoxic activity as a result of systemic immunosuppression.

Cell mediated glioma immune responses are rare[49]. Unlike other tumors, gliomas are unique in that very few reactive TIL can be isolated from tumors[50]. There is also a decrease in the amount of MHC Class I expression on the tumor cell surface[51, 52]. T-cells isolated from gliomas often

have a defective IL-2 receptor and lack the ability to proliferate in response to appropriate stimuli[41].

Given the antigenicity of gliomas and the poor prognosis with traditional methods of treatment a number of researchers have attempted cellular immunotherapy as a means of altering tumor progression.

Glioma Immunotherapy

Cellular immunotherapy, which involves *ex vivo* activation and expansion of lymphocytes before subsequent reintroduction into the host, has the potential to circumvent the glioma induced immune system suppression and to potentiate the host anti-tumor immune response. Early studies of adoptive cellular immunotherapy involved the use of lymphokine activated killer (LAK) cells[53]. LAK cells, derived from large granular lymphocytes found within the peripheral blood, were shown to have non-specific cytolytic activity against autologous and allogeneic tumor cells *in vitro*[54]. Cytolytic activity is dependent on stimulation with IL-2[16]. Through a mechanism which is still unclear, these cells exhibited selective lysis of tumor cells and remained relatively non-toxic to normal tissue cells[55]. *In vivo* adoptive immunotherapy studies with LAK cells and IL-2 showed success in a variety of animal tumor models leading

to clinical trials[56]. However, it was noted that LAK cells showed poor migration into the CNS[57]. Therefore direct LAK cell infusion into the tumor cavity following resection was attempted[58, 59, 60]. These therapeutic protocols resulted in multiple severe side effects and mixed clinical outcomes. Overall, these clinical trials failed to show a consistent increase in patient survival over standard treatment methods. In addition, the systemic side effects associated with LAK and IL-2 administration limited the feasibility of LAK immunotherapy[61]. The factors influencing clinical outcome are also unclear. Although tumors of a similar pathological grade and location were treated, one cannot distinguish the effectiveness of surgical resection from LAK treatment.

Cellular immunotherapy has recently centered on the use of antigen specific cytotoxic cells. Early studies with patients with melanoma found that lymphocytes harvested from within tumor lesions had the ability to lyse autologous tumor when expanded *ex vivo* and reintroduced into the tumor bearing host[62]. The antigen specificity of these tumor infiltrating lymphocytes (TILs) led to similar attempts for gliomas[53]. Glioma infiltrating lymphocytes, however, were predominately of the T-suppressor phenotype as a consequence of glioma elaborated immunosuppressive factors[63]. There was

also conflicting data on the ability of glioma derived TIL to lyse autologous tumor[64]. In a study of adoptively transferred IL-2 expanded TIL cells in mice, hepatic metastases showed regression but little effect was shown on primary brain tumors[65]. Interestingly however, recent studies have shown effective adoptive immunotherapy treatment of glioma bearing rats using spleen effector cells from animals immunized with a known antigenic glioma[66]. T-lymphocytes from immunized animals were stimulated *in vitro* with autologous tumor and IL-2. Immunotherapy with these effector cells resulted in animals rejecting autologous intracranial tumors. Thus, adoptive cellular immunotherapy may require the use of antigen specific sensitized lymphocytes to mediate cytotoxicity.

Although a majority of glioma infiltrating lymphocytes are of the tumor suppressor phenotype and have mixed cytolytic ability against autologous tumor, up to 14% of cells were CD56+ and 4% were CD16+[67] which are known cell surface markers on NK cells. Recently, NK cells negatively selected by flow cytometry were shown to have partial cytotoxic activity against established glioma cell lines and fresh surgical specimens without prior IL-2 stimulation[68]. A potential benefit to immunotherapy with NK cells is the lack of systemic side effects associated with IL-2

administration. Patient trials with NK cell immunotherapy are pending.

Another type of immunotherapy protocol is designated active nonspecific immunotherapy or immunization. This treatment method involves the use of agents to augment the host immune response. Agents which enhance immune response generation or adjuvants are injected within the tumor as a means of inducing an inflammatory response or as a means of up-regulating a tumor specific response. Clinical trials have been conducted utilizing bacille Calmette-Guerin (BCG)[69], purified protein extracts of Streptococcus pyogenes (OK-432)[70], Corynebacterium Parvum[71], levamisole[72] and viruses such as mumps[73] and rabies[74] as a method to stimulate the host immune response. These trials have not been successful in altering patient survival[53] most likely a consequence of host immunosuppression although increased lymphocyte and monocyte infiltrates into tumor were reported[75].

Cancer Gene Therapy

Identification of numerous oncogenes and tumor suppressor genes and greater understanding of their molecular and cellular interactions has fundamentally changed our perception of cancer. With this increased knowledge into the genetic basis of cancer it has become possible to alter tumor growth kinetics by gene modification[10, 76]. Direct gene therapy for cancer is based on the premise that modification of cancer cells in a patient by the transfer of an exogenous gene could result in multiple new treatment options. Tumor growth characteristics could be altered by the transfer of tumor suppressor genes[77] or anti-sense oncogene constructs[78]. Another option is to transfer genes resulting in tumor cell death such as toxin genes or genes inducing apoptosis[79]. Unfortunately, no gene transfer mechanism exists that allows for specific targeting of disseminated cancer or that can guarantee gene transfer to all cells in a localized tumor.

Suicide Genes

Due to the limitation of gene transfer to disseminated cancer cells, suicide gene therapy has focused on the treatment of localized focal deposits of cancer[80]. Suicide genes were first developed as a safety precaution for retrovirus vector based gene transfer used in the treatment of metabolic disorders[81]. Retroviruses integrate randomly within the host cell genome leading to the possibility of insertional mutagenesis as a consequence of provirus integration within a tumor suppressor gene or protooncogene[82]. If a suicide gene was included within the retrovirus vector, then a malignancy resulting from retrovirus gene transfer could be controlled.

The two suicide genes extensively researched are the herpes simplex thymidine kinase gene[83, 84] and the cytosine deaminase gene[85]. Both genes involve the cellular nucleotide utilization pathways[85, 86]. The most predominant suicide gene used is the herpes simplex thymidine kinase gene (HSVTK)[87]. Herpetic infections are controlled by the antiviral agent Ganciclovir® (GCV)[88]. The herpes simplex virus thymidine kinase gene product phosphorylates GCV to produce GCV-triphosphate which acts as a DNA chain terminator and inhibits DNA polymerase activity[89]. Early experiments by Moolten et al. showed that transfer of the

HSVTK gene to cancer cells rendered them sensitive to GCV cytotoxicity *in vitro*[90]. Further studies have shown that transplanted tumor cells engineered to express the HSVTK gene could be eliminated by GCV administration[91].

Bystander Effect

The largest limitation for direct gene therapy remained the need to modify all cancer cells within a localized deposit of cancer. Interestingly, it was noted *in vitro* that when mixtures of HSVTK containing tumor cells and non-HSVTK containing cells were mixed together, there was no diminished cytotoxicity by GCV[92]. Certain tumors showed complete regression *in vivo* when only 50% of cells were modified by HSVTK gene transfer[93]. This phenomenon of amplified GCV toxicity to non-modified cells has been termed the bystander effect. The bystander effect appears to be mediated by the transfer of the toxic nucleotide analogue GCV-phosphate via gap junctions[94]. The evidence for gap junction mediated transfer of toxic metabolites stems from an early observation that the bystander effect is dependent on cell density[95]. Invariably, cells growing in suspension show poor or nonexistent bystander effect while cells with good bystander effect require cell to cell contact. To confirm the requirement for cell contact, R. Touraine has

conducted elegant experiments using transwell plates where non-transduced and HSVTK transduced cells are physically separated but share extracellular media. In these experiments no toxicity to non-transduced cells was detected suggesting that conferred toxicity is not mediated by a secreted substance from HSVTK positive cells[96]. Regardless of the mechanism, the bystander effect allows GCV to exert toxicity to untransduced cells.

The existence of the bystander effect makes direct gene transfer of the suicide gene HSVTK a feasible treatment option since only a fraction of the tumor mass needs to be modified for GCV to exert its antineoplastic effect[92]. Based on these early experiments, direct gene therapy for cancer was attempted[97]. The largest problem was the lack of an effective *in situ* gene delivery method.

Retrovirus Gene Therapy

The first viral gene therapy vectors with demonstrated ability to transfer exogenous genes were retroviruses based on the Moloney Murine Leukemia Virus[98]. To make these vectors, a cell line (PA317) was created containing all genes except for the cis elements required for packaging the viral genome[99]. This packaging cell line produced empty retrovirus virions until transfected with nucleic acid

containing the viral packaging signal and flanked by the viral long terminal repeats[100].

Unfortunately there are many disadvantages associated with *in situ* retrovirus gene transfer. Retrovirus vectors can be produced only at low titers of approximately 10^5-10^6 infectious virions per ml[101]. Furthermore, the transgene expressing retrovirus cannot be concentrated from the packaging cell supernatant[102]. Retrovirus transduction is a receptor mediated process and therefore is dependent on the surface expression of the retrovirus receptor[101]. The majority of tumor cells have poor transduction efficiencies with retrovirus containing supernatant without additional manipulations[102]. Therefore, retrovirus containing supernatant for gene transfer to tumor cells *in vivo* is impractical. Additionally, retroviruses are inactivated at 37°C by human serum[80]. With these limitations, effective tumor transduction would require multiple infusions of vector which is not feasible.

To overcome these limitations, vector producing cells were utilized *in situ*[93]. Vector producing cells are cells which continuously produce and secrete retrovirus particles. A murine fibroblast retrovirus producer cell line PA317 was created to deliver HSVTK gene containing retroviruses[100]. This vector producer cell line has shown some success in

modifying the progression of tumors in rats[93, 97]. Based on these early data, clinical trials of patients with glioblastoma multiforme were attempted[103]. To overcome many of the limitations associated with retrovirus based gene transfer, alternative viral vectors were sought.

Adenovirus Vectors

Adenoviruses were discovered by Rowe et al. in 1953[104] during attempting to cultivate epithelial cells from patient adenoids. These viruses were quickly identified as one of the prime etiological agents responsible for acute viral respiratory disease as well as ocular, gastrointestinal, and urinary diseases[105]. Since their original discovery at least 41 strains infecting humans have been discovered which are further subdivided into a variety of classes by their antigenic determinants[106].

The early impetus for studying adenoviruses arose from clinical needs. However, the ease with which these viruses could be propagated in culture led to multiple studies into viral structure[105], function[105], replication[107] and transformation[108]. Besides increasing our understanding of viral pathogenesis, a great amount of information was generated on the regulation and function of adenovirus genes as models for eukaryotic transcription, RNA processing and

translation (See review by Kremer and Perricaudet)[109]. In fact, more information has been gathered on adenovirus than any other virus with the exception of the Human Immunodeficiency Virus in recent years.

Adenoviruses are non-enveloped icosahedral double stranded linear DNA viruses with a genome of 36 kilobases. The genome is divided into early genes and late genes on the basis of their activation during viral replication[107]. Early genes encode proteins involved in replication of the viral genome while the late genes encode the viral structural proteins.

Most replication deficient adenovirus vectors are made from adenovirus serotype 5 and are non-oncogenic in humans[110]. These vectors are made replication deficient by deletion of the E1a and E1b genes[111]. These genes encode transcription factors necessary for the activation of early region promoters[107]. Without E1 function, proteins required for viral genome replication are not transcribed at levels sufficient to maintain the lytic viral life cycle[112]. In order to grow E1 deleted recombinant adenoviruses, a human kidney cell line, 293, which has a sheared piece of adenovirus integrated into its genome is utilized[113]. This integrated genetic fragment produces E1 proteins which can transcomplement E1 function[113]. Recombinant adenovirus

vectors are made by introducing a transgene cassette into the deleted E1 region of adenovirus.

The advantages of adenovirus meditated gene transfer are many. In ratio to the amount of adenovirus produced, recombinant virus creation and propagation is not labor intensive. Recombinant viruses depending on the transgene being expressed, can be concentrated to titers of greater than 10^{11} particle forming units (pfu) per ml. Additionally, unlike retroviruses, adenoviruses do not integrate into the host cell genome[114]. Non-integration limits their feasibility as a mechanism for permanent and corrective gene therapy[115], however this is an advantage for cancer gene therapy. As a safety issue, there is a diminished probability for insertional mutagenesis to occur resulting in transformation of the target cell. Adenoviruses also have a wide host range[110] and therefore are suitable for gene transfer to numerous cell types. All of these features make adenovirus a suitable vector for cancer gene therapy.

Glioma Tumor Model

Malignant gliomas have been chosen as a tumor model for direct gene therapy for several reasons. These tumors tend to grow as solid focal tumors and are non-metastatic[4]. Therefore, treatment of these primary CNS tumors before progressive brain infiltration has the possibility to result in cure with long term survival and decreased morbidity. The prodrug GCV has very few systemic side effects at the doses necessary for its toxic metabolite to exert its anti-neoplastic effect[116]. Also, GCV readily crosses the blood-brain barrier[97, 103].

Materials and Methods

Cells:

All cells were kept at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Biofluids) supplemented with 10% Fetal Calf Serum (FCS) (Hyclone), 2mM L-Glutamine and 100 units/ml Penicillin and 100 ug/ml Streptomycin (BRL). Cells were passaged when approximately 70% confluent by 2 minute incubation in Trypsin/EDTA (BRL) followed by a single wash step in FCS containing serum. Cells were then replated at 1/6 density in the appropriate size tissue culture dishes. All cell lines are described in the body of this manuscript. They were obtained from American Type Culture Collection (ATCC, Rockville, MD) except for the following:

Cells	Type	Species	Source
9L	Gliosarcoma	Fisher Rat	Dr. E. Oldfield (NIH)
RT2	Astrocytoma	Fisher Rat	Dr. R. Merchant (MCV)
p494	Glioma	Fisher Rat	Dr. R. Touraine (NCHGR/NIH)
MesII-25	Mesothelioma	Fisher Rat	Dr. R. Touraine

205	Fibrosarcoma	C57Bl Mouse	Dr.S. Rosenberg (NIH)
KB	Kidney	Human	Dr. P. Seth (NIH)

Animals:

Female Fisher 344 rats obtained from the National Cancer Institute (Frederick, MD) were used in all studies. Animals were housed in Building 49 of NIH under Protocol #G-94-8 and #G-94-11 complying with NIH approved standards for the humane treatment of animals. All animals were under the supervision of a staff veterinarian. All invasive protocols were veterinarian approved. The maximum allowed progression for subcutaneous growing tumors was 400 mm^3. Supportive care was given when necessary.

Animals were housed at a maximum of 3 per cage and given water and Purina Laboratory Chow *ad libitum*. Immunologically mature animals of approximately 150-200 g (8 weeks) were used in all experiments involving subcutaneous tumor transplantation. In these studies, all animals were weight and age matched by experiment.

Subcutaneous Tumor Transplantation:

The ability of the various glioma cell lines to produce subcutaneous tumor was predetermined. To produce a tumor of

approximately 75 mm^3 in 7 days required a tumor dose of 3 X 10^6 9L, and 2 X 10^6 RT2 cells 117. Cells were injected in a volume of 100 ul Phosphate Buffered Saline (PBS) (Biofluids) subcutaneously on the rat dorsal surface just lateral to the midline using a 1 ml syringe and a 25 gauge needle. The needle was left within the injection site for a few seconds and then pulled out slowly in order to limit needle tract placement of cells. A successful inoculation was determined by formation of a raised dermal welt. All injections were performed using aseptic technique.

Recombinant Adenovirus Injection into Tumor and GCV Administration:

The tumor area was prepared using sterile technique. The recombinant adenovirus was injected in a 100 ul volume of PBS with a 1 ml syringe and 25 gauge needle. The needle was inserted subcutaneously 1 cm inferior to the tumor and passed into the most superior aspect of the tumor. The viral solution was then injected as the needle was repositioned within the tumor mass to insure maximum exposure of cells.

The highest GCV dose tolerable in 150-200g rats without developing extensive pathology was 300 mg/kg/day 118. GCV

(Syntex Corporation), prepared in PBS on the day of injection, was administered to animals 48 hours after tumor injection. Animals received 1 ml of GCV solution containing 150 mg/kg/dose intraperitoneally twice a day for 5 days. Daily injections were conducted 10 hours apart. GCV administration often resulted in renal and intestinal pathology accompanied by dehydration (NIH Pathology Division, personal communication). Animals suffering from weight loss or diarrhea were given intraperitoneal injections of 3 ml of normal saline (Biofluids) to augment fluid loss.

Animal Euthanasia and Spleen Removal:

At various time points after tumor treatment, animals were euthanized in a CO_2 gas chamber. Subsequently, the spleens were removed by aseptic technique through a longitudinal incision on the animal's left dorsal surface. The spleens were placed into DMEM-10% FCS and the capsule broken by forcep teasing. Spleen cells were elaborated by mincing with the nub of a sterile 6 cc syringe and collected in a 50 ml conical tube. Red blood cells were removed by incubation in 5 ml of ACK lysing buffer (NIH media unit) for 5 minutes at room temperature. Cells were washed twice in PBS before additional manipulation.

Chromium Release Assay as a Measure of Cytotoxicity:

Cytotoxicity was assessed by the ability of spleen effector cells to lyse various tumor target cells. To determine the amount of target cell lysis, target cells were labeled with ^{51}Cr. Log phase growing target cells were harvested from tissue culture dishes and single cell suspensions made. Cells were counted using a Coulter counter and 5 X 10^5 cells were centrifuged at 800 g in DMEM-10% FCS. The medium was aspirated from these pellets and the cells were resuspended in the remaining volume of medium, approximately 50 ul. 200 uCi of ^{51}Cr (Sodium Chromate in Sterile Saline; Amersham Biochemicals) solution was added to target cell suspensions and the cells were incubated for 2 hours in a 5% CO_2 incubator at 37°C. During target cell incubation, the various spleen effector cell dilutions which will yield effector to target ratios of 100:1, 50:1, 25:1 and 12.5:1 were prepared by making a stock solution of 5 X 106 cells per ml and serially diluting 1:2. In a 96 well U-bottom plate, 100 ul of these solutions was added to the appropriate wells in triplicate. Target cells were centrifuged and washed 3 times in DMEM-10% FCS. To reduce spontaneous leakage of ^{51}Cr the cells were incubated for an additional 45 minutes after the final wash. Target

cells were washed again in DMEM-10% FCS and resuspended in 10 ml. 100 ul of this final suspension was added to all wells containing effector cells. Additionally, 12 wells received target cells only. These wells serve to measure spontaneous and maximum leakage. The plates were incubated at 37 °C , 5% CO_2 for 4 hours. One hour before harvesting 10 ul of 8% Triton X-100 was added to 6 wells containing just target cells as a measure of maximum ^{51}Cr leakage. Plates were harvested using a Skatron harvesting system (Skatron, Virginia). ^{51}Cr release into supernatant was counted using an ICN Gamma Counter.

Cytotoxicity as measured by ^{51}Cr release from labeled target cells was expressed as percent corrected lysis for a concentration of effector cells using the mean cpm (counts per minute) per triplicate wells. The formula used was as follows:

Corrected % Lysis = 100 X (effector cell sample ^{51}Cr released - spontaneous target ^{51}Cr released) / (maximum target ^{51}Cr released - spontaneous target ^{51}Cr released).

Secondary Stimulation Of Potential Cytotoxic Effector Cells:

Optimum CTL lysis usually requires *in vitro* stimulation of primed effector cells with antigen. In this protocol, effector cells were coincubated with irradiated (5000 rad)

tumor cells at an effector to stimulator ratio of 150:1, 50:1, and 5:1 to determine optimum CTL activity generation. Effector cell cytolytic activity was titrated against tumor stimulation, and then the effects of low dose IL-2 (10 units) stimulation were assessed. In our tumor stimulation model, for the weakly immunogenic 9L tumor, low dose IL-2 was often not effective to generate significant cytolytic activity from splenocytes. Pilot studies in our laboratory have shown increased cytolytic activity when effector cells were cocultured with stimulators at the predetermined optimal concentration with 10% Rat T-stim (RTS, Fisher Chemicals).

