HUMAN ANTI-GLIOMA MONOCLONAL ANTIBODIES FROM PATIENTS WITH NEUROLOGICAL TUMORS

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ABSTRACT

The current management of malignant gliomas is unsatisfactory compared to other solid tumors. Expected median survival is less than one year with even the best of care. At some point in their illness, most patients with neurological tumors are capable of mounting an immune response to their disease. This study focused on the humoral immune response of brain tumor patients by preparing human-human B cell hybridomas from autologous peripheral blood lymphocytes and a human myeloma-like cell line, designated as TM-H2-SP2. Eighteen fusions were successfully performed, and 15.8% of all microwells screened contained human immunoglobulin with anti-tumor activity. Five hybridomas, designated as BT27/1A2, BT27/2A3, BT32/A6, BT34/A5, and BT54/B8 were selected for detailed study. All five produced monoclonal IgM in a range of 2.4-44 μg/ml, had a similar (but not identical) pattern of reactivity against a panel of human tumor cell lines, and did not react with normal human astrocytes. All five human monoclonal antibodies (HmAbs) recognized a subpopulation of tumor cells based on multiparameter flow cytometric studies. Cell sorting experiments suggested that the identified subpopulation may share certain properties with hypothetical tumor stem cells. Preliminary antigen characterization indicated that the HmAbs are directed to cell surface glycolipids. These HmAbs possess certain properties of reactivity that suggest potential roles for them in the future diagnosis and clinical management of human malignant gliomas.

RÉSUMÉ

Le traitement des gliomes malins est présentement moins satisfaisant que celui des autres tumeurs solides. La survie moyenne est inféieure à douze mois, même avec les meilleurs soins possibles. A un certain stade de la maladie, la plupart des patients souffrant de tumeurs neurologiques sont capables de réaction immunologique envers leurs tumeurs. Le but de cette recherche est d'étudier la réaction humorale chez des patients ayant des tumeurs cérébrales. Des hybridomes humains-humains ont été préparés par la fusion de leukocytes du sang périphérique avec un myélome humain, TM-H2-SP2. Parmi les 18 fusions qui ont réussi, 15.8% des micropuits examinés contenaient de l'immunoglobuline humaine présentant une activité anti-tumorale. Cinq hybridomes, nommés BT27/1A2, BT27/2A3, BT32/A6, BT34/A5, et BT54/B8 ont été choisis pour des études plus détaillées. Tous produisent de l'IgM monoclonale à une concentration de 2.4 à 44 µg/ml, et réagissent d'une manière semblable (mais pas identique) avec un pannel de lignées tumorales humaines, mais pas avec des astrocytes humains normaux. Des études de cytofluorométrie utilisant de multiples paramètres ont démontrées que les anticorps produits par ces cinq hybridomes identifient une sous-population de cellules tumorales. Des expériences de triage cellulaire suggérent que la sous-population en question partage certaines propriétés théoriques avec les cellules tumorales souches. Des résultats préliminaires suggèrent que les antigènes reconnus par ces anticorps seraient des glycolipides de la surface cellulaire. Nous pensons que ces anticorps pourraient jouer un rôle dans le diagnostic et le traitement des gliomes malins chez l'homme.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AEC	3-amino-9-ethylcarbazol
ALP	alkaline phosphatase
AST	astrocytoma
B-lymphocyte	lymphocyte derived from bone marrow
BBB	blood-brain barrier
BCG	bacile de Calmette-Guérin
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BSA	bovine serum albumin
°C	degree centigrade
C3	complement protein C3
CDC	complement-dependent cytotoxicity
CFE	colony forming efficiency
cGy	centiGray
cis-platinum	*cis*-diamminedichloroplatinum (II)
CNS	central nervous system
CO_2	carbon dioxide
Con A	concanavalin A
CSF	cerebrospinal fluid
CT scan	computed tomography scan
dH_2O	distilled water
ddH_2O	double-distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTA	diphtheria toxin-A
EAE	experimental allergic encephalomyelitis
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EEG	electroencephalogram
ELISA	enzyme-linked immunosorbent assay

Fab	antigen binding fragment obtained with papain digestion
$F(ab')_2$	antigen binding fragment obtained with pepsin digestion
Fc	crystallizable fragment obtained with papain digestion
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G-6-PD	glucose-6-phosphate dehydrogenase
GBM	glioblastoma multiforme
GFAP	glial fibrillary acidic protein
GMEM	glioma-mesenchymal extracellular matrix
HAT medium	hypoxanthine-aminopterin-thymidine medium
HCl	hydrochloric acid
HGPRT	hypoxanthine-guanosine phosphoribosyl transferase
HLA	human leukocyte antigen
HmAb	human monoclonal antibody
H_2O_2	hydrogen peroxide
HT medium	hypoxanthine-thymidine medium
hrs	hours
HuIFN-αLy	human lymphoblastoid interferon
IA	immune adherence
IF	immunofluorescence
IFN	interferon
IFN-β	human fibroblast interferon-β
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgGEAC	IgG erythrocyte-antibody-complement complex
IgGFc	crystallizable fragment of the IgG molecule
IgM	immunoglobulin M
IL-2	interleukin-2
im	intra-muscular