Secondary stimulation of effector cells were maintained in 24 well plates with lymphocytes at a concentration of 5 X 10^5 cells/well and irradiated (5000 rads) tumor cells at 5 X 10^5 cells/well for 6 days.

Fluorescence Activated Cell Cytometry Analysis

FACS analysis were conducted according to the recommended procedure by Becton Dickinson on a Becton Dickinson FACScan. Analysis was conducted using the Becton Dickinson software Lysis II for the Hewlett Packard or CellQuest for the Macintosh.

All antibodies were obtained from Pharminigen.

Antibody staining was conducted in staining media (PBS supplemented with 0.5% Bovine Serum Albumin and 0.25% NaN_3). 1 X 10^6 cells were centrifuged in Falcon 2052 tubes, medium aspirated and 0.5 ug of directly conjugated antibody was added to the small volume of staining medium remaining. Cells were placed on ice for 20 minutes and then washed twice with 2 ml of staining media. Finally, cells were analyzed by FACS analysis recording 10000 events. If an indirect conjugated antibody was used, after 1 wash in 2 ml of staining media, the second antibody was added. Following a 10 minute incubation, cells were washed twice in 2 ml staining media and then analyzed. Non-antibody stained cells were used to gate relevant events. As a negative control, cells were stained with a similar isotype antibody directed against a non-cellular marker.

ß-Galactosidase Activity Determination and Measurement Of Recombinant Adenovirus Transduction Efficiency:

In order to determine the optimal adenovirus concentration for infectivity, a recombinant adenovirus expressing the E. Coli nuclear ß-galactosidase gene (AV-LacZ) was created. By using ß-galactosidase as a reporter gene, infection of target cells with AV-LacZ can be measured and correlated to adenovirus infectivity. This assay relies upon the hydrolysis of fluorescein di-ß-D-galactoside

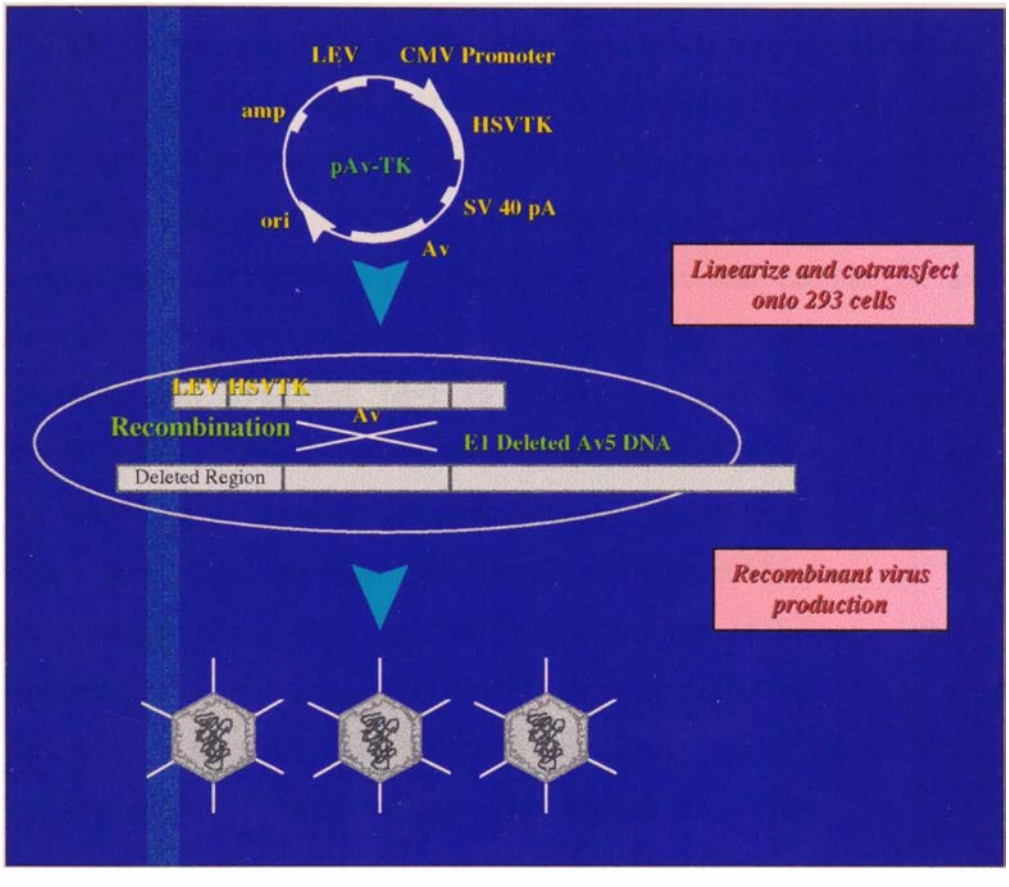
(FDG)(Molecular Probes) inside cells with ß-galactosidase activity and the subsequent detection of fluorescein by FACS which is the most sensitive method existing. The method of MacGregor and Nolan was used[119]. Briefly, cells were transduced in 0.5 ml of DMEM-2% FCS with different multiplicity of infections (MOIs) of AV-LacZ. Thirty sVII hours later, cells were harvested from tissue culture plates and loaded with FDG by a 1 min. hypotonic shock at 37°C. After 1 min, Cells were returned to isotonicity by dilution in ice cold media and kept on ice until analysis. Fluorescein passes through cell membranes two hundred times faster at 37°C then at 4°C[120]. By keeping the reaction on ice, ß-galactosidase activity proceeds without leakage of the detectable cleaved fluorescein molecule allowing for discrimination between the ß-galactosidase positive and negative sub-populations. If the FDG labeled cells were brought to above 15°C, the fluorescein leaks through the cell membrane and into other cells including ß-galactosidase negative cells resulting in a population which stains homogeneously for ß-galactosidase expression when analyzed by FACS[121]. To control for this possibility, the cell lines 205 and 205 G1NBGSVNA (gift from J. Ramsey, NIH) were used. The 205 G1NBGSVNA is a murine fibrosarcoma which constitutively expresses the nuclear ß-galactosidase gene

and positively selected in media containing 1 mg/ml G418 (BRL). 205 is the non-transduced parent line. The 205 G1NBGSVNA cell line stains 100% positive for ß-galactosidase gene expression by FDG loading and flow cytometric analysis (data not shown). By conducting mixture studies, the leakage of fluorescein can be detected.

Recombinant Adenovirus Creation:

The method of Berkner was utilized[122]. Creation of the adenovirus shuttle plasmid pAVS6 has been reported[123]. Figure 1 diagrams the steps in creation of the recombinant adenovirus expressing HSVTK. The plasmid pAVS6-HSVTK (gift of J. Ramsey, NIH) was linearized by Not I (New England Biolabs) digestion and cotransfected on 293 cells with the Cla I cut fragment of Av-dl327 (gift of J. Ramsey, NIH). Two weeks later, infectious recombinant adenovirus vector plaques were picked, expanded, and screened for the HSVTK sequence by polymerase chain reaction. Positive recombinants were grown on 293 cells. AV-LacZ was similarly created using the ß-galactosidase gene.

Figure 1 - Creation of a Recombinant Adenovirus Expressing The Herpes Simplex Thymidine Kinase Gene



Diagramtic representation of AV-HSVTK Creation. The Plasmid pAVS6-HSVTK was linearized by Not I restriction enzyme digestion and cotransfected onto 293 cells with the Cla I cut fragment of Av-dl327.

Recombinant Adenovirus Purification and Propagation:

90% confluent 293 cells were infected in DMEM-2% FCS with the recombinant adenovirus at 1-10 particle forming units (pfu) per cell. After a 1.5 hour incubation, the cells were grown in DMEM-10% FCS and incubated at 37°C, 5% CO_2 until cytopathic effects were detected (approximately 24-36 hr). Cells were harvested from tissue culture dishes and centrifuged at 800 x g. Cells were lysed by 4 cycles of freezing and thawing and this crude viral lysate was centrifuged at 4000 x g. The supernatant was collected and overlaid on a two step Cesium Chloride (CsCl, BioWhittaker Corporation) gradient (d=1.25 over d=1.40) in ultra-clear SW-41 tubes. These tubes were then centrifuged using a SW-41 rotor in a Beckman ultracentrifuge for 1 hour at 35000 rpm. The viral band at the interface was collected and spun on a second 1.33 density CsCl gradient for 18 hours. The opalescent virus band was collected and dialyzed across a 10 mM Tris, 1 mM MgCl, 10% glycerol buffer. Virus was recovered from dialysis bags and stored at -70°C.

Bystander Effect Measurement:

The bystander effect was measured by the ability of various mixtures of HSVTK positive and HSVTK negative cells to proliferate in the presence of increasing concentrations

of GCV. In the present study, 70% confluent cells growing on 60 mm^2 tissue culture dishes were washed once in PBS and then 1 ml of DMEM-2% FCS containing the infectious dose of virus either AV-TK or AV-LacZ was added. After 1.5 hours, the DMEM-2% FCS was aspirated into a bleach containing bucket, cells were washed twice in PBS and then placed in DMEM-10% FCS. After 36 hours, cells were harvested and mixed with non-transduced cells to yield mixtures containing 100% transduced cells, 20% transduced cells, 5% transduced cells and 0% transduced cells. Actual transduction percentage was determined by FACS analysis of ß-galactosidase expression from the cells infected with AV-LacZ. 10^4 cells of these various mixtures were added to individual wells of 96 well flat bottomed plates and a cross titration of 0 mM, 0.5 mM, 5 mM, and 50 mM GCV was added. The plates were placed in a 37^0C, 5% CO_2 incubator. 48 hours later ^3H-Thymidine uptake was measured.

Tritium Uptake Measurement:

0.5 uCi of ^3H-thymidine (NEN) was added to each well and plates were incubated overnight. The next day, media was aspirated from wells and the plates were washed using a Skatron plate washer. 50 ul of 50% PBS diluted trypsin-EDTA was added to wells and the plates were harvested on a Tomtec

Cell Harvester. ß-plate filters were dried in a microwave for 3 minutes and read on a LKB ß-plate counter.

Cell Irradiation:

Cells were prepared at a concentration of 10^6 / ml in DMEM-10% FCS. The appropriate radiation dosage was given using a ^{137}Cs γ-irradiator (Building 10, NIH).

Animal Immunization:

As a positive control, animals were immunized against the various glioma cell lines by a modification of the method of Holladay et al. 48. Briefly, 5 X 10^6 tumor cells were irradiated (dosage given in text) in a Cesium irradiator. These tumor cells were combined with 25 ug BCG and this cell-adjuvant mixture was administered only once to animals by either subcutaneous or intraperitoneal injection.

Limiting Dilution:

T-lymphocytes were obtained from spleens of AV-TK treated-tumor ablated animals by column fractionation (Accurate). A stock dilution of cells was made at a concentration of 20 cells/ml. Aliquots of 200, 100, 50, and 5 ul/well were placed in 96 well flat bottom plates. These dilutions corresponded to 4, 2, 1, and 0.1 cells/well

respectively. Splenocytes from naive control animals were harvested and irradiated at 2500 rads. 25 ul of a 400 cells/ml concentration of these cells was added to the previous lymphocyte containing plates. Syngeneic tumor cells were irradiated (5000 rads) and 25 ul of a 400 cell/ml stock solution was added to each well. Plates were incubated at 37°C and 5% CO_2. Twenty four hours later, 25 ul of RTS (Rat T-stim, Fisher Chemical Company) was added to each well. Medium was replaced every 3 days. In 2 weeks, half the number of cells from each well were transferred to fresh plates. ^{51}Cr labeled syngeneic tumor cells were added to each well of these mirror plates and chromium release was measured in a four hour assay. Chromium release of 15% greater than control was considered a positive reaction. Plates with at least 37% negative reactive wells were considered to have been started from a monoclonal culture. Reactive clones were harvested from the original plates and grown by repeated 5 day stimulation with irradiated autologous tumor cells.

Statistical Analysis:

Data was analyzed on an IBM compatible computer using Prism (GraphPad Corporation, San Diego CA), a statistical analysis software program. The appropriate test utilized is

given in the body of the text in association with the data.

Adoptive Transfer:

Adoptive transfer was conducted by injection of bulk populations of spleen derived lymphocytes from donor animals to recipient animals. Animal sacrifice and lymphocyte derivation is previously described. Donor animals are stated in the body of the text. Recipient animals were naïve 150-200g female Fisher rats which received intraperitoneal inoculations of lymphocytes in 3 ml of PBS. The number of lymphocytes transferred is stated in the accompanying figure legends. Adoptive transfer was conducted on the day of the donor animal sacrifice. These lymphocytes were not *in vitro* stimulated prior to transfer.

Tumor Infiltrating Lymphocyte Derivation:

Tumors were excised from animals using aseptic technique. The skin was carefully dissected away from the tumor parenchyma. The remaining tumor was cut into smaller fragments and placed in a triple enzyme solution consisting of Hanks Buffered Salt Solution (Biofluids) containing 0.1% collagenase (Sigma), 0.002% deoxyribonuclease (Sigma) and 0.01% hyaluronidase (Sigma). After 6 hours, the single cell suspension was centrifuged at 1000 X g and placed in fresh

PBS. This solution was carefully overlaid on Lympholyte-M (Organon Teknica) and centrifuged at 800 X g for 20 min. The cells remaining at the density interface were aspirated, washed 3 times in PBS and then stained according to the staining procedure described previously.

Results

For viral gene transfer to be effective, the recombinant virus must have the ability to bind, penetrate, and effectively express the transgene without undo toxicity to the target cell.

In the past few years, researchers have used recombinant adenoviruses to infect many cell types including neurons and glial cells[124], muscle[125], liver[123], lung[126], and tumor cells[127]. While the receptor seems to be ubiquitous, its expression varies with cell type[111]. Most cell types express adenovirus receptors as exhibited by their ability to be infected by adenoviruses with the exception of lymphoid lineage cells[128].

The gene transfer or transfection efficiency is dependent on the number of viral receptors and the infectious dose of virus[110]. The adenovirus receptor has not been cloned, therefore its expression on cell types cannot be quantified. Cells such as HeLa, KB, and 293s are known to infect quite well with wild type and recombinant adenovirus (data not shown). In order to determine

transfection efficiency and gene expression in a variety of tumor types, cell lines were infected with increasing log doses of a recombinant adenovirus expressing the ß-galactosidase gene (AV-LacZ). ß-galactosidase activity was then quantified by loading with FDG, a fluorescent substrate for the ß-galactosidase enzyme and analyzed by flow cytometry. The highest dose used was a multiplicity of infection (moi) of 100. Safety studies conducted during human clinical trials utilizing recombinant adenovirus have been limited to a total dose of 10^{10} pfu[129]. Greater doses have the potential to lead to acute toxicity. We estimated that given the number of cells in an advanced glioma in our subcutaneous model, an moi of 100 would be the maximal tolerable dose. The results of this experiment are given in Table I.

TABLE I - *IN VITRO* TRANSDUCTION EFFICIENCY OF CELLS INFECTED WITH A RECOMBINANT ADENOVIRUS VECTOR EXPRESSING THE ß-GALACTOSIDASE MARKER GENE

Percent Transduced (%)

MULTIPLICITY OF INFECTION		**0**	**10**	**100**
Cell line	Murine			
C57FB	(Normal Fibroblast)	0	6.30	18.12
EL4	(Leukemic cell)	0	0.20	0.10
38	(Colon Adenocarcinoma)	0	4.27	28.64
205	(Fibrosarcoma)	0	9.33	36.26
	Rat			
9L	(Gliosarcoma)	0	1.00	27.51
C6	(Glioma)	0	4.35	36.34
RT2	(Glioma)	0	7.70	41.58
F98	(Glioma)	0	Not Done	40.15
p494	(Glioma)	0	7.10	26.34
DiTNCL	(Transformed Astrocyte)	0	55.17	100.0
Mes II 25	(Mesothelioma)	0	14.80	21.30
Fat7	(Nasal Carcinoma)	0	6.40	54.70

(Table I continued on next page)

TABLE I - *IN VITRO* TRANSDUCTION EFFICIENCY OF CELLS INFECTED WITH A RECOMBINANT ADENOVIRUS VECTOR EXPRESSING THE ß-GALACTOSIDASE MARKER GENE

Percent Transduced (%)

MULTIPLICITY OF INFECTION		**0**	**10**	**100**
Cell line	Human			
KB	(Cervical Carcinoma)	0	100.0	100.0
DBTRG	(Human Glioblastoma)	0	100.0	100.0
NB4	(Human Glioma)	0	100.0	100.0

Cell lines were obtained and grown as indicated in Materials and Methods. 5 X 10^5 cells were plated in 35 mm^2 wells of a 6 well plate and infected for 1.5 hours in 1 ml of DMEM-2% FCS with AV-LacZ at the given MOI. Cells were harvested 36 hours post infection and the ß-galactosidase activity measured as per Materials and Methods. Results represent the combined average of 3 experiments.

KB cells which are known to infect with adenovirus at high efficiency were used as a positive control to insure AV-LacZ infection. At a low moi of 10, these cells stained 100% positive indicating transfer of the ß-galactosidase gene by our recombinant virus (Table I). In comparing the effectiveness of AV-LacZ transduction, a few trends were noticed. Rodent cells, either murine or rat, infected with varying efficiency depending on the cell type. The lymphoid

cell line EL4 was not susceptible to adenovirus infection. This phenomenon was not limited to rodent cells. Human lymphocyte cell lines also infected poorly with adenovirus[128]. Of the rat cells, all infected with an efficiency of 25% - 40% at an moi of 100 with the exception of the SV40 T-antigen transformed rat astrocyte cell line DiTNCL. SV40 genes are known to complement adenovirus[130]. Hence, the increased gene expression in this cell line may be a consequence of SV40 upregulation of adenovirus replication. Of importance, the rat glioma cell lines 9L, RT2 and P494 infected at levels sufficient to be valid tumor models to paradigm the human situation.

A second noticeable trend was that human tumor cell lines infected with higher efficiency in comparison to the murine or rat cell lines. This result was not unexpected. Murine adenoviruses have been isolated which retain the ability to infect human cells[105]. Analysis of capsid genetic sequences of murine and human adenovirus shows significant but not complete homology (data not shown). Therefore, AV-LacZ, derived from a human adenovirus serotype was more likely to have greater affinity for the human receptor in contrast to its rodent counterpart, thus explaining the difference in transduction efficiency.

If this difference in transduction efficiency is

consistent for *in situ* transduction, these results indicate that our rat tumor models were acceptable but underestimated the eventual transduction efficiency which could be expected with human tumors. The actual *in vivo* significance of this difference in transduction efficiency was uncertain. Upon direct intratumoral injection, the exposure of cells to adenovirus will be inversely proportional to the distance from the site of adenovirus inoculation[127]. Certain cells will receive high moi of adenovirus while others may receive a significantly lesser amount. Also, cells within a tumor foci are more densely packed than in culture. This cell density may limit adenovirus exposure and transduction efficiency.

As stated earlier, upon infection, cells can harbor multiple replicates of the adenovirus[131]. Therefore, the level of gene expression within a given cell is varied. In order to analyze intracellular gene expression, cells were infected with AV-LacZ and analyzed by flow cytometry. The FACS-Gal assay is unique in that cells can be analyzed not just for AV-LacZ infection but the assay allows for the level of gene expression to be indirectly quantified. This assay measures the fluorescence of cells after conversion of a non-fluorescent substrate by the adenovirus transferred ß-galactosidase gene product to a fluorescent marker[132]. The

intensity of fluorescence is an indirect correlation of ß-galactosidase gene expression. The transduction efficiency values measured in Table I represented the number of cells showing positive fluorescence. In Figure 2, gene expression levels as indicated by fluorescence intensity for the human cell line DBTRG are depicted. This cell line was chosen because it was 100% infected at a relatively low doses of adenovirus (moi=10, Table I). Therefore, increasing the infectious dose of the recombinant adenovirus (moi=100) allowed for comparison of gene expression while transduction efficiency stayed constant.