iv	intravenous
kbp	kilobase pairs
KCl	potassium chloride
kg	kilogram
LAI	leukocyte adherence inhibition
LAK	leukocyte activated killer
L-cell	large cell
log	logarithm to base 10
$\log_2$	logarithm to base 2
L-PHA	leukoagglutinating phytohemagglutinin
m	meter
M	molar
mm^2	square millimeter
mAb	monoclonal antibody
MeV	mega electron-volt
MHC	major histocompatibility complex
mg	milligram
ml	milliliter
MRI	magnetic resonance imaging
MW	molecular weight
NaCl	sodium chloride
NaN_3	sodium azide
NK	natural killer
nm	nanometer
NNP	4-hydroxy-3,5-dinitrophenacetic acid
OD	optical density
p	probability
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PEG	polyethylene glycol; pneumoencephalogram
PGE_2	prostaglandin E_2
PHA	phytohemagglutinin
PMSF	phenylmethylsulfonyl fluoride
PRPP	5-phosphoribosyl-1-pyrophosphate

LIST OF FIGURES

Chapter 1: Introduction

Chapter 3: Derivation of Human Anti-Glioma Monoclonal Antibodies from Patients with Neurological Tumors

Chapter 4: Characterization of Human Monoclonal Antibodies to Glioma Cell Surface Antigens

PWM	pokeweed mitogen
q	each
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
RITC	rhodamine isothiocyanate
rpm	revolutions per minute
S-cell	small cell
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SG	specific gravity
SLMC	spontaneous lymphocyte-mediated cytotoxicity
TBS	Tris-buffered saline
TGF-β_2	transforming growth factor β_2
TIL	tumor infiltrating lymphocyte
T-lymphocyte	lymphocyte of thymic derivation
TM-H2	TM-H2 human myeloma-like cell line
TM-H2-SP2	TM-H2-SP2 human myeloma-like cell line
U/m^2	units per square meter
v/v	volume for volume
w/v	weight for volume
w/w	weight for weight
αMEM	alpha minimum essential medium
µg	microgram
µl	microliter
µm	micrometer
>	greater than
<	less than
#	number
%	percent
≈	approximately equal to

Appendix: Biotinylation of Human Monoclonal Antibodies

LIST OF TABLES

Chapter 1: Introduction

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Chapter 4: Characterization of Human Monoclonal Antibodies to Glioma Cell Surface Antigens

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PREFACE

The experiments described in this thesis were carried out in the laboratory of Dr. Gerald Price, McGill Cancer Centre, and in the division of hematology, Royal Victoria Hospital, under the supervision of Dr. Price. The human myeloma-like cell line, TM-H2-SP2, used in this study was originally described by Sullivan, et al. (1982), and was provided by Dr. Price.

The Southern blots reported in section 3.8 were contributed by Delbert Dorscheid, a graduate student in Dr. Price's laboratory. Some of the labelling of primary tumor explant cultures described in section 4.6 was carried out by John Guy, under the direction of the candidate. Immunohistochemical labelling of cultured glioma cells (section 4.7) was performed with the assistance of Ms. Gene Shematek, of Dr. Sullivan's laboratory. Christopher Schlachta, a summer student in Dr. Price's laboratory, and Richard McKenzie, flow cytometry technician in Dr. Price's laboratory, performed flow cytometric studies and immunochromatography with antibodies BT34/A5 and BT54/B8 (sections 4.5 and 4.9, respectively). Flow cytometric analysis of propidium iodide stained glioma cells (section 5.5) was performed with the assistance of Sandeep Mayer and Richard McKenzie. The biotinylation of partially purified antibody described in the Appendix was carried out by

Victor Sandor. All other experimental procedures were carried out by the candidate.

The initial purpose of the present study was to derive and characterize human anti-glioma monoclonal antibodies from patients with malignant gliomas and other neurological tumors. In a subsequent phase, attention was focused on the biological significance of the tumor cell subpopulation identified by the monoclonal antibodies.

This thesis consists of six chapters. Chapter 1 introduces the topic to be investigated, and summarizes the relevant literature. Chapter 2 outlines the material and methods used in the thesis. Chapters 3 to 5 describe the experimental results obtained, and chapter 6 offers some concluding remarks and future perspectives. This is followed by a bibliography on the subject.

1 INTRODUCTION

Malignant gliomas are the most common of all primary brain tumors. They are also among the most difficult to manage clinically. Depending on their intracranial location, they can grow to a substantial size before causing any symptoms. Their biological behaviour is notoriously aggressive, although they rarely metastasize outside of the CNS.

Surgery and radiotherapy alone are seldom curative; with the best of care, median survival is under one year. Moreover, despite major efforts to introduce new adjunctive therapies, the prognosis for patients with malignant gliomas has remained essentially unchanged for the past thirty years.

Many adjunctive therapies, including immunotherapy, are either presently ineffective, or carry an unacceptable risk of serious morbidity. With the development of murine hybridomas by Köhler and Milstein (1975), however, tumor immunodiagnosis and immunotherapy with monoclonal antibodies (mAbs) has emerged as a promising area of investigation.

Studies with murine mAbs have already contributed substantially to the understanding of glioma biology and heterogeneity; however, these reagents may not be ideally

suited for clinical applications in humans for a variety of reasons. In contrast, human mAbs (Croce, et al. 1980; Olsson and Kaplan, 1980) offer the possibilities of greater specificity and biological compatability than their murine counterparts.

1.1 Pathology of Malignant Gliomas

1.1.1 Description and Classification

Malignant gliomas are neuroepithelial tumors which commonly arise in the cerebral hemispheres. As a group, they include the anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic ependymoma, and glioblastoma multiforme. These tumors all possess certain microscopic features in common such as dense cellularity, increased numbers of mitotic figures, nuclear pleomorphism with hyperchromasia, and cellular pleomorphism. In glioblastoma multiforme the above features are present, as well as necrosis with pseudopalisades, capillary endothelial proliferation with pseudorosette formation, and mesenchymal proliferation.

Classification of these tumors is based on one of two systems. The histogenic method, proposed by Bailey and Cushing (1926), is based on the resemblance of tumor cells to primitive neuroepithelial precursors, and views tumors as arising from immature precursor cells. In contrast, the morphologic method of Kernohan and Sayre (1952) grades

tumors according to morphologic features shared with normal adult cells, from which they are thought to arise by a process of "de-differentiation". In the Kernohan system, astrocytomas are graded on a scale of I to IV, with grade IV equivalent to the glioblastoma multiforme of Bailey and Cushing. Both methods attempt to correlate histological appearance with clinical prognosis, and have been refined and incorporated into present-day classification systems (Russell and Rubinstein, 1977; Zülch, 1975).

Malignant gliomas arise most frequently in the cerebral hemispheres, particularly in the frontal and temporal lobes, although they may occur in any region of the CNS (Escourolle, et al. 1971). There is a predilection for the periventricular region and subcortical white matter. Approximately 20% of glioblastomas exhibit evidence of having arisen from a pre-existing astrocytoma, and 3% are multicentric (Russell and Rubinstein, 1977).

1.1.2 Growth and Vascularization

Growth by microscopic invasion is a characteristic feature of malignant gliomas. Even macroscopically well-circumscribed tumors exhibit this property (McComb and Bigner, 1984). Typically, there is infiltration in and around the white and grey matter, blood vessels, pia, and ependyma. In its most extreme form, this leads to the condition known as diffuse gliomatosis cerebri. In

addition to local invasion, malignant gliomas frequently gain access to the leptomeninges and ventricular system, with subsequent spread throughout the neuraxis. This manifests itself clinically as spinal subarachnoid seeding and meningeal gliomatosis. Extraneural metastases, however, are extremely rare. The most common extraneural sites for glioblastoma multiforme are lungs, lymph nodes, bone, liver, and pleura in descending order of frequency (Liwnicz and Rubinstein, 1979; Pasquier, et al. 1980). Access to extra-meningeal tissue sometimes occurs through a former craniotomy site, although this is rarely observed.

Malignant gliomas are well vascularized tumors, and this is especially the case in glioblastoma multiforme. But aside from quantitative changes in tumor vasculature, there are important qualitative changes as well. The concept of a blood-brain barrier arose from experiments done in the 19th century in which certain intravenously administered dyes were noted to stain all the organs of small animals except for the brain. Since then, many ultrastructural and functional details of brain capillaries have been elucidated, and it is now known that these vessels are different from those located anywhere else in the body (Goldstein and Betz, 1986).

The physical basis for the blood-brain barrier's selectivity resides in the capillary endothelial tight junctions and the paucity of pinocytic vesicles capable of

transporting nutrients and other substances. Molecules which can passively cross the blood-brain barrier have to be lipid soluble, uncharged, and possess a low molecular weight. Active transport systems exist for minerals and nutrients. In malignant gliomas, however, there is breakdown or total absence of the blood-brain barrier due to widening of endothelial cell junctions, capillary fenestration, increased numbers of pinocytic vesicles, irregularities of the basement membrane, and absence of glial investiture (Long, 1970; Hirano and Matsui, 1975; Weller, et al. 1977). The resulting capillary 'leakiness' forms the basis for diagnostic imaging with radiocontrast and radiolabelled substances, allowing these products to pass freely into the tumor parenchyma.

The pathogenesis of malignant gliomas, like many other solid tumors, is thought to involve an initial avascular phase followed by a phase of intense vascular proliferation and rapid growth. Brem (1976) transplanted live glioma cells into the rabbit cornea, an immunologically priviledged site, and observed ingrowth of new capillary vessels from the corneal limbus.