Figure 2 - Comparitive Fluorescence Intensity of Human Glioma Cells Transduced With An Adenovirus Vector Expressing The β-Galactosidase Gene And A Retrovirus Transduced Fibrosarcoma

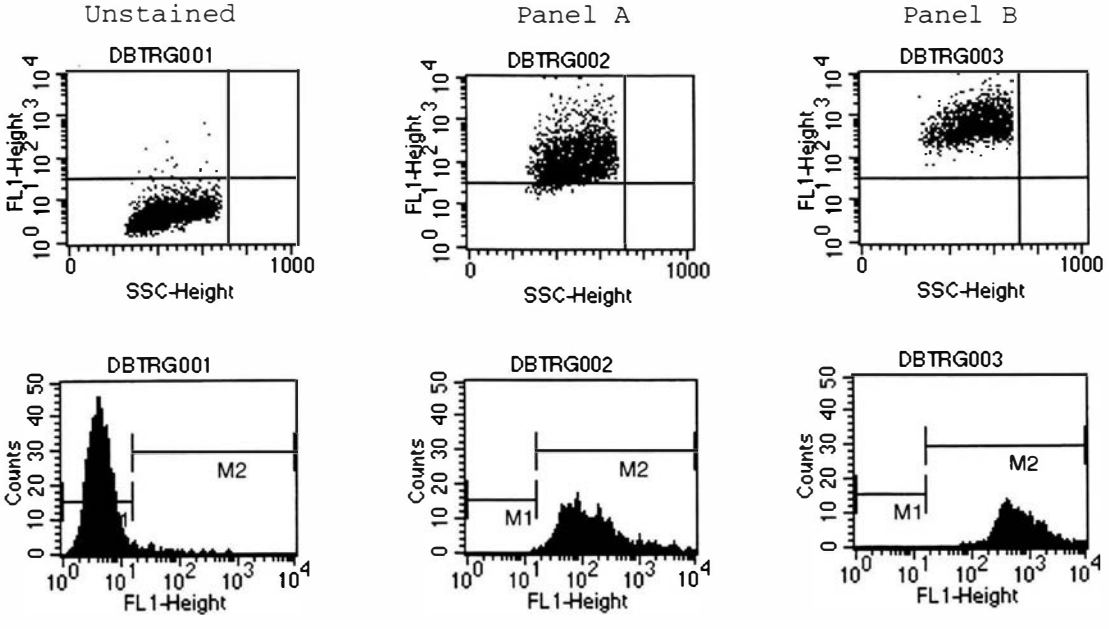


Figure Legend on Accompanying Page

Figure 2 - Comparative Fluorescence Intensity Of Human Glioma Cells Transduced With An Adenovirus Vector Expressing The ß-Galactosidase Gene And A Retrovirus Transduced Fibrosarcoma

Cell Line	Mean Fluorescent Intensity
DBTRG - AV-LacZ MOI 10	243.04
DBTRG - AV-LacZ MOI 10	810.78
205 G1NBGSVNA	241.00

Fluorescence of the cell line DBTRG when loaded with FDG as indicated in Materials and Methods is shown. Both dot plots (top row) and fluorescence histograms (middle row) are given. Positive fluorescence for dot plots is indicated on the y-axis. Cytoplasmic complexity is noted on the x-axis. Mean fluorescent intensity is given in the table.

The staining control is given in the bottom row which shows fluorescence histograms of the cell line 205 and 205G1NBGSVNA.

At an moi of 10, 100% of cells showed positive fluorescence for ß-galactosidase activity (Panel A). Similarly, at the higher infectious dose of virus (moi=100, Panel B), 100% of cells fluoresced positively. However, the cells were shifted upwards indicating greater fluorescent intensity. This increase in fluorescence represents greater ß-galactosidase activity in these cells. This phenomenon is also represented in the fluorescence histograms (middle row). Marker 1 (M1) represents baseline fluorescence of unstained cells. Marker 2 (M2) encompasses the cells after FDG loading. The mean fluorescent intensity (MFI)

demarcated by Marker 2 is given in the accompanying table. In comparing the attainable MFI with that of a 100% retrovirus transduced cell line, 205 G1nBGSVNA, a marked difference was seen. When 100% of cells are retrovirus transduced (205-G1nBGSVNA), the MFI was 241. This level of ß-galactosidase activity was comparable to cells transduced with AV-LacZ at a low moi of 10 (MFI=243). At an moi of 100, much greater enzyme activity is exhibited by the adenovirus transduced cells (MFI=810).

These data are significant for representing a distinct advantage of adenovirus vectors over retrovirus vectors. A single cell may be multiply infected with adenovirus and may harbor 1000 copies or greater of the recombinant virus[131]. In contrast retrovirus infectivity is limited by an unknown mechanism to a few integrated copies per cell[80]. Thus, the level of adenovirus mediated gene expression was not attainable with a retrovirus vector system. The high copy number of adenovirions permissable within a cell allowed for greater gene expression which for the recombinant adenovirus expressing the HSVTK gene ultimately allows for greater conversion of the GCV prodrug to its toxic analogue. Increased GCV toxicity is a distinct advantage of adenovirus HSVTK gene transfer. For all the cell lines represented in Table I increasing the adenovirus infection dose increased

transduction until cells were 100% saturated after which an increase in gene expression was observed (data not shown).

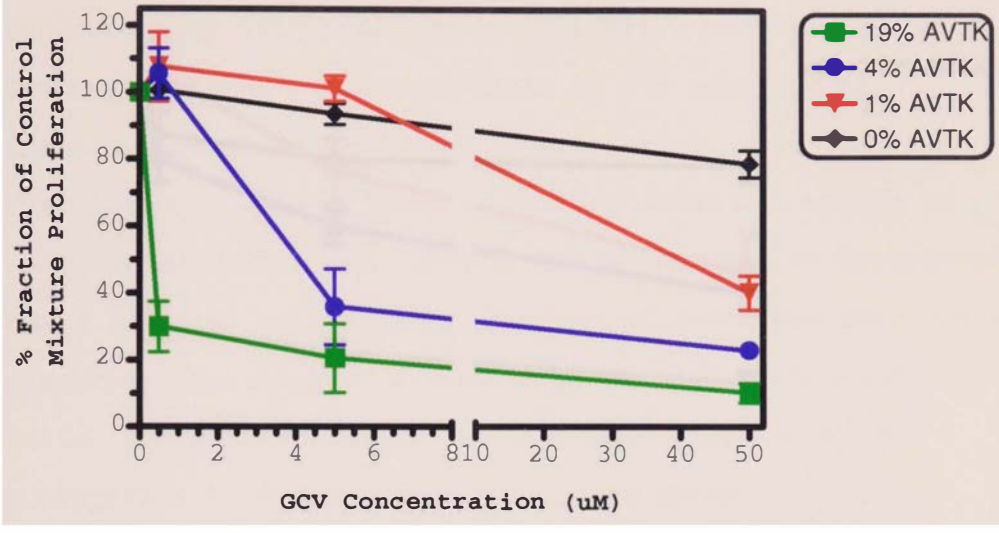
Despite the excellent transduction efficiency of human gliomas by recombinant adenovirus (Table I), another possible limitation to viral gene therapy was the method of adenovirus delivery. HSVTK gene transfer confers susceptibility to GCV toxicity to tumor and normal cells[133]. In order to minimize toxicity to normal cells, the preferred method of delivery has been direct injection of recombinant adenoviruses directly into the tumor bed[93, 133]. However, this method is unlikely to infect all cells within a tumor focus. In addition, our results from Table I indicated that adenovirus gene transfer even at high multiplicity of infections was not adequate to confer suicide gene susceptibility to 100% of glioma cells (9L and RT2). As a viable tumor treatment regimen, effective gene transfer to all cells within a tumor focus must occur in order to prevent tumor reoccurrence. Transfer of the HSVTK gene in combination with GCV overcomes this requirement for gene transfer to every tumor cell by taking advantage of the metabolic cooperativity of tumor cells by the bystander effect[134]. In 1990, Moolten et al. showed effective tumor killing when as few as 10% of the tumor cells expressed the

HSVTK gene[91]. The mechanism of GCV susceptibility when only a fraction of the tumor mass was genetically modified was through transfer of the toxic phosphorylated GCV from a genetically modified cell to an adjacent non-modified cell through gap junctions (see Literature Review).

In Figures 3 and 4, the bystander effect associated with AV-TK gene transfer to rat glioma cell lines is reported. The susceptibility of these gliomas to bystander killing *in vitro* was measured by transduction of glioma cells (moi=100) and then placing mixtures of transduced and nontransduced cells in increasing GCV concentrations. Cell proliferation was measured 48 hours later by ^{3}H-thymidine uptake. For a cell line exhibiting a poor or nonexistent bystander effect, the percent of control proliferation is equivalent to the percent of transduced cells. For example, a cell mixture containing 50% HSVTK gene modified cells translates to a 50% loss of proliferative potential since only gene transferred cells are susceptible to GCV toxicity. A singular problem with assessment of bystander effect mediated by AV-TK gene transfer related to differential cell transduction. At an moi of 100, 9L and RT2 are not 100% transduced (Table I). Therefore, the serial dilutions of adenovirus transduced cells with untransduced cells was not directly reflective the actual percent of transduced cells.

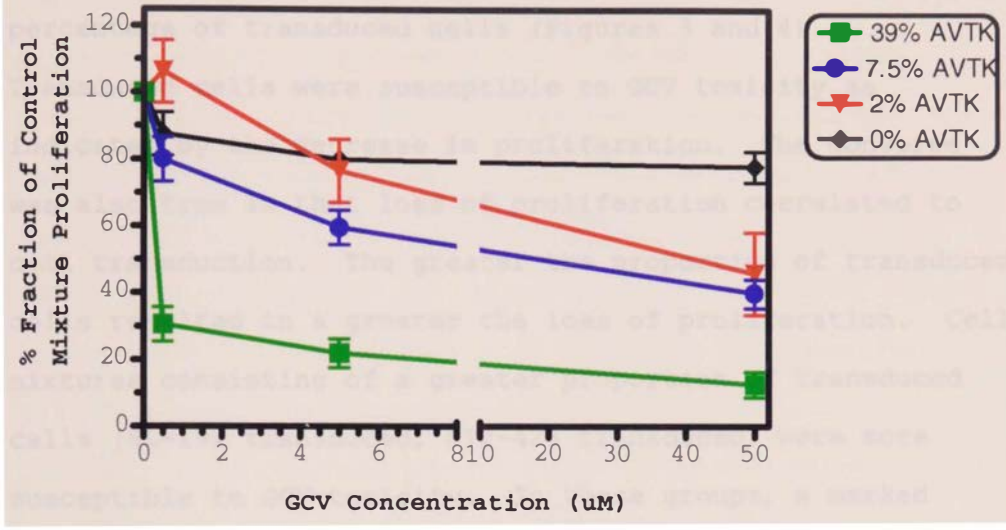
Therefore, the actual number of transduced cells within a mixture was determined by parallel infections of cells with AV-LacZ at an moi of 100. Transduction efficiency was determined by FACS analysis and bystander mixtures were normalized using these data.

Figure 3 - In Vitro Bystander Effect Associated With AV-TK Infection of 9L Glioma Cells



70% confluent 9L cells plated in 60 mm^2 dishes were infected with AV-TK at a moi of 100. 36 hours post infection, cells were harvested and serially diluted with noninfected 9L cells. 10^4 cells were plated in triplicate in 96 well flat-bottom plates (Falcon) and placed in increasing log concentrations of GCV. 36 hours later, cells were pulsed overnight with ^{3}H-Thymidine and uptake measured per Materials and Methods. The data points represent the cell mixture proliferation values normalized to the individual control cell mixtures at 0 uM GCV. Actual transduction efficiency was determined by parallel infections of 9L cells with AV-LacZ at a moi of 100 and determination of transduction percentage by flow cytometry per Materials and Methods.

Figure 4 - In Vitro Bystander Effect Associated With AV-TK Infected RT2 Glioma Cells



70% confluent RT2 cells plated in 60 mm^2 dishes were infected with AV-TK at a moi of 100. 36 hours post infection, cells were harvested and serially diluted with noninfected RT2 cells. 10^4 cells were plated in triplicate in 96 well flat-bottom plates (Falcon) and placed in increasing log concentrations of GCV. 36 hours later, cells were pulsed overnight with ^{3}H-Thymidine and uptake measured per Materials and Methods. The data points represent the cell mixture proliferation values normalized to the individual control cell mixtures at 0 uM GCV. Actual transduction efficiency was determined by parallel infections of RT2 cells with AV-LacZ at a moi of 100 and determination of transduction percentage by flow cytometry per Materials and Methods.

The bystander effect for the 9L and RT2 glioma cell lines was dependent on two factors; the dose of GCV, and the percentage of transduced cells (Figures 3 and 4). Transduced cells were susceptible to GCV toxicity as indicated by the decrease in proliferation. The converse was also true in that loss of proliferation correlated to cell transduction. The greater the proportion of transduced cells resulted in a greater the loss of proliferation. Cell mixtures consisting of a greater proportion of transduced cells (9L-19% transduced, RT2-42% transduced) were more susceptible to GCV toxicity. In these groups, a marked decrease in cell proliferation was observed even at the lower GCV concentrations (0.5 and 5.0 uM).

Interestingly, both glioma cell lines exhibited a good bystander effect. A 60% or greater loss of proliferative ability was seen with 9L and RT2 at the 5 uM GCV concentration when as few as 8% or less of cells were transduced. In comparing the cell lines, RT2 exhibited greater bystander mediated killing than 9L. At all doses of GCV, 9L required twice the number of HSVTK gene expression to exhibit the loss of proliferative potential seen with RT2. Given these data, both tumor cell lines were potential models to further explore the *in vivo* efficacy of AV-TK.

The biodisposition of GCV in rats is unknown. Therefore, the *in vitro* dose corresponding to *in vivo* tumor exposure to GCV is not available. In humans, the FDA approved dose of GCV for the treatment of herpetic infections is 5 mg/kg/day[135]. Our results clearly indicated that tumor killing was enhanced by increasing GCV dose. If HSVTK gene transfer is to become a viable treatment option, the maximum tolerable dose of GCV without undo systemic toxicity needs to be determined.

For rats, the maximum tolerable dose was determined to be 300 mg/kg/day when given intraperitoneally split into two doses, 10 hours apart 118. Woo and colleagues have shown effective glioma regression with adenovirus mediated HSVTK gene transfer at doses ranging from 50 mg/kg/day to 200 mg/kg/day[136].

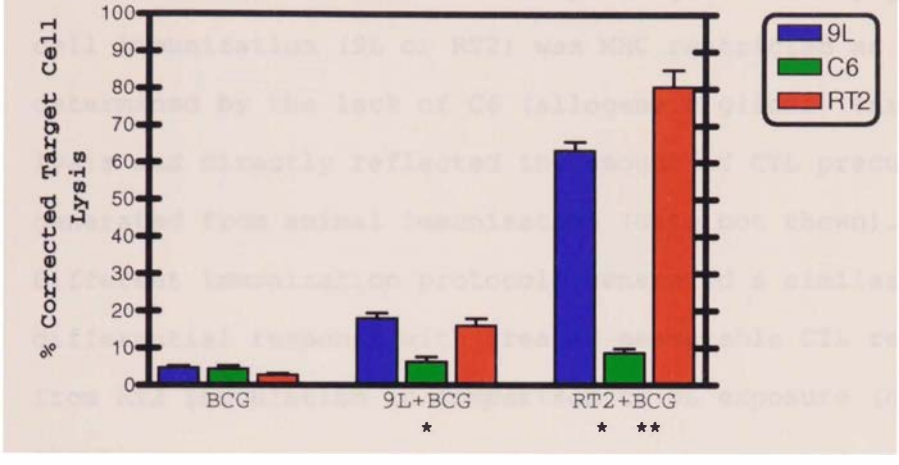
The ability of AV-TK to mediate tumor regression in different tumor models was tested. AV-TK has been used to effectively regress tumors in a variety of animal models including glioma[137], lymphoma[138] and mesothelioma[139]. Interestingly, of these studies, only in immunocompetent animals was AV-TK able to abrogate tumors. Treatment of human tumors in nude animals resulted in a significant

decrease in tumor volume, however, tumors continued to progress after the GCV administration period ended[137]. In animal models, repeat administration of adenovirus vectors was not successful due to generation of a strong humoral immune response after a single virus exposure[123]. Based upon the observation that tumor elimination was only possible in immunocompetent animals, the role of the host immune response in augmenting AV-TK mediated tumor regression was studied.

The success of Woo and colleagues[137] in earlier experiments with the rat glioma 9L prompted further research. Another rat glioma model described extensively in the literature is RT2[48]. Both cell lines express antigens to which cellular immune responses can be generated[48]. Previously, the immunogenicity of rat gliomas has been characterized by TIL isolated from subcutaneous or intracranial implants of tumor cells[36]. In these studies, host survival and frequencies of CTL precursors correlated with tumor immunogenicity and the number of CTL precursors was reflective of the cytolytic immune response generated after animal immunization. Therefore, we evaluated the ability of these cell lines to generate a cellular immune response after immunization of animals. Different immunization protocols for generating immune responses were

tested in our laboratory (data not shown). Figure 5 shows the cytolytic response of splenocytes from animals immunized subcutaneously with 5 X 10^6 irradiated tumor cells and 25 ug of *Mycobacterium Bovis* (BCG) as an immune adjuvant.

Figure 5 - Cellular Immune Response From Animals Immunized With Rat Glioma Cell Lines



Immunization Regimen

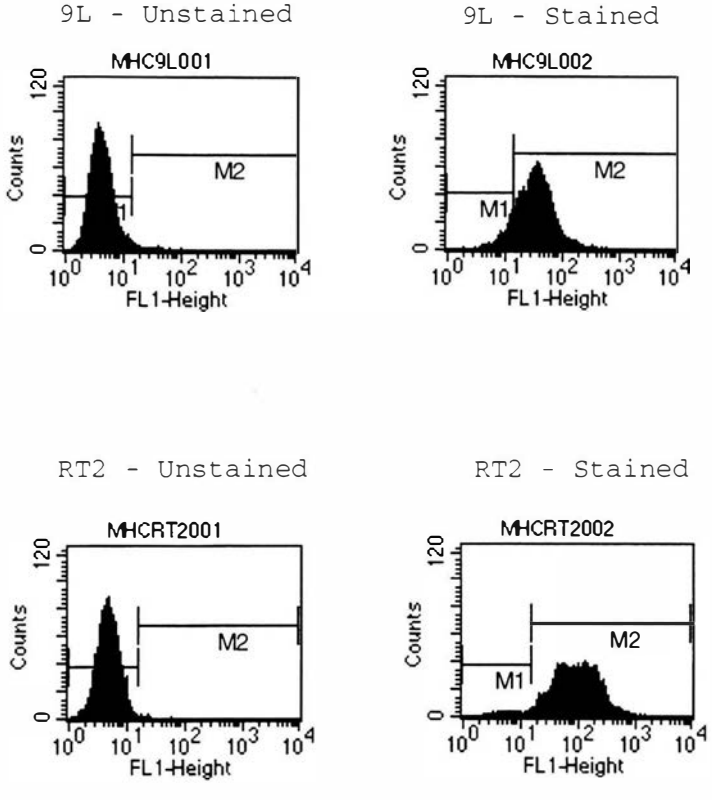
Fisher rats were immunized by subcutaneous injection of 5 X 10^6 glioma cells and 25 ug BCG or BCG alone (x-axis). Target cells included the syngeneic Fisher rat derived 9L and RT2 cells and the allogeneic Wistar-Furth rat derived C6 cells. Splenocytes from 3 animals were harvested 14 days after immunization and secondarily stimulated for 6 days *in vitro* with irradiated cells to which they they had been immunized. Target cell lysis (y-axis) was measured in a 4 hour ^{51}Cr release assay. Lysis data is given for an effector to target ratio of 100:1. Data was analyzed by Friedman test for repeated one way analysis of variance. The 9L+BCG and the RT2+BCG immunization groups (*) were significant ($p<0.05$) in comparison to the BCG immunization group. Additionally, the RT2+BCG group (**) was significant ($p<0.05$) in comparison to the 9L+BCG group.