Growth factors which have been implicated in the pathogenesis of malignant gliomas are epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). The c-*erb B* oncogene codes for a cell membrane protein which is homologous to the EGF receptor. Using human cDNA

probes, Lieberman et al. (1985) were able to demonstrate a 6-60 fold amplification in the EGF receptor gene in 4 of 10 primary human malignant gliomas. This observation has been extended by Wong, et al. (1987) who studied 63 malignant gliomas, and found that in 24 (43%) the EGF receptor gene was amplified >8-fold and was also frequently rearranged.

Similarly, enhanced production of the c-*sis* oncogene product, which resembles the β-chain of PDGF, has been demonstrated in cultured human glioma cells (Nister, et al. 1984), but not directly from primary tumors. These studies would suggest that molecular events surrounding the amplification and expression of growth factor-related oncogenes may play an important role in the pathogenesis and stromal reaction to malignant gliomas (Westermark, et al. 1985).

1.1.3 Heterogeneity and Progression

Malignant gliomas have long been recognized to be heterogeneous tumors (Shapiro and Shapiro, 1984). Indeed, they demonstrate both inter- and intra-tumor variability. In the strictest sense, the term heterogeneity should refer to differences attributable to distinct cell lineages or stem lines, and not 'secular' differences, which may be governed by cell cycle state and other epigenetic phenomena (Heppner, 1984). The apparent existence of seperate lineages within a single tumor would appear to contradict

the clonal origin hypothesis (see section 1.1.4), if it were not for the phenomenon of tumor progression. The latter process can be defined as the evolution of tumor cell populations over time, and the generation of increasingly anaplastic sublines as a result of an inherent genetic instability (Nowell, 1976). Tumor progression, therefore, may be regarded as a dynamic force behind tumor heterogeneity.

Cell lineage heterogeneity in primary malignant gliomas was demonstrated by Shapiro, et al. (1981) who prepared chromosomal 'reference sets' from 8 primary tumors, then plated the dissociated single cells from the same 8 tumors by limiting dilution and studied the karyotypes of the individual clones. Each tumor was found to contain from 3 to 21 identifiable subpopulations, and 7.6 to 25% of the respective clones had karyotypes which could be identified in the reference set.

Progression of benign astrocytomas *in vivo* is a widely accepted clinical phenomenon, and it is estimated that 20% of all glioblastomas originate from a pre-existing benign tumor (Russell and Rubinstein, 1977). Evidence for tumor progression *in vitro* was contributed by Mark, et al. (1977), who reported that a 64 chromosome subline of the human glioma line U-251MG replaced, over a period of several months, the 60 chromosome stemline which had been continuously observed in cultures for over 7 years.

Variations in glioma DNA content after multiple passages *in vitro* has also been noted. Flow cytometric analyses of primary malignant gliomas have revealed the presence of predominantly diploid and often tetraploid populations (Frederiksen, et al. 1978; Kawamoto, et al. 1979; Wolley, et al. 1979), whereas analysis of permanently cultured glioma cell lines has predominantly demonstrated a near-triploid or near-tetraploid karyotype (Bigner, et al. 1985).

Loss of specific genetic sequences on chromosome 10p has been demonstrated by James, et al. (1988), using the technique of restriction fragment length polymorphism (RFLP) analysis. These investigators studied 53 unrelated patients with glial tumors of varying histological grades, and observed loss of constitutional heterozygosity for loci on chromosome 10p in 28 of 29 cases of glioblastoma multiforme. This high degree of correlation between loss of genetic sequences on a particular chromosome and emergence of the malignant phenotype strongly favors a common molecular pathway of tumor progression for most glial tumors.

Another phenomenon which appears to be an example of tumor progression is the loss of glioma differentiation markers in high grade gliomas, and after successive passages of differentiation marker positive glioma cells *in vitro* or in athymic nude mice. Diserens, et al. (1981)

reported the loss of the astrocytic marker glial fibrillary acidic protein (GFAP) after the first 10 passages *in vitro* of an established human glioma line, LN-18. Similarly, Jones, et al. (1981) reported the loss or diminished expression of GFAP in primary malignant gliomas passaged in nude mice.

The clinical implications of glioma cellular heterogeneity are particularly relevant, since heterogeneity may well be the major determinant of therapeutic resistance (Bigner, 1981). In a study of irradiated glial cells from normal brain and cultured astrocytomas, Gerweck, et al. (1977) were able to demonstrate relative radioresistance in 2 of 3 malignant astrocytomas, evident as an initial 'shoulder' on the survival curve.

Using a clonogenic cell assay and biopsy material from patients with malignant gliomas, Rosenblum, et al. (1983) were able to predict in a prospective manner therapeutic resistance to the drug BCNU with 100% accuracy. Clinical response to the drug, however, was predictable in only 42% of cases. The authors speculated that most tumors consist of subpopulations of cells which are: 1) sensitive to BCNU at all doses, 2) resistant to BCNU at all doses, or 3) resistant at clincal (*in vivo*) doses but sensitive at very high (*in vitro*) doses. By administering BCNU intra-arterially via selective supra-ophthalmic catherization, it

is now possible to kill tumor cell populations which would otherwise be resistant to conventional intravenous doses (Emrich and Villemure, 1988).