A very strong cytolytic response (82%) at an effector to target ratio of 100:1 was measured from RT2 immunized

animals. In contrast, 9L immunized animals had a considerably diminished response (18%). No significant cytotoxicity against glioma targets was measured by BCG immunization alone. The CTL response generated by glioma cell immunization (9L or RT2) was MHC restricted as determined by the lack of C6 (allogeneic glioma) target cell lysis and directly reflected the amount of CTL precursors generated from animal immunization (data not shown). Different immunization protocols generated a similar differential response with greater measurable CTL response from RT2 inoculation in comparison to 9L exposure (data not shown).

To further determine parameters affecting antigenicity, surface MHC Class I expression was quantified. The MHC class I gene products are known to play a role in tumor immunity. Both qualitative and quantitative changes in MHC expression can result in altered anti-tumor immune responses. Downregulation of MHC Class I by viral gene products is known to enhance tumorigenicity by abrogating CTL mediated tumor reactivity. Therefore, quantification of MHC class I expression may further corroborate the increased immunogenicity of RT2 over 9L. In Figure 6, the surface MHC Class I expression of 9L and RT2 is depicted.

Figure 6 - Surface MHC Class I Expression of the 9L and RT2 Glioma Cell Lines



Glioma cell lines growing at 70% confluency were harvested and stained with a FITC labelled antibody against MHC Class I gene products (Pharminigen). Cells were immediately analyzed by flow cytometry. Panels on the left represent unstained cells. Panels on the right are after antibody staining. Fluorescence is detected on the x-axis. M1 = gate set from unstained control cells. M2 = M1 exclusionary gate.

Previously we reported that both 9L and RT2 tumor cells expressed antigens against which cellular immune responses were generated (Figure 5). However, these cell lines varied in their ability to generate reactive CTL. A possible explanation can be derived from comparing their surface MHC class I expression (Figure 6). The MFI upon staining with antibody RT1A^{1} (Pharmingen) for MHC expression was almost three thousand times greater for RT2 versus 9L (Figure 6). Interestingly, generation of a cytolytic response upon animal immunization correlated to the difference in MHC expression. RT2 cytotoxicity from immunized animals was approximately 4 times greater than measured for 9L at an effector to target ratio of 100:1 in repeat experiments. Possible causes include greater tumor antigen presentation in the context of MHC Class I, greater engagement of T-Cell receptors with MHC or increased probability of lymphocyte and tumor interaction.

On the other hand, this difference in antigenicity may not be related to MHC expression. CTL activity measurement is not an exact process and MHC expression may vary by cell lines *in vitro* when grown *in vivo*. Furthermore, generation of a cellular immune response is dependent on many other factors including antigen presenting cells.

Regardless of the mechanism, RT2 was more immunogenic

than 9L as defined by greater *in vitro* CTL response generation. Given the differences, however, the efficiency of AV-TK treatment to mediated tumor eradication was studied using both cell lines. It was our opinion that the difference in tumor immunogenicity would allow for a determination of the role of host immunity in tumor regression.

In Table II, AV-TK , mediated ablation of subcutaneous gliomas was compared between cell lines and tumor sizes.

TABLE II - SUBCUTANEOUS GLIOMA ABLATION EFFICIENCY BY ADENOVIRUS MEDIATED HERPES SIMPLEX THYMIDINE KINASE GENE TRANSFER AND GANCICLOVIR ADMINISTRATION

Tumor:		9L		RT2	
Treatment	Tumor Size	50-100 mm^3	100-150 mm^3	200-250 mm^3	300-350 mm^3
No Virus	+ GCV	0/9	0/9	0/6	0/6
	- GCV	0/9	0/9	0/6	0/6
AV-LacZ	+ GCV	0/9	0/9	0/6	0/6
	- GCV	0/9	0/9	0/6	0/6
AV-TK	+ GCV	0/9	0/9	0/6	0/6
	- GCV	**14/15 ***	15/29	**12/12 ***	**9/9 ***

Subcutaneous tumors of various sizes stated above were injected with 2 X 10^9 pfu of the recombinant virus given in column 1. Control animals received sham injections of vehicle. Thirty sVII hours after injection, certain groups of animals were given injections of GCV (column 2) as per Materials and Methods. Table values represent animals with complete tumor ablation without tumor relapse as defined by non-palpable tumor 3 months from the initial date of tumor injection. Results are the combined averages of 3 experiments. Results in bold (*) are statistically significant ($p<0.05$) by chi square analysis when compared to the treatment groups: AV-TK - GCV, AV-LacZ $\pm$ GCV and No Virus $\pm$ GCV.

In Table II, tumor ablation was contingent on HSVTK gene transfer and GCV administration. Total ablation of

tumors did not correlate to tumor regression. *In situ* treatment of subcutaneous tumors by intratumoral injections of AV-TK followed by GCV administration resulted in tumor regression in 100% of animals (data not shown). No tumor regression was seen in other control groups. Tumor progression was not altered in animals treated with AV-LacZ either with or without GCV treatment suggesting that neither adenovirus or GCV is acutely cytostatic or cytotoxic independently. Also, transfer of the HSVTK gene alone (AV-TK without GCV) was not sufficient for tumor regression or tumor eradication. Tumor regression was dependent on both thymidine kinase gene transfer and GCV administration.

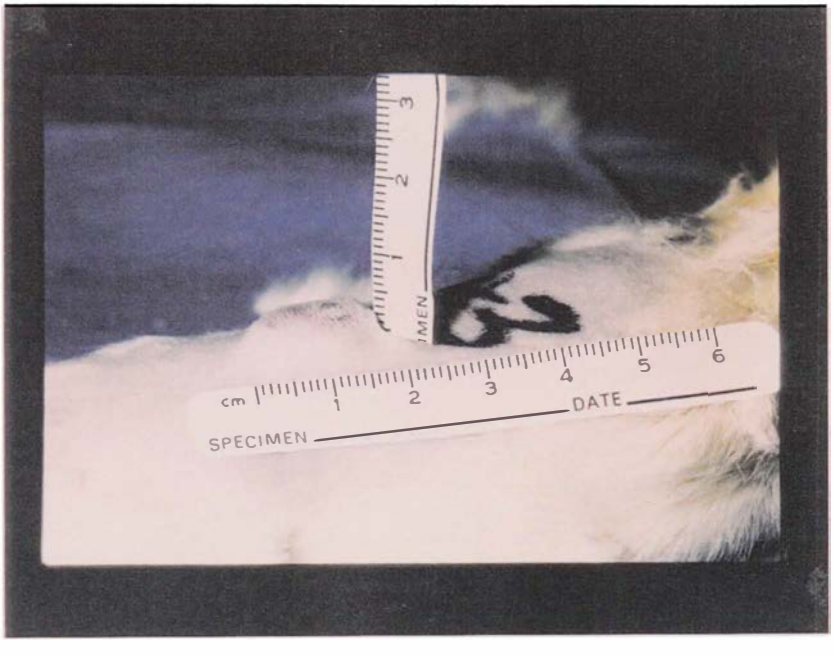
Concerning tumor abrogation, of the animals bearing 100-150 mm^3 9L tumors, 20 of 29 animals initially showed complete tumor elimination but 5 animals had tumor reoccurrence. The final treatment efficiency was approximately 50%. Tumor size did have a bearing on AV-TK treatment effectiveness for 9L tumors. Treatment of smaller tumors (50-100 mm^3) resulted in a greater cure rate (14/15 animals) which was statistically significant in comparison to the other treatment groups while the 50% tumor ablation effeciency for the larger 9L tumors was not.

In contrast, treatment of RT2 tumors was more efficacious. Tumors of 300-350 mm^3 (twice the size of 9L

tumors) were effectively eliminated with HSVTK gene transfer plus GCV administration. Of the animals with eliminated RT2 tumors, relapse was never observed. Therefore, AV-TK plus GCV was more effective for the treatment of the more immunogenic RT2 gliomas.

In Figure 7, a series of panels illustrating the course of an exceptionally large RT2 tumor that regressed upon AV-TK and GCV administration is shown.

Figure 7A - RT2 Tumor Regression After Treatment With AV-TK Intratumoral Injection And GCV Administration

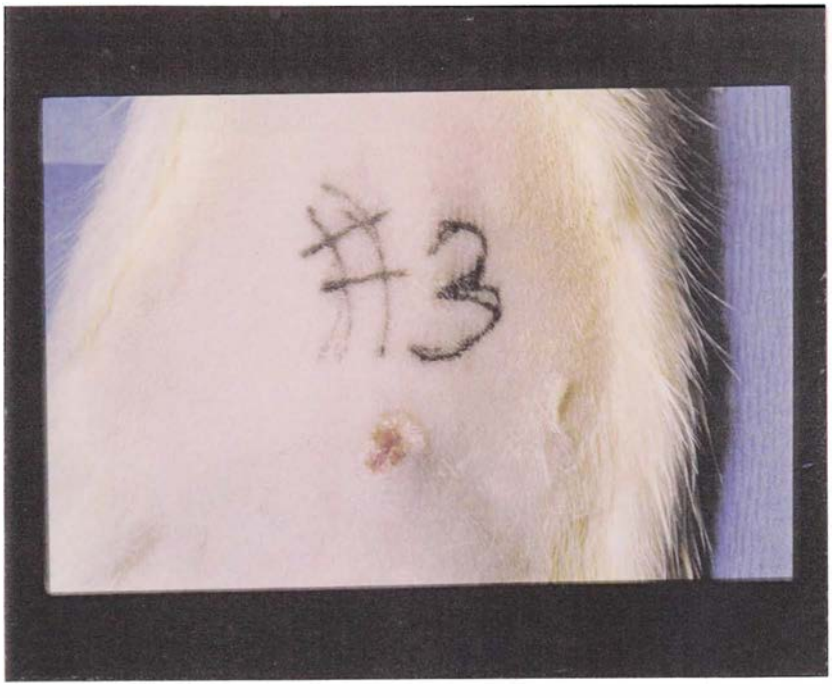


2 X 10^6 RT2 glioma cells were injected subcutaneously on the dorsal back 1 cm from the midline. 21 days later, this tumor of approximately 1800 mm^3 was injected with 2 X 10^9 pfu of AV-TK in 100 ul PBS. 48 hours after injection, this animal was given intraperitoneal injections of 150 mg/kg/dose twice a day, 10 hours apart, for 5 days.

Figure 7B - RT2 Tumor Regression After Treatment With AV-TK Intratumoral Injection And GCV Administration

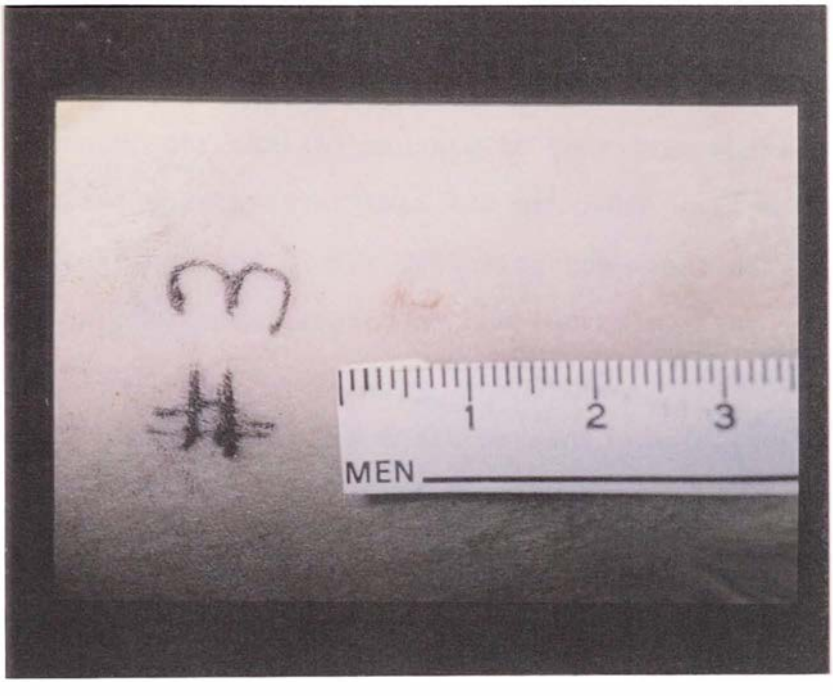
2×10^6 RT2 glioma cells were injected subcutaneously on the dorsal back 1 cm from the midline. 21 days later, this tumor of approximately 1800 mm^3 was injected with 2×10^9 pfu of AV-TK in 100 ul PBS. 48 hours after injection, this animal was given intraperitoneal injections of 150 mg/kg/dose twice a day, 10 hours apart, for 5 days. This photograph was taken 1 week after AV-TK injection and 3 days from the end of the GCV administration period.

Figure 7C - RT2 Tumor Regression After Treatment With AV-TK Intratumoral Injection And GCV Administration



2 X 10^6 RT2 glioma cells were injected subcutaneously on the dorsal back 1 cm from the midline. 21 days later, this tumor of approximately 1800 mm^3 was injected with 2 X 10^9 pfu of AV-TK in 100 ul PBS. 48 hours after injection, this animal was given intraperitoneal injections of 150 mg/kg/dose twice a day, 10 hours apart, for 5 days. This photograph was taken 3 weeks after AV-TK injection and 18 days from the end of the GCV administration period.

Figure 7D - RT2 Tumor Regression After Treatment With AV-TK Intratumoral Injection And GCV Administration



2 X 10^6 RT2 glioma cells were injected subcutaneously on the dorsal back 1 cm from the midline. 21 days later, this tumor of approximately 1800 mm^3 was injected with 2 X 10^9 pfu of AV-TK in 100 ul PBS. 48 hours after injection, this animal was given intraperitoneal injections of 150 mg/kg/dose twice a day, 10 hours apart, for 5 days. This photograph was taken 4 weeks after AV-TK injection and 25 days from the end of the GCV administration period.

The tumor represented was originally 1800 mm^3 (Figure 7A). This animal was part of an experiment evaluating survival of rats with subcutaneous tumors. Therefore, the tumor size was permissible under veterinarian approved exemption to our earlier mandate to sacrifice animals when tumor sizes were greater than 400 mm^3. One week following AV-TK treatment and 3 days following the final GCV injection, the tumor regressed considerably. The tumor mass was erythematous and inflamed (Figure 7B). However, a majority of the tumor was still present. In Figure 7C, the tumor at 3 weeks from adenovirus injection is depicted. The tumor mass further regressed to become scar tissue and only a residual ulcerated lesion was visible. After tissue remodeling, this scar remained as a granulomatous lesion (Figure 7D).

As depicted in Figure 7, residual tumor remained at the end of the GCV dosing period (Figure 7B and 7C) and was present as far as 3 weeks after AV-TK injection into the tumor. Many animals with tumors of this size continued to exhibit tumor regression and eventually resulted in complete tumor elimination (data not shown). The course of this tumor serves to illustrate the involvement of host immunity in augmenting tumor regression.

Using radiolabeled GCV in bystander effect studies, H.

Ishii has shown that GCV effects *in vitro* occured very rapidly[140]. Transfer of the phosphorylated GCV metabolites occurs in a matter of hours. If this datum is extrapolated to the *in vivo* situation, as a conservative estimate one may assume that any transduced cell has been killed by 7 days from the end of the GCV dosage period (approximately 10 days from the time of adenovirus administration). The serum half-life of GCV is approximately 6 hours[135]. Therefore, any further killing of residual tumor after this period cannot be explained by GCV mediated toxicity. An explanation of this phenomenon must depend on host augmentation of tumor lysis. Prior studies in athymic nude mice showed significant regression of human transplanted tumor cell lines after AV-TK and GCV treatment[137]. However, despite a substantial decrease in tumor volume, residual tumor remained and eventually progressed. Treatment of tumors in immunocompetent animals, has shown increased infiltration of macrophages and lymphocytes at the injection site[141]. These results indicate that *in vivo* bystander killing in the absence of complete tumor transduction may have an immune component. Our working hypothesis was that AV-TK and GCV reduced the tumor burden sufficiently allowing for a host anti-tumor immune response to be generated (Figure 7). To test this hypothesis, the ability of AV-TK with GCV to

mediate 9L tumor regression in nude rats was evaluated.

TABLE III - SUBCUTANEOUS 9L GLIOMA ABLATION EFFICIENCY BY ADENOVIRUS MEDIATED HERPES SIMPLEX THYMIDINE KINASE GENE TRANSFER AND GCV ADMINISTRATION

Treatment	Prodrug Administration	Animals Rejecting Tumor
AV-TK	+ GCV	0/11
	- GCV	0/3
AV-LacZ	+ GCV	0/3
Vehicle	+ GCV	0/3
Vehicle	- GCV	0/3

Subcutaneous 9L tumors were grown by inoculation of 3×10^6 9L cells on the dorsal surface of nude rats. These tumors (100-150 mm^3) were injected with 2×10^9 pfu of the recombinant adenovirus stated in the left column in a total volume of 100 ul PBS. Animals were subsequently dosed with GCV as stated in the Materials and Methods for 5 days. Results represent the number of animals with complete tumor ablation defined as non-palpable tumor as determined 2 months from the time of adenovirus inoculation. Results are not statistically significant in comparison to each other.

Tumors were started on the dorsal surface of animals by 9L cell inoculation. After 21 days, tumors of approximately 100-150 mm^3 were treated with recombinant virus or vehicle injection and then separated into groups receiving either GCV or PBS injections. Animals were observed for 4 months and tumor volumes were measured on a weekly basis.

Interestingly, animals receiving AV-TK injection coupled with GCV administration showed partial tumor regression (data not presented). Tumors diminished in volume to approximately 50% of their original size over the first 2 weeks from the time of adenovirus injection. However, after this initial period of tumor regression, tumor volumes proceeded to increase over the subsequent weeks necessitating animal euthanasia after 2 months. In contrast, tumor regression was not observed in the other control groups. Tumor volumes in the other groups increased over time until animals were sacrificed.

Similar to the observations made in immunocompetent animals (Table II), tumor regression was dependent on HSVTK gene transfer and prodrug (GCV) administration. Quite differently however, treatment of subcutaneous tumors in these athymic animals by AV-TK and GCV was ineffective in mediating complete tumor elimination (Table III). Whereas in our earlier experiments, tumors of the same size were ablated by AV-TK and GCV treatment in immunocompetent animals (Table II), none of the 9L tumors of athymic animals were eradicated by HSVTK gene transfer. This difference in treatment efficiency was attributed to the lack of an anti-tumor immune response in the athymic animals. These results indicate the importance of the immune response in

facilitating tumor ablation. *In vitro*, 9L cells are considered to have a relatively good bystander effect (Figure 3) mediated by the transfer of toxic GCV metabolites. The poor treatment efficiency *in vivo* of 9L tumors suggests that upon intratumoral injection of AV-TK, the GCV associated bystander effect was insufficient to result in complete tumor regression. *In vivo*, HSVTK gene transfer needed to be augmented by host immunity in order to be maximally effective as a treatment regimen.

To further study the involvement of host immunity in tumor eradication, animals with ablated gliomas were given secondary challenges with lethal (3×10^7) and tumorigenic (3×10^6) doses of tumor cells by inoculation on the opposite flank (Table IV). As a positive control for tumor formation, naïve animals were similarly given tumor challenges.