1.1.4 Clonal Origin and Stem Cell Nature of Human Tumors

The pathogenesis of malignant gliomas from normal glial precursors likely involves a stepwise series of genotypic and phenotypic changes, culminating in the emergence of a single transformed clone. Studies using the X-linked enzyme glucose-6-phosphate dehydrogenase (G-6-PD) have now firmly established the clonal origin of most human tumors (Fialkow, 1976).

G-6-PD exhibits several different phenotypes (isoenzymes) which can be distinguished on the basis of electrophoretic migration. Certain female individuals are heterozygous for G-6-PD, and express two distinguishable isoenzymes with an equal frequency, in accordance with the Lyon hypothesis of X-chromosome inactivation (Lyon, 1961). In those heterozygotes who develop tumors, an equal prevalence of both isoenzymes in the tumor is indicative of a multiclonal origin, whereas the expression of only one isoenzyme would be consistent with a monoclonal origin.

Extensive studies of tumors arising in G-6-PD heterozygotes have revealed a monoclonal origin in all cases where it could be established that tumor sampling was

adequate and the number of normal contaminant cells was minimal. The only exceptions are genital warts, which are known to be caused by the papovavirus (even multicentric uterine fibroids have been shown to originate each from a single clone).

Support for the monoclonal nature of human tumors also comes from studies of colorectal tumors of females using RFLP analysis of X-linked loci (Fearon and Vogelstein, 1987). Both adenomas and carcinomas of either familial or spontaneous etiology exhibited a monoclonal pattern of X chromosome inactivation. In the study by James, et al. (1988), the pattern of loss of constitutional heterozygosity for chromosome 10 loci in glioblastomas was likewise monoclonal, in spite of the heterogeneity which exists between cells. These studies add strong support to the stem cell/clonal expansion theory of tumor growth.

The stem cell model of tumor growth (Mackillop, et al. 1983) (Fig.1.1) postulates the existence of three tumor cell populations: 1) stem cells (undifferentiated and capable of infinite self-renewal), 2) 'end' cells (relatively differentiated but not capable of self-renewal, making up the bulk of the tumor), and 3) transitional cells (progressive states of differentiation between stem and 'end' cells, and capable of only limited proliferation).

The model proposes that under normal mitotic conditions, a stem cell can divide to form more stem cells

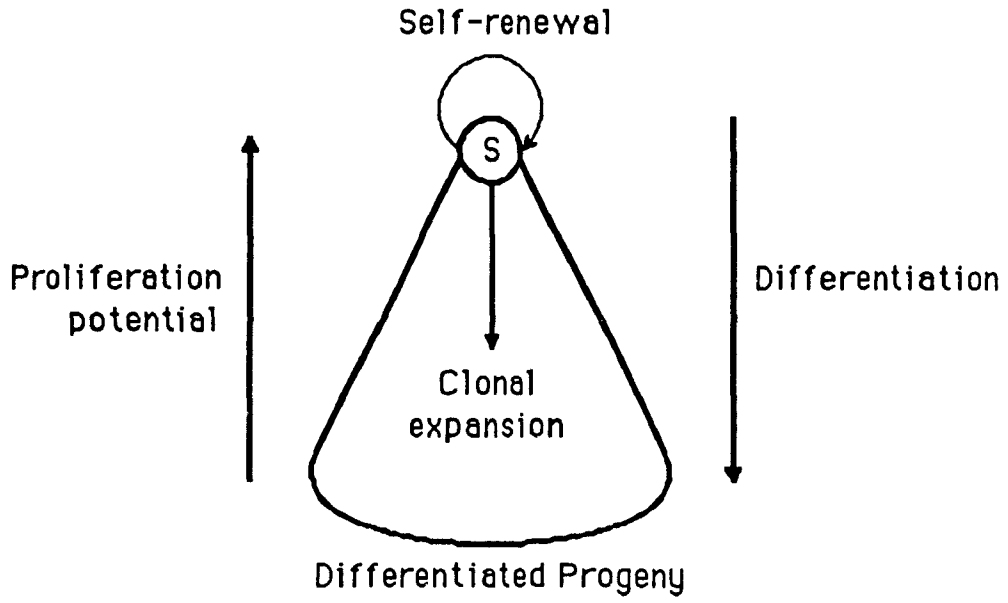


Figure 1.1: Stem cell hypothesis of tumor growth

Tumor stem cells (S) are thought to exist as a separate compartment capable of infinite self-renewal, represented in this diagram by the circular arrow. Their high proliferative potential is at the extreme end of one spectrum, represented on the left hand side of the diagram, while their low rate of cellular differentiation is at the extreme end of another spectrum, represented on the right. Once a stem cell divides, it becomes committed to forming either more stem cells, or more differentiated progeny capable of only limited self-renewal. The latter will increase in number at a much higher rate than the tumor stem cell, through the process of clonal expansion, and form the bulk of the tumor cell mass. This is represented by the widening skirt below the stem cell compartment. In normal tissues, the probability of a stem cell dividing and forming two more stem cells is exactly 0.5, whereas in neoplastic tissues, it is >0.5. Tumor masses will enlarge because of an ever-expanding stem cell compartment. As the tumor undergoes progression to a more anaplastic state (e.g. from a grade I to a grade IV astrocytoma), the tendency for the transformed stem cells to form more differentiated progeny decreases. Microscopically there will be a greater proportion of immature-looking cells; however, this is <u>not</u> the result of 'de-differentiation'. This merely represents the dynamic shifts in tumor cell populations. Adapted from Mackillop, et al. (1983).

with the potential for self-renewal, or divide to form differentiated cells with only limited proliferative potential. In order to maintain a stable number of stem cells, the probability of stem cell self-renewal versus differentiation in non-neoplastic tissues would be exactly 0.5. In malignant tissues, however, the probability of stem cell self-renewal would be >0.5.

According to the model, a tumor enlarges because of an ever-expanding stem cell compartment, which in turn generates increasing numbers of differentiated progeny. As a tumor undergoes progression, the proportion of stem cells increases, resulting in a more anaplastic phenotype. The model also predicts that residual stem cells can regenerate the original tumor heterogeneity after subcurative therapy.

Support for the stem cell origin of malignant gliomas comes from studies by Lewis (1968) on mitotic activity in the adolescent primate brain. In the mature mammalian CNS, most glia and all neurons can be regarded as non-proliferating end cells. The few dividing cells which are encountered are probably glial stem cells. In perfusion-fixed brains of young monkeys, the major site of mitotic proliferation was found to be the subependymal layer of the lateral ventricles, corresponding to the fetal periventricular germinal matrix. Subependymal cells are the embryonic source of bipotential glial progenitor cells, which in turn differentiate into either type II astrocytes

or oligodendrocytes (Raff, et al. 1983). Malignant transformation of bipotential glial progenitor cells probably gives rise to both astrocytomas and mixed oligo-astrocytomas; indeed, the subependymal zone is the most common site of origin of these tumors in the human.

1.2 Current Management of Malignant Gliomas

1.2.1 Conventional Therapy

Conventional therapy of malignant gliomas consists of surgical resection followed by megavoltage radiotherapy (Fig.1.2). Systemic chemotherapy with BCNU is only marginally beneficial (Walker, et al. 1978), and of no proven advantage over combinations of drugs (Salcman, 1985; Levin, et al. 1985). The main indication for surgical 'internal decompression' of malignant gliomas is the relief of life-threatening elevated intracranial pressure. In addition, surgical tumor debulking will confirm the diagnosis and 'set the stage' for other therapeutic modalities by removing necrotic and hypoxic tissue. In surgically accessible lesions, there is no longer any justification for a small craniotomy or burr-hole biopsy which is followed by no definitive therapy. Nor is there any justification for performing lobectomies for 'internal decompression', unless they harbor discrete lesions (Ransohoff, 1983). For lesions of the basal ganglia, thalamus, and brain stem, which are considered to be

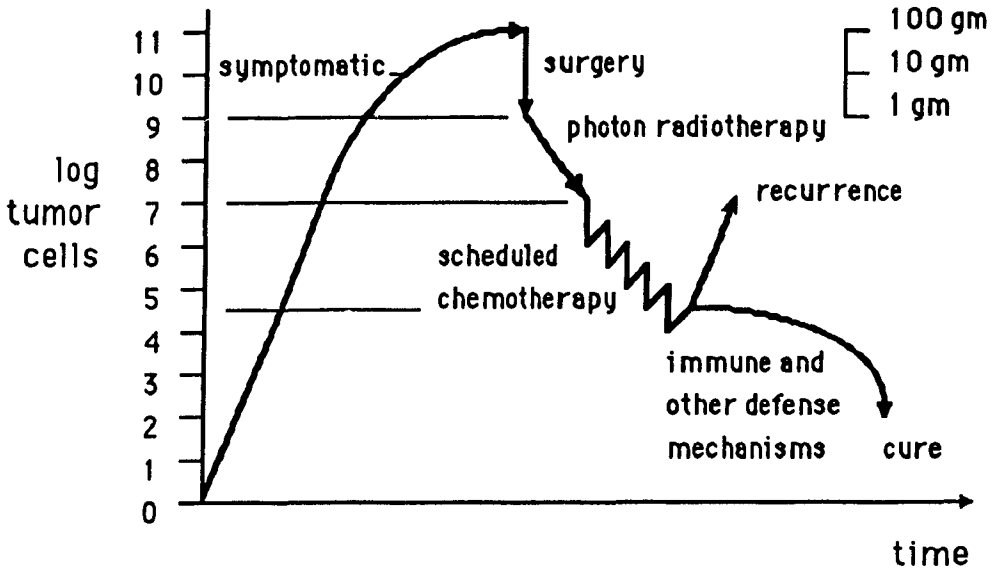


Figure 1.2: Therapy of malignant gliomas

In the initial phases of growth, malignant gliomas are thought to increase in size exponentially, as represented by the straight line. Once the tumor cell mass has reached $\approx 10^8$ cells, growth begins to slow down slightly. In most cases, the tumor will produce symptoms once it has reached a size of 10^{10} cells, or ≈10 gm. By the time the patient undergoes surgery, the tumor cell mass may have doubled in size. A 99% debulking procedure, which is technically impossible in most cases, will reduce the tumor burden back down to between 10^8 and 10^9 cells. Conventional radiotherapy (5000 cGy) may reduce tumor burden by 2 more logarithms, and chemotherapy with BCNU, a further 2 logarithms. This still leaves the patient with $\approx 10^5$ malignant cells for immune and other defense mechanisms to eradicate. Mobilization of the patient's immune system would therefore be important in eliminating the last tumor cells from the body. Adapted from Sano (1983).