TABLE IV - RESISTANCE TO TUMOR FORMATION IN ANIMALS WITH ABLATED SUBCUTANEOUS GLIOMAS BY AV-TK AND GCV ADMINISTRATION

Initial Ablated Tumor	Secondary Tumor Challenge	Dose	Animals Forming Tumor
9L	9L	3×10^6	**0/15 ***
		3×10^7	**0/12 ***
	MesII25	3×10^6	3/3
RT2	RT2	3×10^6	**0/8 ***
		3×10^7	**0/6 ***
	MesII25	3×10^6	3/3
None	9L	3×10^6	30/30
		3×10^7	15/15
	RT2	3×10^6	12/12
		3×10^7	12/12
	MesII25	3×10^6	3/3

Animals with approximately 200 mm^3 tumors (column 1) ablated by AV-TK injection and GCV administration per Materials and Methods were inoculated with tumorigenic or lethal doses (column 3) of rat glioma cells or the sygeneic mesothelioma cell line MesII-25 on the opposite flank from the initial tumor site. This secondary challenge of tumor cells was given at 1 month from the time of AV-TK injection of the initial ablated tumor. These animals were observed for 3 months. Tumor formation (column 4) was defined as palpable

tumor at least 1 month from the time of secondary tumor inoculation. Results represent the combined values of 2 experiments. Results in bold (*) are statistically significant ($p<0.001$) by chi-square analysis when compared to naïve control animals challenged with either 9L or RT2 tumors.

In Table IV animals with an ablated subcutaneous 9L or RT2 glioma generated an anti-tumor immune response capable of preventing engraftment of tumor cells. 100% of animals with an ablated glioma by AV-TK and GCV treatment rejected a secondary inoculum of tumor cells. However, this anti-tumor response was tumor specific as determined by the secondary challenges with the MHC Class I expressing syngeneic mesothelioma cell line MESII-25. Animals with previously ablated 9L or RT2 tumors failed to reject the mesothelioma cell line and were only able to reject tumor cells to which they were previously exposed (Table IV). Development of anti-tumor immunity was not contingent on the immunogenicity of the initial glioma as animals with ablated 9L tumors were as resistant to secondary tumor challenges as the animals cured of RT2. Animals have been observed for over 1 year since the time of AV-TK treatment and all animals remain tumor free. These animals are continuing to be observed.

Next, the kinetics of tumor immunity was studied.

Particularly of interest was the time period over which these animals exhibited tumor immunity. In the previous experiment animals were challenged with tumor cells 1 month after treatment of the initial tumor. To address if the observed anti-tumor response was possibly limited in its time course, animals with ablated tumors were secondarily challenged with tumor cells as in the previous experiment. However, the timing of the secondary tumor challenge was varied from the time of initial tumor eradication.

TABLE V - RESISTANCE TO TUMOR FORMATION IN ANIMALS WITH ABLATED SUBCUTANEOUS GLIOMAS BY AV-TK AND GCV ADMINISTRATION

Initial Ablated Tumor By AV-Tk And Gcv Treatment	Secondary Tumor Challenge	Animals Forming Tumor Upon Rechallenge At 1 Month	Animals Forming Tumor Upon Rechallenge At 2 Months	Animals Forming Tumor Upon Rechallenge at 6 Months
9L	9L	**0/15 ***	**0/3 ***	**0/9 ***
RT2	RT2	**0/8 ***	0/3	**0/6 ***
None	9L	15/15	15/15	15/15
None	RT2	3/3	6/6	6/6

Animals with approximately 200 mm^3 tumors (column 1) ablated by AV-TK injection and GCV administration per Materials and Methods were inoculated with tumorigenic doses of the tumor cells to which they had previously cured (column 2) on the opposite flank from the initial tumor site. This secondary

challenge of tumor cells was given at 1 month (column 3), 2 months (column 4) or 6 months (column 5) from the time of AV-TK injection of the initial tumor. These animals were observed for 3 months. Results (column 3, 4, and 5) represent tumor formation as defined by palpable tumor at least 1 month from the time of secondary tumor injection. Results in bold (*) are statistically significant ($p<0.05$) by chi square analysis when compared to their corresponding controls challenged with the same tumor cell line.

Animals exhibited long standing tumor immunity. For periods up to 6 months after ablation of the original tumor, animals rejected engraftment of the autologous tumor cells (Table V). This long term tumor immunity was not dependent on the immunogenicity of the original tumor as 9L ablated animals rejected secondary tumor engraftment as well as RT2 ablated animals. Long term tumor resistance is significant since in most cases of tumor reoccurrence the secondary tumor focus is a consequence of seeding from the original site. As tumor metastases often share the same antigenic epitope, development of tumor immunity following treatment of the initial tumor may prevent distant metastases. This hypothesis is currently being investigated.

Given AV-TK treated 9L ablated animals to rejected engraftment of the minimum tumor forming dose or (3×10^6 cells) or a lethal dose (3×10^7 cells) of tumor cells for a period of at least 6 months from the time of original tumor

exposure, involvement of the host immune response was indicated. There are three major types of lymphocytes reported to have cytotoxic anti-tumor activity both *in vitro and in vivo*. These cell populations are NK cells, LAK cells and CTL[14].

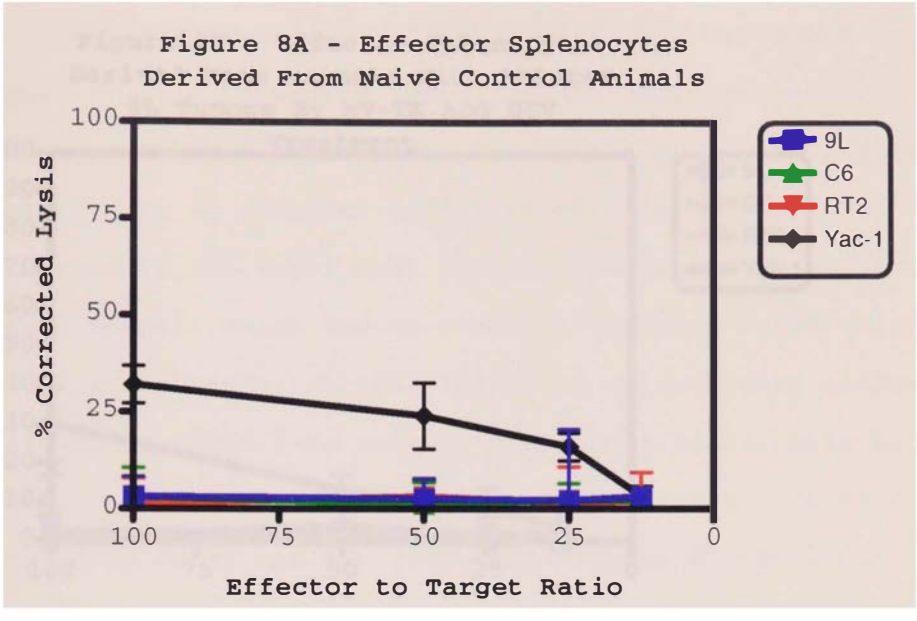
NK cells are normal circulating cells involved in immune surveillance 14. This group of lymphocytes has the intrinsic ability to recognize and destroy tumor cells without previous exposure. In humans they are CD3-, CD16+, and CD56+, and are a subset of the large granular lymphocytes[14]. There is increasing evidence that the second group of lymphocytes, LAK cells, are not a separate lineage but represent activated NK cells[17]. LAK cells are produced by stimulating normal NK cells with high dose IL-2 (>1000 Units/ml). They are characterized by their ability to lyse a variety of fresh and cultured target cells without antigen specificity[18]. As a consequence their cytotoxic potential is not MHC restricted. Lastly, CTL are a subset of T-lymphocytes with surface CD8+ expression which kill target cells expressing specific antigen[18]. Their purpose in immune surveillance is to recognize foreign peptides derived from intracellular antigens associated with self MHC class I molecules and to lyse these cells[13]. A majority of

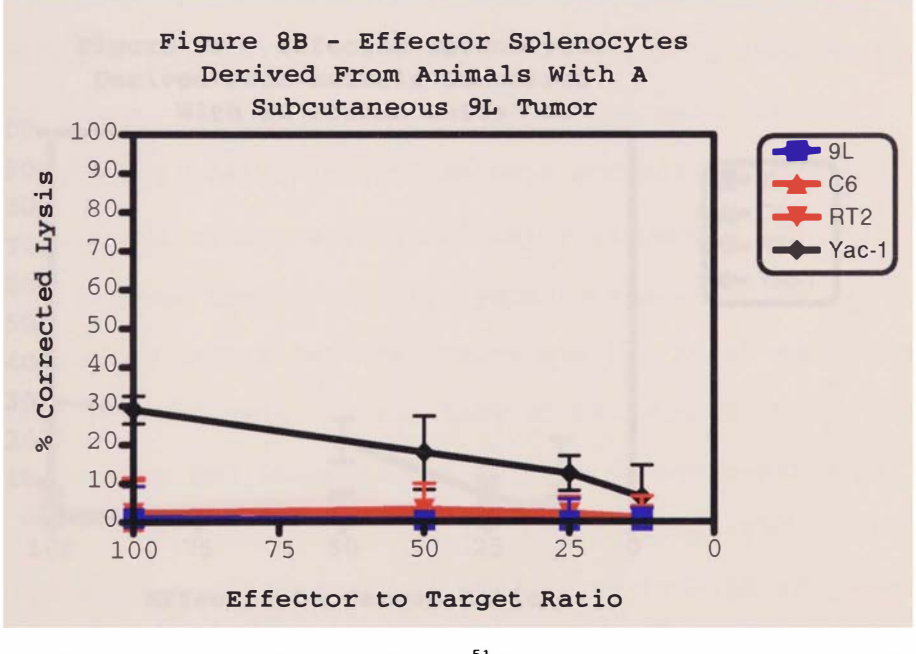
circulating CTL are not fully differentiated and activation requires antigen recognition along with cytokine stimulation usually provided by CD4+ regulatory lymphocytes[14].

To determine the cell type responsible for conferring tumor immunity in our animals, *in vitro* cytotoxicity assays were conducted. Cytotoxicity was measured by the ability of splenocytes to lyse ^{51}Cr labeled target cells. In order to differentiate between the three cytotoxic cell types, cytotoxicity was measured directly from freshly harvested splenocytes and also from cells secondarily stimulated with irradiated autologous tumor cells and RTS. NK cells require no secondary stimulation and their activity can be detected from the freshly harvested immune cells. In contrast, CTL responses only occur *in vivo* and to detect CTL activity *in vitro* requires secondary stimulation along with regulatory lymphocytes for antigen presentation. The irradiated autologous tumor cells provide the antigen and RTS provides the necessary cytokine stimulation. The target cells included the autologous tumor cells (9L or RT2) to which the animal had been primed, C6 an allogeneic glioma in order to determine MHC restricted cytotoxicity and MatB, a syngeneic unrelated adenocarcinoma to determine tumor specificity. LAK cell activity would be manifest as lysis of the syngeneic tumor cells as well as the allogeneic C6 target

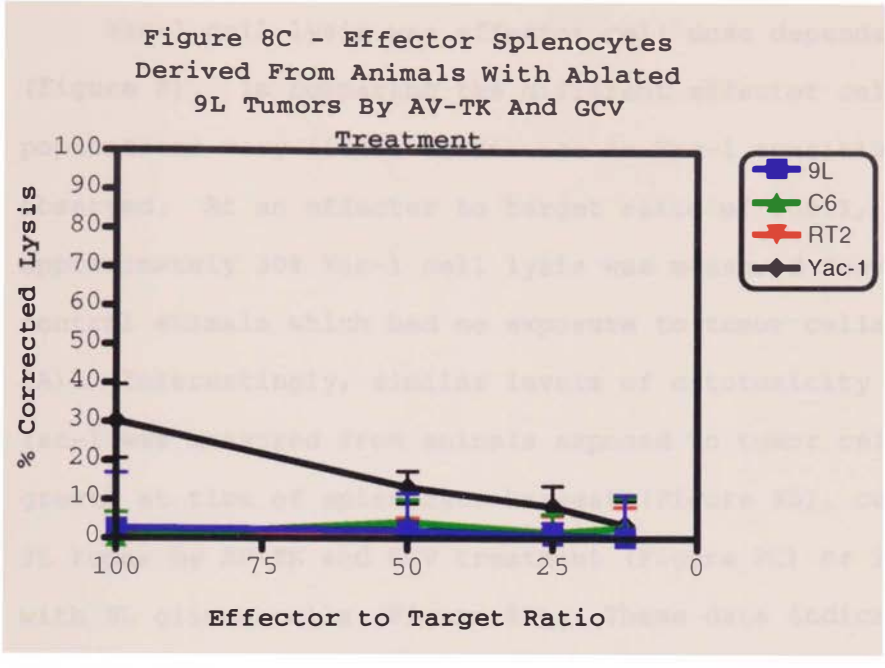
cells and MatB target cells. In contrast, CTL activity would exhibit as specific lysis of only the tumor cells used for immunization.

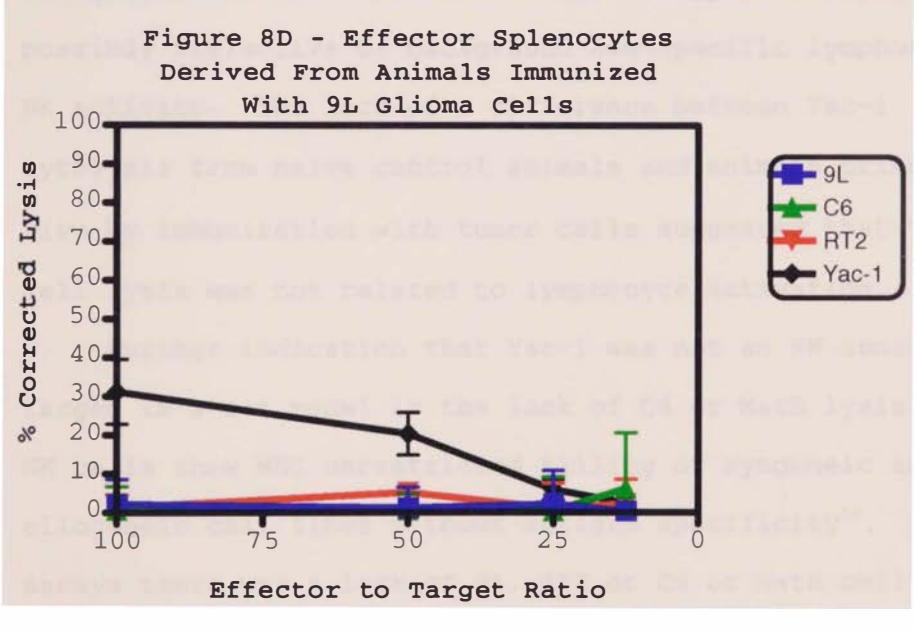
Figure 8 represents the results of a variety of *in vitro* cytolytic assays from freshly harvested splenocytes to measure NK cell activity. In addition to the normal glioma targets, Yac-1, a target cell known to be sensitive to NK cell cytolysis was included. Although the mechanism for NK cell susceptibility for this cell line is unknown, it has been used extensively as an indicator of NK cell activity in murine CTL assays. Due to the lack of a rat NK sensitive target, other researchers have similarly used Yac-1 in rat studies[48]. In these studies Yac-1 cell lysis from rat effector cells reflected lymphocyte activation without specificity for NK cells.





Cytotoxicity measured by 4 hour ^{51}Cr release assays against glioma targets without secondary stimulation. Splenocyte derivation is given in the title.





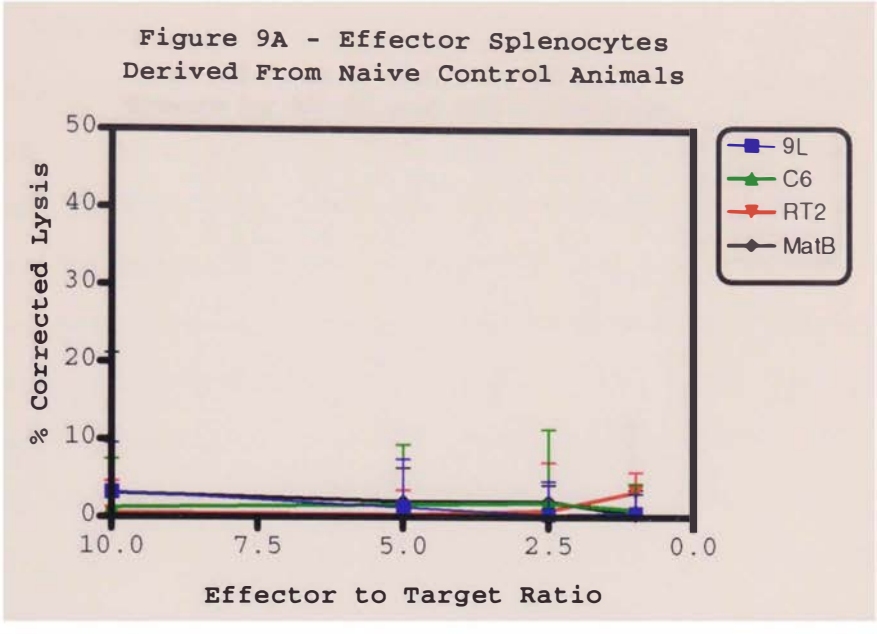
Cytotoxicity measured by 4 hour ^{51}Cr release assays against glioma targets without secondary stimulation. Splenocyte derivation is given in the title.

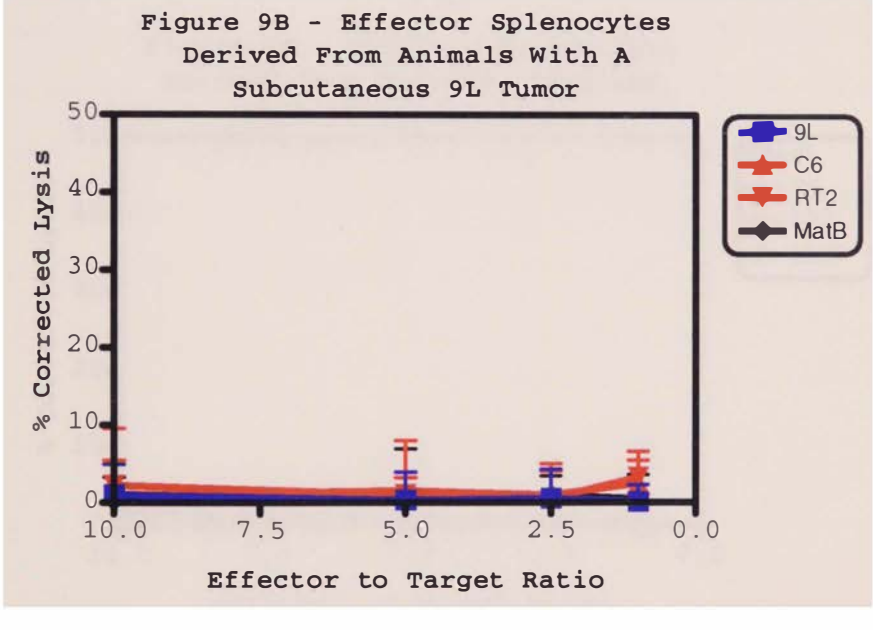
Yac-1 cell lysis was effector cell dose dependent (Figure 8). In comparing the different effector cell populations very little difference in Yac-1 sensitivity was observed. At an effector to target ratio of 100:1, approximately 30% Yac-1 cell lysis was measured from naive control animals which had no exposure to tumor cells (Figure 8A). Interestingly, similar levels of cytotoxicity against Yac-1 was measured from animals exposed to tumor cells by growth at time of splenocyte harvest (Figure 8B), cured of 9L tumor by AV-TK and GCV treatment (Figure 8C) or immunized with 9L glioma cells (Figure 8D). These data indicated that Yac-1 lysis was non-specific. This non-specific lysis was possibly reflective of background non-specific lymphocyte or NK activity. The lack of a difference between Yac-1 cytolysis from naive control animals and animals primed *in vivo* by immunization with tumor cells suggested that Yac-1 cell lysis was not related to lymphocyte activation.