surgically inaccessible targets, CT-guided stereotaxic biopsy is diagnostic in more than 90% of cases (Ostertag, 1980).

As an adjunctive modality in the therapy of malignant gliomas, radiotherapy is of proven benefit (Walker, et al. 1980; Salcman, 1980; Bloom, 1982). Its effect appears to be dose-related (Walker, et al. 1979), limited primarily by the intrinsic sensitivity of normal tissues. The usual course involves 5000-6000 cGy given in 170-200 cGy daily fractions, 5 days a week, for 5-6 weeks. Under hypoxic conditions, such as in the necrotic centre of a tumor, the sensitivity of biological systems to ionizing radiation is decreased by as much as two thirds. This has prompted clinical studies of radiation sensitizing agents such as metronidazole and misonidazole, which have been shown to be effective *in vitro*. In one randomized trial (Urtasun, et al. 1982), survival curves for patients receiving irradiation plus a nitroimidazole were identical to those receiving conventional radiotherapy alone.

A more promising approach involves the stereotaxic implantation of a radiation source (usually ^{125}I or ^{192}Ir) directly into the tumor bed, so-called interstitial brachytherapy. The rationale for this method is twofold: higher radiation doses can be delivered directly to the tumor volume, and lower dose rates will allow more time for normal tissues to repair sublethal damage, minimizing the

harmful effects on surrounding brain (Gutin and Bernstein, 1984).

Systemic administration of carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU] is of little benefit in the treatment of malignant gliomas. Although it is well tolerated when given in the usual dose (80 mg/m^2 i.v. once daily x 3 days, q. 6-8 weeks), systemic inactivation limits its clinical usefulness when administered by the intravenous route. To improve drug delivery and benefit from the pharmacokinetic 'first pass effect', more direct routes of administration have been proposed.

Initial trials of intracarotid *cis*-platinum [*cis*-diamminedichloroplatinum (II)] infusion were associated with a high incidence of ophthalmic, auditory, and CNS toxicity (Stewart, et al. 1982). Recent refinements involving selective arterial catheterization of major cerebral vessels have considerably improved morbidity (Hochberg, et al. 1985). Another means of increasing BCNU delivery is with megadose intravenous administration followed by bone marrow rescue (Fingert and Hochberg, 1984; Kessinger, 1984). Using either method, a few partial remissions have been observed, although the exact role these techniques will play in the management of malignant gliomas remains to be determined.

Special mention should be made of the role of corticosteroids in the management of malignant gliomas.

Although their exact mechanism of action is unknown, they are thought to reduce peritumoral edema by inhibiting the enzyme phopholipase A_2, thus limiting the conversion of membrane-bound arachidonic acid to prostaglandins and leukotrienes, which mediate changes in capillary permeability. Clinically, these agents are invaluable in the palliation of symptoms of raised intracranial pressure, although their effect on the patient's immune system may not always be desirable (see section 1.3.5).

1.2.2 Survival

Survival in malignant gliomas is notoriously poor; despite this, it remains incompletely studied. The beneficial effects of radical surgery and radiotherapy are so taken for granted that it is now considered unethical to include as a treatment arm patients to receive surgery alone. From a study of 17 historical reports in the glioblastoma literature, Salcman (1980) concluded that the median survival time for patients receiving surgery alone was 16 weeks, for patients receiving surgery plus radiotherapy 37 weeks, and for those receiving surgery plus radiotherapy plus chemotherapy 40 weeks (Fig.1.3). It was also noted that survival curves for all three treatment combinations were exponential in shape and converged at around 18 to 24 months. Five year survival was estimated to be zero percent, irrespective of treatment.