Further indication that Yac-1 was not an NK sensitive target in a rat model is the lack of C6 or MatB lysis. True NK cells show MHC unrestricted killing of syngeneic and allogeneic cell lines without antigen specificity[16]. In our assays there was a lack of 9L, RT2 or C6 or MatB cell lysis. Possible causes are either poor or nonexistent NK cell mediated cytotoxicity from rat splenocytes or a lack of NK

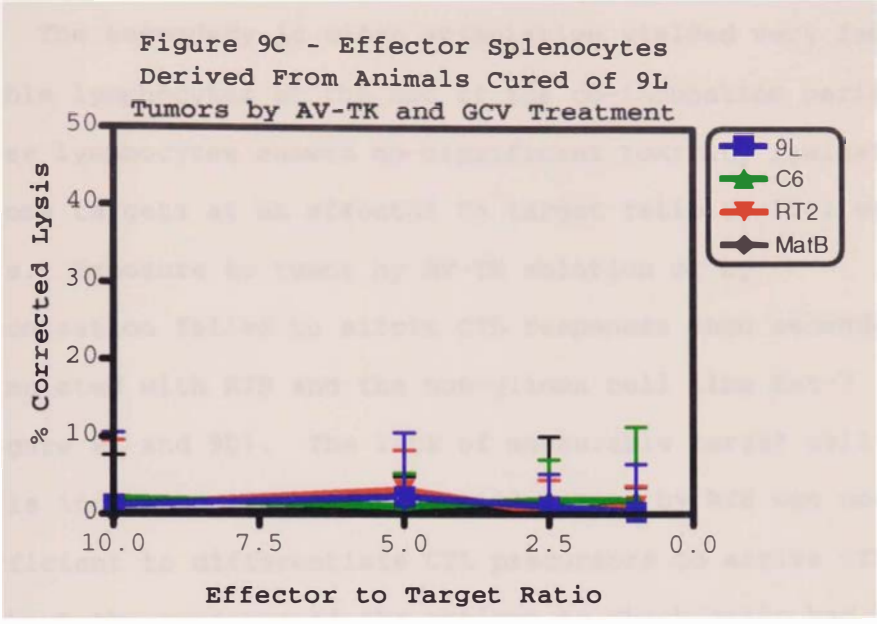
cell sensitivity of the target cells. Similar results were obtained from animals exposed to RT2 tumors (data not shown).

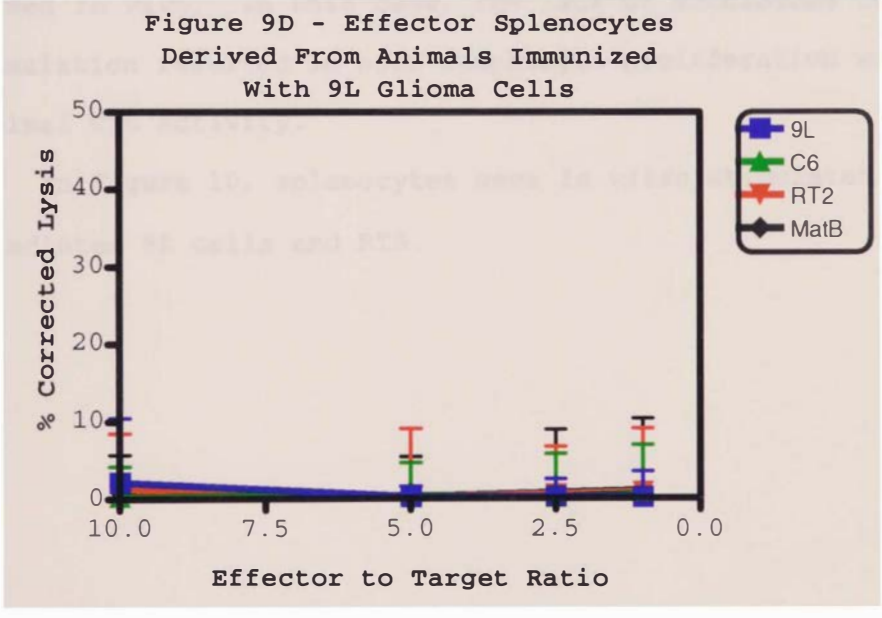
The data presented in Figure 8 was consistent with reports by Holladay et al.[48] In their model, immunization with 9L cells failed to exhibit cytotoxic activity without secondary *in vitro* stimulation with 9L tumor cells. Others have similarly shown that lymph node and spleen derived lymphocytes are rich in tumor specific CTL precursors without measurable CTL activity unless stimulated with antigen and cytokines[142]. The most frequently employed secondary stimulation protocol uses low dose IL-2 (10 Units/ml) and irradiated autologous tumor cells. However, pilot studies in our laboratory have shown augmented CTL activity after secondary stimulation with autologous tumor cells and RTS (data not shown). RTS is a cytokine rich medium derived from Con A stimulated rat splenocytes. Hence, our secondary stimulation protocol was a modification of the method of Holladay et al.[38], replacing IL-2 with media containing 10% RTS. To account for non-specific RTS stimulation of effector cells, Figure 9 represents CTL assays after a 6 day *in vitro* stimulation of effectors with an unrelated cell line, the syngeneic nasal carcinoma Fat-7, and RTS.





Cytotoxicity measured by 4 hour ^{51}Cr release assays against glioma targets after a 6 day secondary stimulation of splenocytes with Fat-7 and RTS. Splenocyte derivation is given in the title.

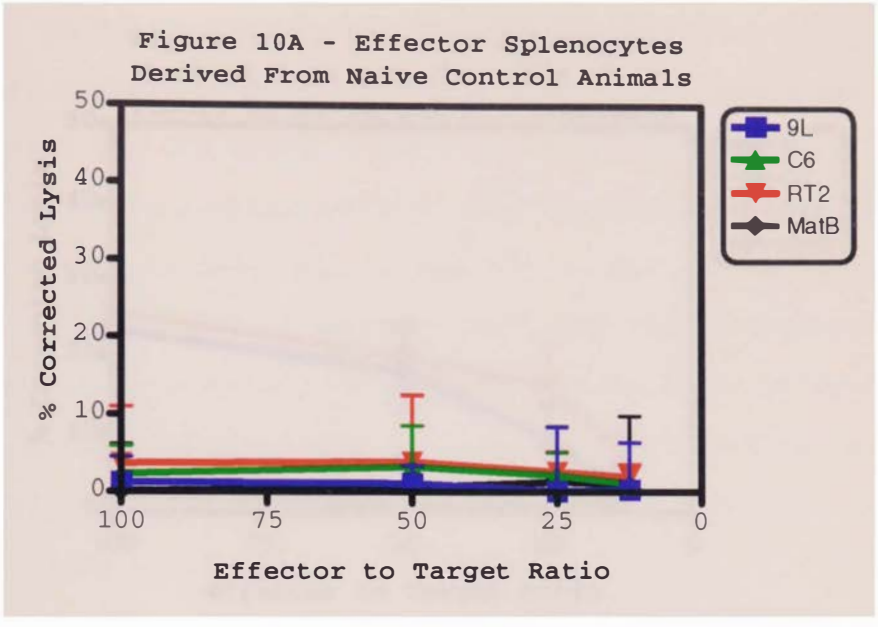


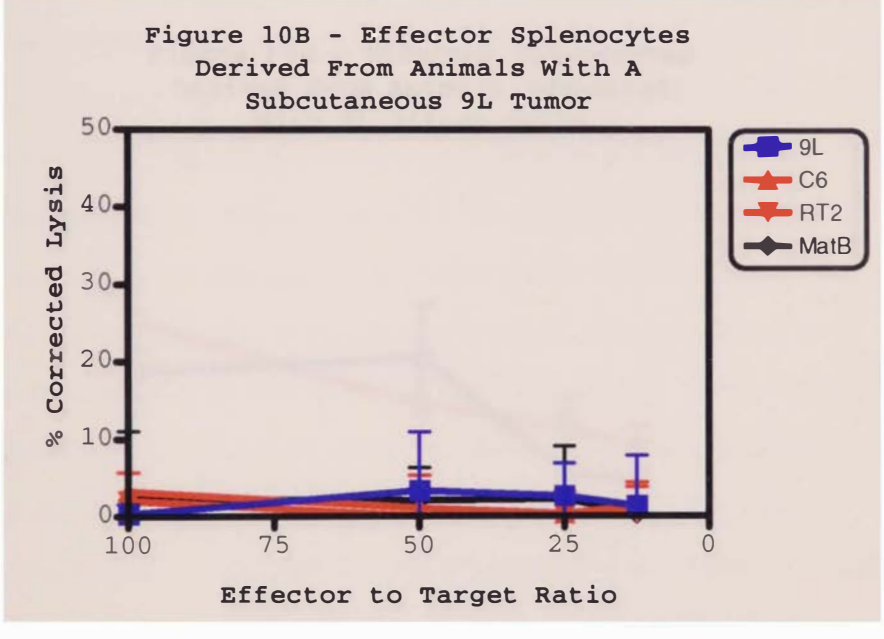


Cytotoxicity measured by 4 hour ^{51}Cr release assays against glioma targets after a 6 day secondary stimulation of splenocytes with Fat-7 and RTS. Splenocyte derivation is given in the title.

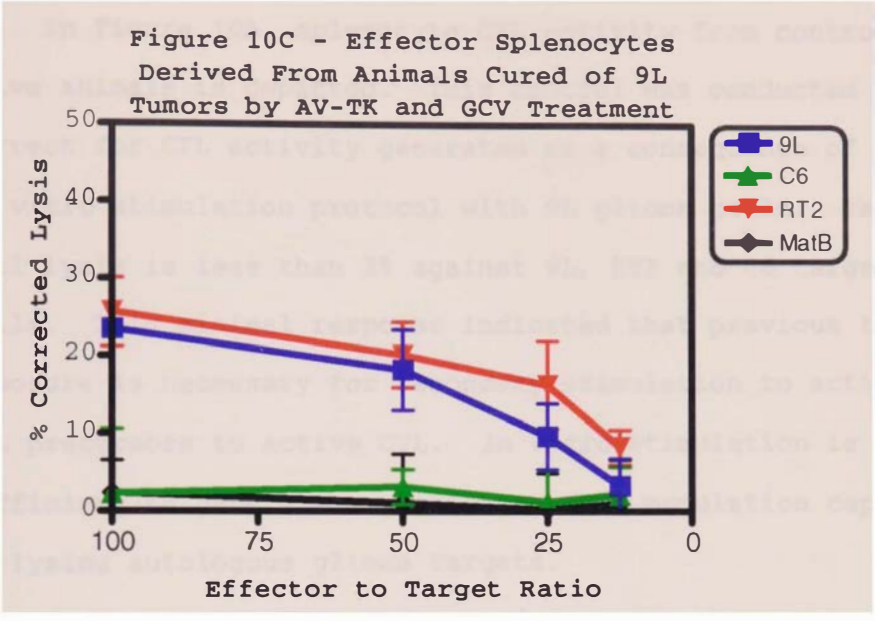
The secondary *in vitro* stimulation yielded very few viable lymphocytes at the end of the co-incubation period. These lymphocytes showed no significant toxicity against glioma targets at an effector to target ratio of 10:1 or less. Exposure to tumor by AV-TK ablation or by immunization failed to elicit CTL responses when secondarily stimulated with RTS and the non-glioma cell line Fat-7 (Figure 9C and 9D). The lack of measurable target cell lysis indicated that cytokine stimulation by RTS was not sufficient to differentiate CTL precursors to active CTL without the presence of the antigen to which cells had been primed *in vivo*. In this case, the lack of autologous tumor stimulation resulted in poor lymphocyte proliferation with minimal CTL activity.

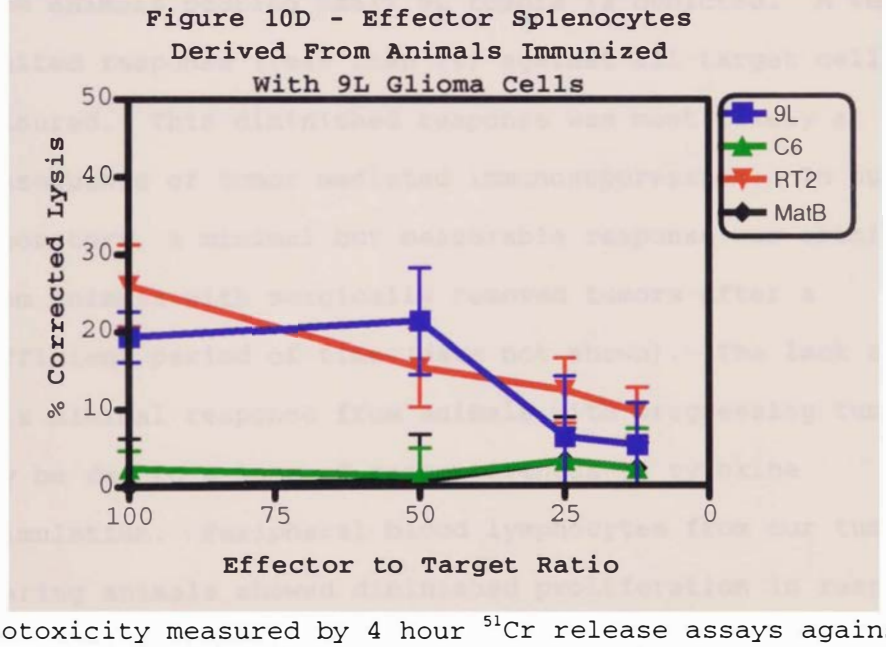
In Figure 10, splenocytes were *in vitro* stimulated with irradiated 9L cells and RTS.





Cytotoxicity measured by 4 hour ^{51}Cr release assays against glioma targets after a 6 day secondary stimulation of splenocytes with 9L cells and RTS. Splenocyte derivation is given in the title.





Cytotoxicity measured by 4 hour ^{51}Cr release assays against glioma targets after a 6 day secondary stimulation of splenocytes with 9L cells and RTS. Splenocyte derivation is given in the title.

In Figure 10A, splenocyte CTL activity from control naive animals is depicted. This control was conducted to correct for CTL activity generated as a consequence of the *in vitro* stimulation protocol with 9L glioma cells. Target cell lysis is less than 3% against 9L, RT2 and C6 target cells. This minimal response indicated that previous tumor exposure is necessary for secondary stimulation to activate CTL precursors to active CTL. *In vitro* stimulation is not sufficient to generate a significant CTL population capable of lysing autologous glioma targets.

In Figure 10B, CTL response from splenocytes generated from animals bearing small 9L tumors is depicted. A very limited response (less than 1%) against all target cells was measured. This diminished response was most likely a consequence of tumor mediated immunosuppression. In our laboratory, a minimal but measurable response was obtained from animals with surgically removed tumors after a sufficient period of time (data not shown). The lack of this minimal response from animals with progressing tumor may be due to a loss of responsiveness to cytokine stimulation. Peripheral blood lymphocytes from our tumor bearing animals showed diminished proliferation in response to the mitogens Con A, PHA and IL-2 (data not shown). Similarly, much fewer viable lymphocytes were recovered

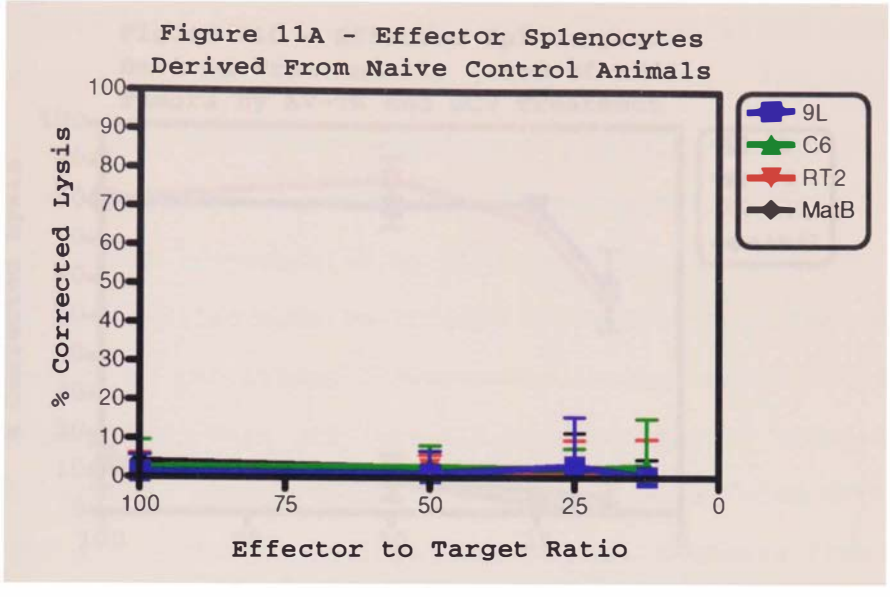
after secondary stimulation in contrast to effectors from immunized animals (Figure 10D). In addition to the loss of cytokine mediated proliferation, CTL precursors from tumor bearing animals may also fail to differentiate into active CTL by antigen stimulation.

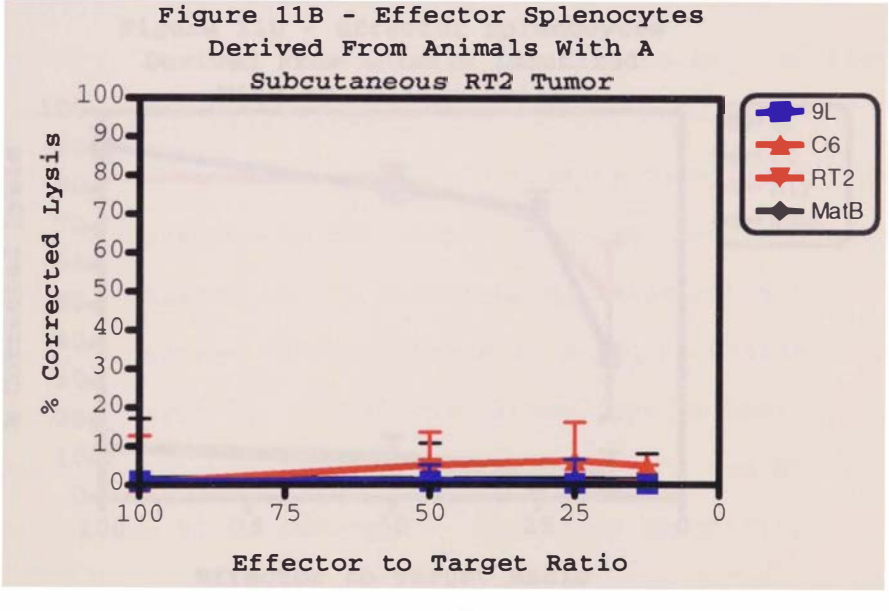
In contrast to Figure 10A and 10B, in Figure 10C effector cells from our AV-TK treated-9L ablated animals showed significant cytolytic killing of 9L and RT2 glioma target cells. Interestingly, this cytotoxicity was not tumor specific but glioma specific. There was limited lysis of the syngeneic breast adenocarcinoma cell line MatB. However, significant lysis of both the syngeneic glioma cell lines 9L and RT2 was measured at effector to target ratios of 50:1 and 100:1. This glioma cross reactivity has been reported[38] and is postulated to be a result of a common glioma antigen expressed by both cell lines. This glioma specific CTL response failed to lyse the allogeneic C6 glioma target cell line. However, this observation suggested CTL cells as immune effector cell population instead of LAK cells which would exhibit MHC unrestricted lysis of C6 cells (data not shown).

Interestingly, the immune response from animals with ablated 9L tumors by AV-TK and GCV (Figure 10C) was similar to that from animals immunized with 9L tumor cells and BCG

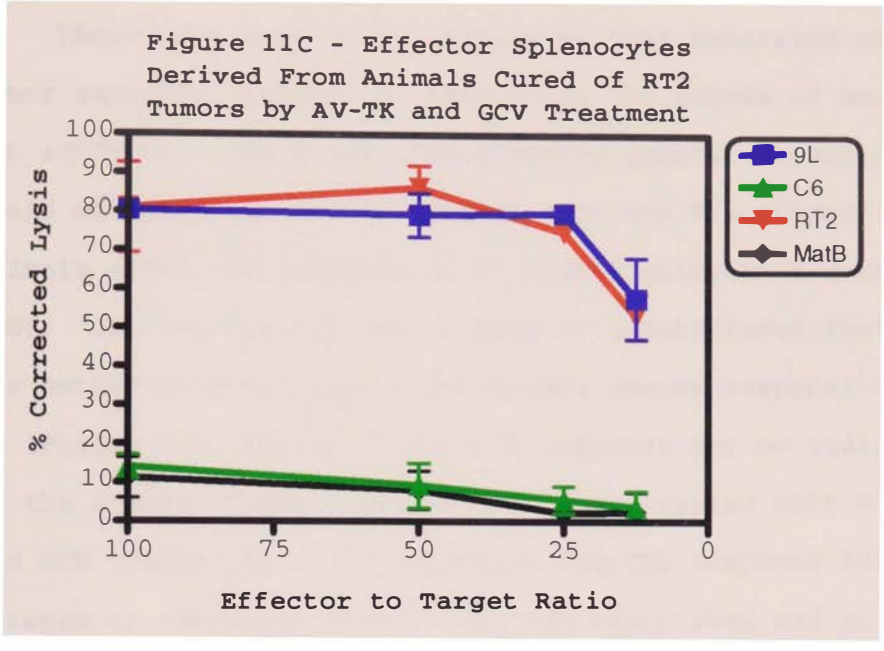
(Figure 10D). The immune response was slightly greater against glioma target cells from immunized animals, however, this difference was not statistically significant. These data suggested that AV-TK plus GCV treatment of tumors was similar to tumor cell vaccination in its ability to generate a CTL immune response.

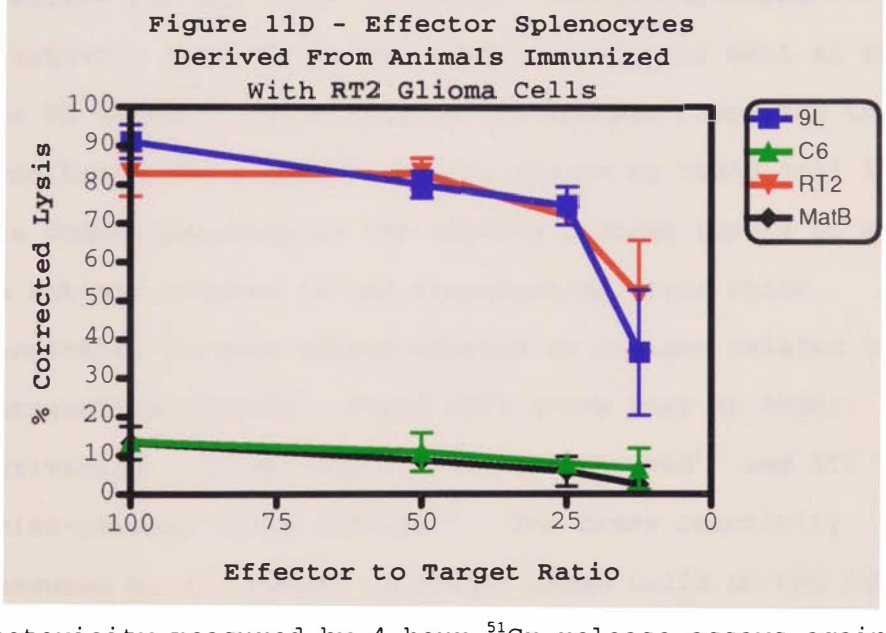
Figure 11 represents a similar study conducted with animals exposed to RT2.





Cytotoxicity measured by 4 hour ^{51}Cr release assays against glioma targets after a 6 day secondary stimulation of splenocytes with RT2 cells and RTS. Splenocyte derivation is given in the title.





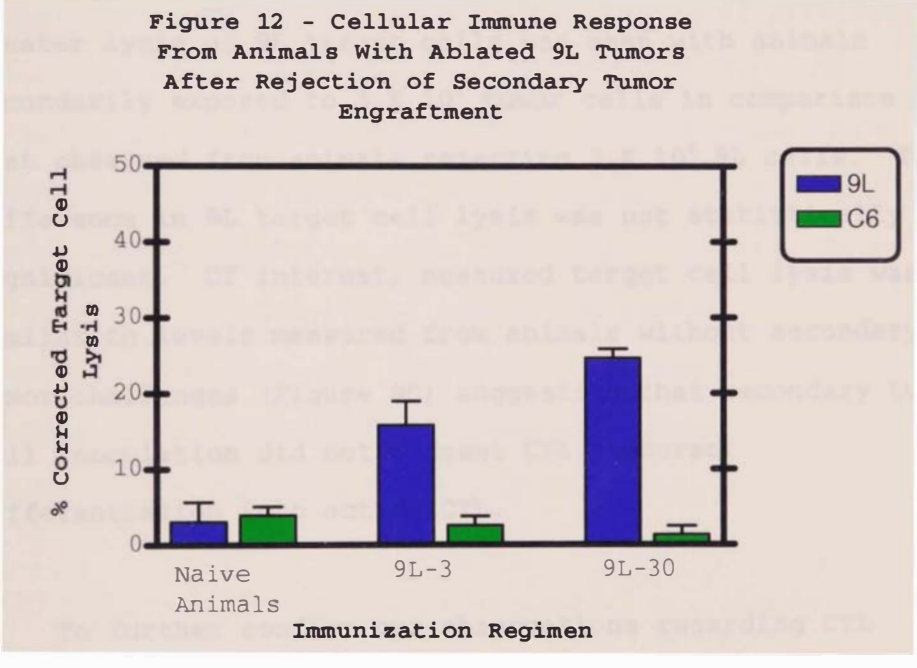
Cytotoxicity measured by 4 hour ^{51}Cr release assays against glioma targets after a 6 day secondary stimulation of splenocytes with RT2 cells and RTS. Splenocyte derivation is given in the title.

These data were quite similar to that generated with 9L tumor exposure (Ficure 10) except for the degree of measured CTL activity. There was significantly greater measured lysis of syngeneic glioma targets from the RT2 primed animals (>80%) as compared to 9L primed animals (Figure 10D). Our earlier studies (Figure 2) demonstrated that RT2 was better at generating a measurable immune response than 9L (Figure 2). This stronger CTL response may be indicative of the greater tumor response rate when treated with AV-TK and GCV (Table III). Of interest, the CTL response from RT2 ablated or immunized animals was MHC restricted and glioma specific but not tumor specific. The glioma cross-reactivity from RT2 primed effector cells as well as from the 9L effector cells (Figure 10) further supported the hypothesis for a common antigen shared by these cell lines. The common paradigm is for virally induced tumors to express an antigen related to the transforming virus while chemically derived tumors express an antigen related to a mutagenized protein. These cell lines vary in their derivation. 9L was ethylnitrosurea derived[143] and RT2 was avian-sarcoma virus induced[144]. The cross reactivity measured by our assays indicated these cells do not follow this paradigm. These cell lines also vary greatly in morphology and growth characteristics. Hence, the common

shared antigen may be glioma specific.

The next addressed question was regarding CTL activity from animals with ablated tumors which rejected secondary engraftment of tumorigenic and lethal doses of tumor cells. We hoped to determine if a second tumor cell exposure would serve to augment the CTL response. Measurement of CTL from animals rejecting a secondary 9L tumor challenge is given in Figure 12.

Figure 12 - Cellular Immune Response From Animals With Ablated 9L Tumors After Rejection of Secondary Tumor Engraftment



Cytotoxicity measured by 4 hour ^{51}Cr release assay against the syngeneic glioma target 9L and the allogeneic glioma C6 target cells. Naïve = Pooled splenocytes from 3 animals never exposed to 9L tumor cells. 9L-3 = Pooled splenocytes from 3 animals with 65 mm^3 subcutaneous 9L tumors ablated by AV-TK and GCV treatment which subsequently rejected

secondary tumor engraftment after subcutaneous inoculation of 3 X 10^6 9L cells. 9L-30 = Pooled splenocytes from 3 animals with 65 mm^3 subcutaneous 9L tumors ablated by AV-TK and GCV treatment which subsequently rejected secondary tumor engraftment after subcutaneous inoculation of 3 X 10^7 9L cells. For the latter 2 groups, splenocytes were harvested 2 weeks after the secondary tumor challenge.

The CTL response from animals cured of initial 9L tumors by AV-TK and GCV treatment with subsequent rejection of tumorigenic and lethal doses of 9L cells was approximately 20% against the 9L glioma target cells. Greater lysis of 9L target cells was seen with animals secondarily exposed to 3 X 10^7 tumor cells in comparison to that observed from animals rejecting 3 X 10^6 9L cells. This difference in 9L target cell lysis was not statistically significant. Of interest, measured target cell lysis was similar to levels measured from animals without secondary tumor challenges (Figure 9C) suggesting that secondary tumor cell inoculation did not augment CTL precursor differentiation into active CTL.

To further confirm our observations regarding CTL mediated lysis for both 9L and RT2 cells, CTL assays were conducted with purified populations of CD8+ and CD4+ cells (data not shown). Bulk splenocytes from animals with ablated tumors by AV-TK and GCV treatment were depleted of

CD4+ or CD8+ cells by co-incubation with antibodies directed against these surface molecules and complement. CTL activity was effectively blocked to approximately 5% of the cytotoxicity of the bulk population when depleted of CD8+ cells. In contrast, cytotoxicity was not effected or enhanced in those populations consisting of CD4+ depleted cells. Incubation of the secondary antibody and complement did not alter cytotoxicity. To further confirm CD8+ cytotoxic T lymphocytes were responsible for target cell lysis, the effector cell showing MHC restricted cytotoxicity was cloned by limiting dilution and phenotyped. FACS analysis identified the effector cell as CD8α+CD8ß+ with abundant αß t-cell receptor (data not shown). This surface phenotype was consistent with CTL cells. In rats, NK cells which do not undergo thymic maturation express CD8α homodimers and lack CD8ß expression. In contrast CTL express CD8α8ß heterodimers. A NK cell marker was not available limiting direct immunophenotyping.

In vitro cytotoxicity unfortunately may not be indicative of the *in vivo* situation. Many unaccountable variables make CTL assays suspect. Measured cyotoxicity can vary depending on the length of secondary incubation[145], the ratio of effector:stimulator cells during the incubation[145],

as well as temperature and length of the CTL assay (data not shown). Lastly, RTS varies with lot. The amount and ratio of the cytokines contained in each lot was not determined.

Of great concern was the weak cytolytic response measured from AV-TK and GCV treated-9L ablated animals (Figure 10C). The actual *in vivo* efficacy of cells exhibiting approximately 20% cytolysis *in vitro* at an effector to target ratio of 100:1 was questionable. To determine the actual *in vivo* efficacy of effector cells a variety of adoptive transfer experiments were conducted.

If tumor immunity was transferred from tumor eradicated animals without secondary stimulation, actual *in vivo* efficacy could be proven. Results of adoptive transfer experiments from AV-TK plus GCV treated 9L and RT2 ablated animals to naive animals is depicted. Splenocytes were given by intraperitoneal injection to naive animals which were subsequently challenged with the minimum tumor forming dose (3×10^6 9L or 2×10^6 RT2) of tumor cells.

TABLE VI - RESISTANCE TO TUMOR ENGRAFTMENT AFTER ADOPTIVE TRANSFER OF UNSTIMULATED SPLENOCYTES FROM ANIMALS WITH SUBCUTANEOUS GLIOMAS ABLATED WITH AV-TK AND GCV TREATMENT

Donor Lymphocytes	Cell Number Transfered	Secondary Tumor Challenge	Animals Rejecting Tumor
Naïve Animals	5×10^7	9L	0/6
	5×10^8	9L	0/5
	5×10^8	RT2	0/6
Animals With a 9L Tumor	5×10^8	9L	0/6
Animals With a RT2 Tumor	5×10^8	RT2	0/6
Animals with 9L Tumors Ablated by AV-TK and GCV Treatment	5×10^7	9L	1/8
	5×10^8	9L	2/6
Animals with RT2 Tumors Ablated by AV-TK and GCV Treatment	5×10^7	RT2	**6/6 ***
	5×10^8	RT2	**8/9 ***

Splenocytes from 3 donor animals (column 1) were pooled and given by intraperitoneal injection to naive animals. These splenocytes were harvested from animals 6 weeks after initial tumor inoculation and 5 weeks from the commencement of AV-TK treatment. Recipient animals were challenged 36 hours later with the minimum tumor forming dose of 9L or RT2 cells on their right dorsal surface approximately 1 cm from the midline. Tumor formation defined as palpable tumor was assessed 2 weeks from the time of tumor challenge. Results represent the combined averages of 2 experiments. Results in bold (*) are statistically significant ($p<0.05$) by chi-square analysis when these groups are compared to adoptive therapy from naïve animals or animals bearing 9L or RT2 tumors.

Transfer of splenocytes from naive or tumor bearing animals did not confer protection to recipient animals from tumor challenge since no animals rejected secondary tumor challenges after adoptive lymphocyte transfer (Table VI). In contrast, adoptive transfer of lymphocytes from animals with ablated 9L tumors by AV-TK and GCV treatment conferred tumor resistance (1/8 and 2/6 animals) but was not statistically significant. In contrast, donor lymphocytes from AV-TK treated-RT2 tumor ablated animals prevented tumor engraftment with higher efficiency than from the AV-TK treated 9L tumor ablated animals (Table VI). With the exception of a single animal receiving 5 X 10^8 donor lymphocytes, all recipient animals were protected from secondary RT2 tumor challenge. This conferred protection by adoptive transfer was not dose dependent as the single animal which failed to reject tumor rechallenge received the larger dose of lymphocytes. The ability of donor lymphocytes from our animals to confer tumor resistance without secondary stimulation indicated that CTL mediated protection of animals from further tumor rechallenge (Table IV) was feasible.

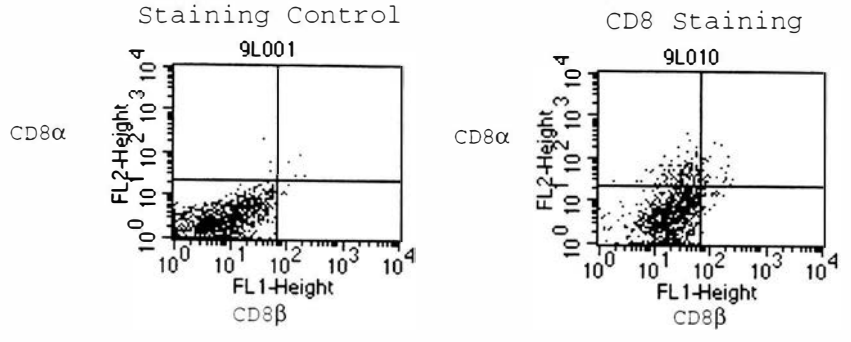
Our, CTL data and phenotypic analysis of the cloned lymphocyte cell line indicated that tumor immunity was mediated by glioma specific CTL. To further confirm the

role of CTL in mediating tumor regression, the phenotypes of TILs from regressing tumors were analyzed. AV-TK treated tumor immune animals were resistant to tumor rechallenge (Table IV). Challenging these animals with 3 X 10^6 cells prevented tumor engraftment with no palpable tumor formation. In contrast, these animals formed a small palpable tumor which regressed over 14 days when given a 3 X 10^7 9L cell challenge. These tumors were completely eliminated by day 21.

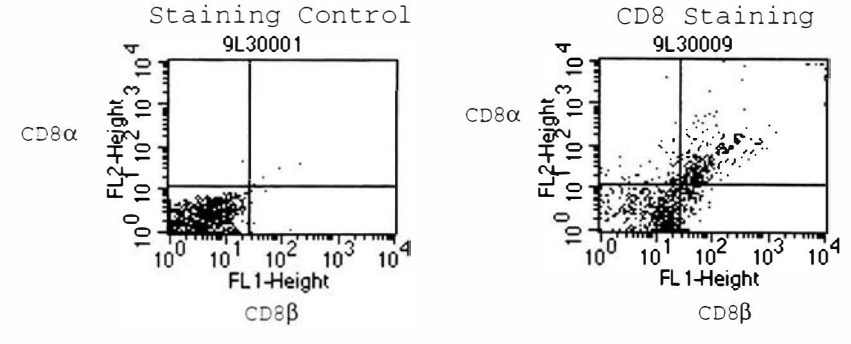
Phenotypic analysis was conducted on lymphocytes harvested from tumors by enzyme digestion at day 10 following a secondary tumor challenge with 3 X 10^7 9L tumor cells. Pilot studies in our laboratory indicated that the ratio and phenotypes of immune effector cells varies by tumor size with large tumors consisting mostly of macrophages while smaller tumors contained CD4+ lymphocytes. As a comparative control, naive animals were given an inoculum of 3 X 10^7 9L tumor cells and TIL were harvested only from tumors approximating the size of tumors from rechallenged animals. Actual FACS staining for CTL is given in Figure 13.

Figure 13 - Analysis Of TIL Phenotypes Isolated From Secondary 9L Tumors Given To Animals With Previously Ablated 9L Tumors By AV-TK Treatment

Naive Tumor Bearing Animals



Secondary Tumors From Animals With Initial Ablated Tumors By AV-TK And GCV Treatment



Tumors from 3 animals were pooled and TIL harvested and stained with antibodies against CD8α and CD8β (Pharminigen). The top panels represent TIL from tumors harvested from naive animals. The bottom panels represent TIL from secondary tumors given to animals cured of initial 9L tumors by AV-TK and GCV Treatment. CD8α positivecells are shown in the upper left quadrant. CD8β positive cellsare depicted in the lower right quadrant. Cells expressing both markers stain in the upper right quadrant.

NK cells express CD8α homodimers[146] while CTL having undergone thymic maturation have surface CD8α8ß heterodimers[146]. Therefore, staining with the antibodies CD8α-PE and CD8ß-FITC (Pharmingen) stained NK cells (CD8α+8ß-) in the upper left quadrant and CTL (CD8α+8ß+) in the upper right quadrant. Quantification of cells in these quadrants showed a significant difference in the cell type and number of TIL between naive tumor bearing animals and our AV-TK treated-tumor rechallenged animals. During normal tumor progression there were few antigen specific CTL. The bulk of CD8+ cells were of the NK phenotype. In contrast, the majority of CD8+ cells (30%) in our rechallenged animals were of the CTL antigen specific variety. The rechallenged animals having been previously exposed to tumor cells generated tumor specific CTL. These CTL cells were known to be protective against further tumor engraftment (Table VI). 3 X 10^7 cell inoculum tumors not harvested in this experiment continued to regress and were eventually eliminated. The greater ratio of CTL composing the TIL from these tumors indicated CTL were involved in tumor regression and ablation.

The previous studies aimed to characterize the immune effector mechanism responsible for tumor immunity. These

studies were important in understanding the role of host immune responses in tumor regression. The next question of interest concerned the role of adenovirus mediated tumor killing to facilitate generation of tumor immunity.

A variety of protocols have been utilized to augment anti-tumor immune response generation including tumor vaccination strategies aimed at generating a systemic immune response capable of mediating tumor regression of distant metastases[147]. For unifocal tumors, researchers have attempted to upregulate cytotoxic tumor specific lymphocytes by injection of tumors with immune adjuvants such as BCG[69] and viruses[73, 74]. Currently, clinical treatment of subcutaneous tumors most often involves surgical removal of the lesion[1]. However in many cases, there was tumor relapse with tumors occurring at foci far distant from the site of the original tumor[6]. In most of these cases, tumor reoccurrence was attributed to the lack of tumor immunity. Henceforth, an experiment was conducted to determine if AV-TK and GCV were beneficial over surgical removal of the neoplasm or tumor vaccination strategies in generating an anti-tumor immune response. Given the difficulty in generating an immune response with 9L tumors, this tumor was chosen as the subcutaneous model.

Varying sizes of subcutaneous tumors were surgically

excised. All surgically treated animals remained tumor free without tumor reoccurrence. Due to the immunosuppressive nature of these gliomas, the immune status of animals was periodically determined by measurement of peripheral blood lymphocyte (PBL) proliferation in response to mitogen stimulation by ^{3}H-thymidine uptake (data not shown). Proliferation of PBL in response to Con A was measured at 5, 10, 14, and 21 days from the time of tumor resection. In comparison to proliferation of PBL from naïve non-tumor bearing animals, a 40% diminution in proliferation was measured from tumor bearing animals up to day 10. Day 14 and day 21 PBL proliferation values were equivalent to values generated by naive control animals indicating normal immune status. Since there was no host impairment, on day 24 from the time of tumor resection, animals were given a tumor innoculum of 3×10^{7} cells on the opposite side from the initial tumor. The results are given in Table VII.

TABLE VII - COMPARISON OF GENE THERAPY TO ALTERNATIVE METHODS FOR THE TREATMENT OF SOLID SUBCUTANEOUS UNIFOCAL TUMOR

Treatment	Average Tumor Size	Animals Rejecting Tumor
Surgical Excision	168 mm^3	0/3
	221 mm^3	0/3
	289 mm^3	0/3
	350 mm^3	0/1
Tumor Cell Vaccination	No Adjuvant	0/6
	+ BCG	**9/9 ***
Animals with Ablated 9L Tumors by AV-TK and GCV Treatment	+ GCV	**15/15 ***

Animals were treated as indicated (column 1). Surgical excision involved dissection of the subcutaneous tumor with 1 cm free margins with skin closure using absorbable 3-0 suture material. Immunization consisted of a subcutaneous injection of 10 million irradiated (3000 rads) 9L cells $\pm$ 25 ug BCG in 100 ul of PBS. AV-TK and GCV treatment of tumors was conducted as described in Materials and Methods. Secondary tumor challenges were conducted on the opposite flank using 3 X 10^6 9L cells in 100 ul of PBS. Tumor formation was assessed 3 weeks from tumor inoculation. Results are a combined average of 3 experiments. Results in bold (*) are statistically significant ($p<0.05$) by chi-square analysis when compared to the pooled surgical excision group or the tumor vaccination without adjuvant treatment group.

Surgical resection of primary tumors failed to generate

tumor immunity. All animals with surgically removed primary tumors developed a tumor upon secondary tumor challenge. The size of the initial tumor had no bearing on this outcome. Researchers have shown that of CTL infiltrating tumor, only a small percentage retain the ability to lyse tumor[15]. The majority of CTL were apparently non-functional as a consequence of tumor secreted factors such as TGF-ß[43]. Of importance, surgical removal of the tumor resulted in measurable *in vitro* cytotoxicity against autologous tumor cells (data not shown) suggesting that memory CTL precursors were generated. However, *in vivo* these CTL had lost the functional activity necessary to reject secondary engraftment of tumor cells. Researchers have thus attempted alternative methods for generating protective tumor immunity.

A recent method for modifying tumor progression has been through vaccination with irradiated or mutagenized tumor cells[147]. This type of treatment was based on generating an anti-tumor immune response sufficient to result in regression of tumor at distant foci. Tumor vaccination strategies were successful in some specific animals models[69] but many concerns over this strategy still existed. Interestingly, immunization of our animals with 1 X 10^7 irradiated 9L cells was not protective against

secondary tumor challenge. For the weakly immunogenic 9L tumor, the presence of non-dividing cells was not sufficient to generate an antitumor immune response (Table VII). Generation of tumor immunity by immunization required 9L cell inoculation with BCG, an immune adjuvant. An immune adjuvant was necessary to prime host immune effector cells to the irradiated 9L cells. The mechanism of this adjuvant effect in this tumor model is unknown.

As expected, animals with an average of 65 mm^3 tumors abrogated with AV-TK and GCV treatment were resistant to secondary challenge of tumor cells on the opposite flank from the site of the initial tumor. These data are significant in that immunization strategies lacked the ability to generate an immune response without the help of an immune adjuvant such as BCG. Recombinant adenoviruses were shown to elicit proinflammatory cytokines such as IL-6 and IL-8 from peripheral blood lymphocytes[148]. Further evidence in mice indicated that serum interferon-γ levels rise upon adenovirus exposure[149]. Earlier in this paper we reported that AV-TK and GCV tumor ablated animals have generated long term tumor specific immunity (Table IV and V). Generation of this tumor immunity may be related to an antiviral immune response generated against the adenovirus with the adenovirus serving as an immune adjuvant. In the

case of AV-TK intratumoral injection, tumor cell lysis by HSVTK gene transfer coupled to possible adenovirus mediated inflammation may together facilitate generation of tumor immunity.

To determine the role of adenovirus-immune system interaction in altering 9L tumor progression, TIL phenotypes were analyzed after adenovirus injection. At day 1, day 4 and day 7 following intratumoral adenovirus injection, tumors were excised, enzymatically disassociated and the TIL were immunophenotyped and quantified. On day 1, a detectable difference in the number or TIL phenotypes was not found within non-treated tumors and those tumors treated with AV-TK or with AV-TK plus GCV (data not shown). In contrast, a significant difference in TIL phenotypes was seen by day 4 (Table X). As stated earlier, pilot studies in our laboratory indicated TIL phenotypes varied greatly depending on tumor size. By day 4, the AV-TK plus GCV treated tumors were considerably smaller than the other treatment groups. A direct comparison was not possible without correcting for the number of tumor cells. Unfortunately, a tumor cell marker was not available Therefore, the goal in this experiment was to determine general trends associated with adenovirus administration.

TABLE VIII - COMPARISON OF TUMOR INFILITRATING LYMPHOCYTE PHENOTYPES AFTER INTRATUMORAL INJECTION OF RECOMBINANT ADENOVIRUS VECTORS

	% CD4+	% CD8+	CD4 : CD8
Control-9L Tumor	29	37	1 : 1.25
9L Tumor-AV-TK Injected	23	44	1 : 2
9L Tumor-AV-TK Injected + GCV Administration	11	31	1 : 3

Animals with approximately 200 mm^3 tumors were injected with vehicle (PBS) or AV-TK. One group received GCV administration as stated in Materials and Methods. The fourth day after tumor injection, tumors were surgically excised, separated from the skin and digested in triple enzyme solution for 6 hours. Viable lymphocytes were obtained by separation on lympholyte rat (Accurate). TIL from 3 animals were pooled, stained and analyzed by FACS. Data was corrected for the total number of lymphocytes by co-staining with a pan-lymphocyte antibody (PX-52, Pharminigen). Similar results have been seen in repeat experiments.

The percentage of positively stained CD4+ and CD8+ lymphocytes were normalized to the total number of lymphocytes by co-staining with a pan-lymphocyte marker (OX-52, Pharmingen). The most significant noticeable trend relates to CD4:CD8 ratio. CD8 is the predominant marker on cytolytic cells and while CD4 represents the group of

regulatory lymphocytes involved in antigen recognition and initiation of the immune response. The AV-TK administered group showed a marked shift in the CD4:CD8 ratio towards CD8+ cells (Table VIII). This increased proportion of CD8 cells in the tumor was possibly a reflection of lymphocyte migration in response to adenovirus induced inflammatory cytokines resulting in more of these cells being attracted to the tumor. This increase in CD8+ lymphocytes was not however, necessarily of any significance *in vivo*. Treatment of tumors with AV-LacZ or AV-TK without concomitant GCV administration failed to regress or alter the progression of any tumors in numerous experiments (Table II). Augmentation of the host immune response was not sufficient to alter tumor progression without significant HSVTK mediated tumor regression. The reactivity of these TIL against autologous tumor was not determined.

More importantly, the AV-TK plus GCV treatment group showed a more dramatic shift of the CD4:CD8 ratio towards the CD8+ cell population. The tumors in this treatment group were considerably smaller than the others due to the GCV mediated toxicity for HSVTK gene transferred cells. This shift was perhaps attributable to additional antigen being present as a consequence of dying cells within the tumor.

In conclusion, intratumoral adenovirus injection altered the phenotype of TILs such that greater numbers of cytotoxic cells were present within the tumor upon. This increase in cytolytic cells reflected either increased migration of lymphocytes to the site of tumor or greater proliferation of normally existing TIL upon reduction of tumor burden. In either case, adenovirus injection into the tumor resulted in changes of the local tumor environment in favor of generating tumor immunity.

DISCUSSION

In situ retrovirus mediated HSVTK gene transfer has proven successful in the eradication of experimental brain tumors in animal models[136, 137] and has shown some efficacy in human clinical trials[103]. These experiments utilize intratumoral injections of retrovirus vector producing cells. There are numerous limitations to this method[103]. Vector producing cells infect tumor cells in close proximity to the injection site with poor efficiency[97]. Additionally, there is the potential for insertional mutagenesis as a consequence of retrovirus integration[102]. In contrast, adenovirus vectors have a number of appealing features making them ideal vectors for cancer gene therapy. These vectors can accommodate genes as large as 6 kb and can be concentrated to very high titers[110]. Of clinical relevance, the hazard of insertional mutagenesis is not a factor. Safety studies using adenovirus vectors have shown minimal probability of integration[111]. Given these advantages, we have evaluated the ability of recombinant adenovirus to infect a variety of normal and tumor cell types across a broad host range using a recombinant adenovirus expressing

the ß-galactosidase gene. Adenovirus infectivity is a receptor mediated process and hence is dependent on adenovirus binding mediated by the structural fiber protein[109]. The adenovirus receptor has yet to be identified however its expression varies by host and cell type. As a consequence, infectivity of recombinant adenovirus, similarly, varied by host and cell type. Rat and mice cell lines did not infect as well as human cell lines. Lymphoid cells were not susceptible to adenovirus infection. In contrast, transduction of tumor cells including mesotheliomas and gliomas was very efficient (Table I). Along with efficient transduction of numerous cultured human and rat tumor cells, adenovirus gene transfer resulted in greater transgene expression than could be achieved with retrovirus transduction (Figure 2). The level of gene expression was dependent on adenovirus dose and transduction efficiency. These results suggested that adenovirus vectors could efficiently transduce tumors and proves the feasibility of adenovirus mediated gene therapy.

As a suicide gene vector, the use of the herpes simplex thymidine kinase gene has an intrinsic advantage. Not all cells in a tumor need to be genetically modified in order to be susceptible to GCV toxicity[92]. Tumors transduced with vectors expressing the HSVTK gene showed considerable

regression when only a fraction of the tumor mass was modified. This phenomenon has been named the bystander effect. Due to the lack of a gene transfer method allowing for genetic modification to all cells within a tumor focus, the bystander effect allows cancer gene therapy using the HSVTK gene to be feasible. We have established subcutaneous rat glioma models (9L and RT2) and evaluated their *in vitro* bystander effect (Figure 3 and 4). Both cell lines exhibited sufficient bystander killing when as few as 8% of the cells were modified by adenovirus mediated HSVTK gene transfer. For a given dose of GCV, RT2 showed greater susceptibility to bystander killing than 9L. Approximately, twice the number of 9L gliosarcoma cells had to be genetically modified to exhibit the bystander susceptibility exhibited by RT2.

Although the mechanism underlying the bystander effect has been explored *in vitro*[95, 96], its role *in vivo* has yet to be determined. Given the difference in bystander effect between the cell lines, we evaluated the efficacy of *in situ* adenovirus mediated HSVTK gene transfer to result in tumor regression and ablation. Confounding evaluation of our results was the difference in immunogenicity of the two glioma cell lines. Tumor immunogenicity reflects the ability of tumor cells to stimulate helper and cytotoxic T-

lymphocytes upon recognition of TAA by these cells. As measured by standard CTL assays, RT2 generated a much stronger cytolytic response from secondary stimulated spleen cells than could be obtained from animals similarly immunized with 9L cells (Figure 5). Given the demonstration that host responses against tumors are predominately through cellular interactions[14], the role of the immune response in augmenting tumor eradication in our experiments must be taken into account.

To assess the importance of bystander effect *in vivo*, adenovirus treatment efficacy was assessed using a 9L tumor model in athymic rats. These nude animals retain NK cell activity but cannot produce a normal T-cell mediated immune response. This deficiency allows for direct evaluation of the role of bystander killing in complete tumor regression. *In vitro*, a 60% decrease in cell proliferation at 5 uM GCV was exhibited when as few as 8% of cells were transduced with AV-TK (Figure 3). However, this level of bystander effect may not be achieved *in vivo*. R. Touraine[96] has conducted *in vivo* bystander studies where animals with 9L tumors composed of a variety of mixtures of 100% retrovirus transduced cells and non-transduced tumor cells are treated with GCV. In these studies, complete tumor regression was reported when as few as 20% of 9L cells are HSVTK modified.

These experiments were conducted in immunocompetent animals, hence, evaluation of these results is difficult due to possible contribution by the host immune system. With GCV exposure, the transduced cells are growth arrested and will eventually perish. These cells upon interaction with antigen presenting cells may elicit an immune response which may be responsible for further tumor regression. In our experiments in athymic animals, treatment of 9L tumors with AV-TK and GCV resulted in partial tumor regression but failed to completely ablate existing tumor (Table III). Others have reported similar results using different tumor models in nude animals[137]. Significant tumor regression was established but complete eradication of an unifocal tumor did not occur. In conclusion, tumor ablation required involvement of the host immune response.

Given the knowledge that host immune responses play a role in tumor eradication, we evaluated the role of tumor antigenicity as a determinant of AV-TK treatment efficacy. The glioma tumor models utilized were the weakly immunogenic 9L and the more immunogenic RT2 (Figure 5). Following treatment of both tumors, regression was dependent on HSVTK gene transfer and GCV administration. No other combination of recombinant adenovirus coupled with GCV dosing resulted in tumor regression or ablation (Table II). The efficiency

of AV-TK and GCV treatment to mediate complete tumor elimination was dependent on tumor antigenicity. Approximately 50% of 9L tumors between 100-150 mm^3 could be eliminated by HSVTK gene transfer. In contrast, 100% of larger RT2 tumors (approximately 300-350 mm^3) were ablated with the same dose of AV-TK. In certain cases, tumors continued to regress during a time frame which could not be explained by AV-TK gene transfer and GCV susceptibility (Figure 7). Therefore, adenovirus mediated transfer of the HSVTK gene requires further interaction of the host immune response in order to be effective.

Currently, retrovirus mediated HSVTK gene transfer is being evaluated in clinical trials[103]. Based on the early success of these trials, future clinical experiments utilizing AV-TK have been proposed. Before HSVTK gene transfer becomes a viable treatment regimen, factors governing tumor susceptibility to gene therapy need to be determined. With all new treatments, there must be a significant advantage over current treatment protocols. Researchers have currently concentrated on bystander effect as the primary determinant to HSVTK effectiveness. Our results evaluating efficacy in nude and immunocompetent animals indicated that tumor antigenicity instead of bystander effect better reflected AV-TK effectiveness as a

treatment option.

Of interest, AV-TK and GCV treatment of subcutaneous tumors was able to protect animals from further tumor engraftment (Table IV). Development of tumor immunity was dependent on complete eradication of the tumor by AV-TK and GCV. This tumor immunity was long-standing with animals able to reject tumor engraftment as far as 6 months from the initial exposure to tumor (Table V). In characterizing this memory immune response we determined that it was mediated by CD8αCD8ß cytotoxic T-lymphocytes. This data indicated that host anti-tumor immune responses played an important role in augmenting and maintaining tumor regression associated with AV-TK and GCV administration. Development of tumor immunity was expected with 9L and RT2 because these tumors express antigens to which protective cellular immune responses are generated 35. The importance of tumor antigenicity and host immunity in tumor ablation has ramifications for the use of immunosuppressive drugs such as steroids used frequently with brain tumor patients. Furthermore, this CTL response was sufficient to protect animals from further tumor engraftment after adoptive transfer (Table VI). The importance of this data relates to tumor reoccurrence. By generating tumor immunity animals were protected from reappearance of distant metastatic tumor.

Lastly, we determined the role of adenovirus gene therapy to augment generation of an anti-tumor immune response. Previously, researchers have attempted to modify tumor progression through upregulation of tumor specific CTL by a variety of methods[70, 71, 72] including direct injection of adjuvants such as BCG[69] and viruses such as mumps[73] and rabies[74]. These protocols have had limited success most likely as a consequence of tumor related immune suppression. Other researchers have attempted genetic modification of tumor cells by the transfer of immunogenic cytokines[10]. The success of these protocols was dependent on the tumor model. However, AV-TK gene transfer is unique in that suicide gene transfer results in substantial tumor regression reducing the tumor burden (Figure 7). In addition, adenoviruses including recombinant adenoviruses are known to interact with the host immune system[150]. Adenoviruses have been shown to upregulate inflammatory cytokines such as IL-6 and IL-8 *in vitro*[148]. In animal models, CTL have been shown to be a determinant in loss of recombinant adenovirus expression[151]. This interaction of adenovirus with the host immune system may additionally potentiate generation of an anti-tumor immune response. Analysis of TIL from adenovirus treated tumors showed a marked shift towards CD8+ lymphocytes (Table VIII). This shift was more dramatic with AV-TK and GCV

treated tumors most likely due to a decrease in tumor burden and increased tumor antigen presentation. Interestingly, adenovirus injection without GCV also resulted in a shift of TIL towards CD8+ T-lymphocytes. However, the exact significance of this shift is unknown. The reactivity of these cells against tumor was undetermined. The increase in CD8+ cells may truly represent an increase in tumor specific CTL or may be directed at adenovirus associated antigens. This virus associated immune response may amplify the anti-tumor immune response generated incidental to tumor cell killing by HSVTK gene transfer. Therefore, AV-TK and GCV treatment may serve to upregulate an antitumor immune response by serving as a tumor vaccination strategy. HSVTK gene transfer effectively decreases tumor burden by conferring GCV toxicity and the adenovirus backbone can increase tumor immunogenicity by serving as an immune adjuvant.

This adjuvant effect is a distinct advantage for the use of adenovirus mediated HSVTK gene transfer for use in the treatment of small focal neoplasms. In comparing this gene therapy regimen to alternative treatment modalities such as surgery and tumor vaccination, these other protocols failed to generate protective tumor immunity (Table VII). Lack of a memory immune response by these traditional

treatment methods translates to greater susceptibility for tumor relapse.

Adenovirus mediated HSVTK gene transfer with concomitant GCV administration has numerous advantages relating to the generation of tumor immunity.

SUMMARY

With greater knowledge into immune system networks and their role in cancer defense, more effective therapeutic strategies can be attempted. Gliomas have an unique interaction with the immune system. The brain has long been considered an immune-privileged organ. However, lymphoid cells are known to cross the blood-brain barrier and migrate into the glioma parenchyma. Furthermore, gliomas are known to express antigens against which cellular immune responses can be generated. The lack of a normal cellular immune response is a consequence of tumor secreted immunosuppressive factors which are known to induce suppressor cells and to negatively regulate the function of infiltrating NK and cytotoxic T-lymphocytes.

Thus in order to control human glial cell cancers requires tumor expression of antigen, an ability to overcome tumor induced immune-suppression and a means to activate and expand tumor reactive lymphocytes. Adenoviral mediated herpes simplex thymidine kinase gene transfer may be able to perform all of these functions. AV-TK significantly reduces tumor volume by conferring sensitivity to GCV. The

diminished tumor burden results in a decrease of secreted immunosuppressive factors. In addition, the adenovirus may serve as an immune adjuvant in the tumor microenvironment. Adenovirus treated tumors cause a marked shift in the phenotypes of tumor infiltrating lymphocytes towards the cytotoxic T-lymphocyte population. In comparison to surgical removal of tumor, or direct adjuvant injection, AV-TK treatment facilitates generation of tumor immunity which is in part responsible for tumor eradication.

Given the success of retroviral mediated herpes simplex thymidine kinase in modifying glioma progression in current clinical trials, the ability of an adenovirus vector to generate an antitumor immune response should dramatically improve the therapeutic efficacy of suicide gene therapy.

CONCLUSIONS

1) AV-TK and GCV administration effectively ablated subcutaneous tumors in immunocompetent animals.

2) The putative HSVTK associated bystander effect is not sufficient to result in tumor eradication without augmentation by the host immune response.

3) Tumor antigenicity is directly related to AV-TK and GCV treatment efficacy.

4) AV-TK and GCV treatment of subcutaneous tumors results in long standing protective immunity against further tumor formation.

5) Tumor immunity induced by AV-TK and GCV treatment is mediated by CD8αCD8ß cytotoxic T-lymphocytes.

6) Adenovirus injection has immunomodulatory properties and may serve as an immune adjuvant.

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