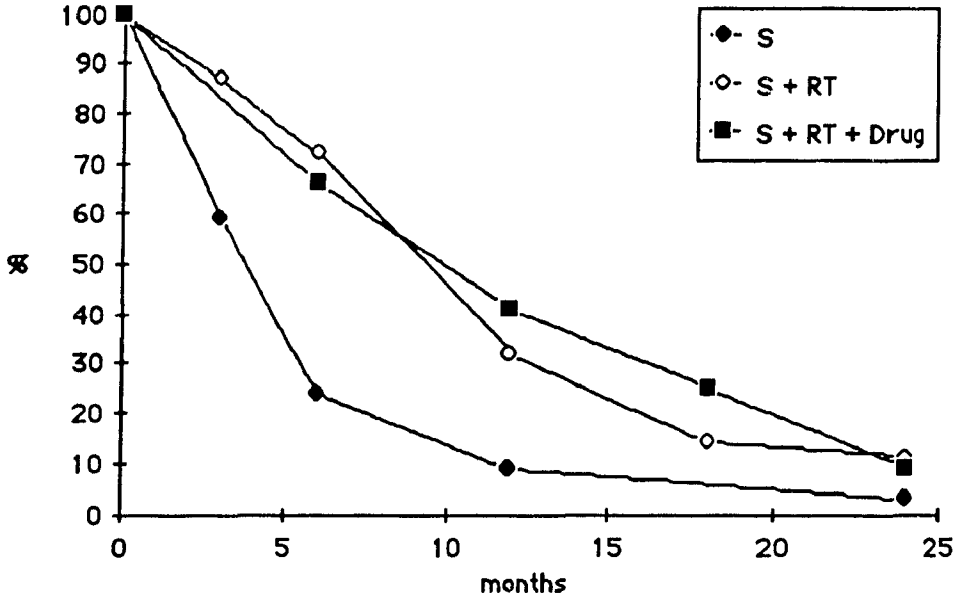


Figure 1.3: Survival in glioblastoma multiforme

This diagram is a comparison of survival data for three individual treatment groups: surgery alone (S), surgery plus conventional radiotherapy (S + RT), and surgery plus conventional radiotherapy plus an antineoplastic drug, usually BCNU (S + RT + Drug). Percentage survival is plotted on the ordinate, and duration of survival (in months), on the abscissa. The addition of conventional radiotherapy will significantly prolong survival in the first 18 months, however, beyond this point all three curves converge. The addition of chemotherapy does not appear to significantly prolong survival at any point along the curve. Adapted from Salcman (1980).

The addition of post-operative radiotherapy is decisive in prolonging survival in the first 18 months. The contribution of chemotherapy does not appear to be significant at any point in the survival curve. These observations have suggested to many investigators that glioblastoma multiforme may be so resistant to cure that adding different treatment modalities would only delay an inevitable and fatal recurrence.

In a study of all malignant gliomas, including glioblastoma multiforme, Byar, et al. (1983) concluded that there were three risk factors which had a highly significant effect on outcome: age, pre-operative performance status (Karnofsky score, Table 1.1), and histological grade ($p<0.00001$ for all three). In general, patients younger than 45 years of age (except those younger than 15 years old), or patients with a high pre-operative Karnofsky score (Karnofsky, et al. 1948), or patients in whom the tumor grade was Kernohan III but not IV (glioblastoma multiforme), all did the best. Other positive predictive factors were presence of headaches, absence of motor symptoms, history of seizures, absence of personality change, duration of symptoms >6 months, and absence of tumor necrosis.

On this basis, the authors were able to group the 358 patients reported by Walker, et al. (1980) into 3 risk categories, and found that the actuarial survival curve of

Table 1.1: Karnofsky scoring system for assessing performance status

Score	*Interpretation*
100	normal: no complaints, no evidence of disease
90	able to carry on normal activity: minor symptoms
80	normal activity with effort: some symptoms
70	cares for self: unable to carry on normal activity
60	requires occasional assistance: cares for most needs
50	requires considerable assistance and frequent care
40	disabled: requires special care and assistance
30	severely disabled: hospitalized, death not imminent
20	very sick: active support care needed
10	moribund: fatal processes are progressing rapidly

Adapted from Karnofsky, et al. (1948)

their best group (mainly younger patients with high Karnofsky scores) closely matched the predicted survival of 25% at 2 years. It is interesting to note that a full 51.5% of patients in this group had the diagnosis of glioblastoma multiforme, suggesting that younger patients with this disease may be more responsive to therapy than previously thought.

Finally, in those patients who do survive longer than 12 months, there is evidence that many are unable to return to an active social life or premorbid employment despite the demonstration of static or diminished tumor volumes on CT scan (Hochberg and Slotnick, 1980). In a study of fourteen patients who had received multimodality therapy of malignant gliomas, Seiler (1980) found three poorly functioning patients whose CT scans demonstrated diffuse leukencephalopathy but no evidence of tumor recurrence. He speculated that the cause of the leukencephalopathy was chemotherapeutic potentiation of radiation necrosis.

1.2.3 Complications

Complications of conventional therapy of malignant gliomas may be divided into three broad areas: complications of surgery, radiotherapy, and chemotherapy. Common surgical complications range from post-operative edema, to hemorrhage, hematoma, hydrocephalus, deep venous thrombosis, or infection. Complications of radiotherapy

are divided into: 1) acute (24 hrs.), 2) early-delayed (4-12 weeks), and 3) late-delayed (6-36 months) radiation encephalopathies. The pathological basis for early-delayed radiation necrosis is thought to be demyelination, whereas the mechanism of late-delayed necrosis appears to involve injury to the cerebral vasculature (Burger, et al. 1979).

Sheline, et al. (1980) concluded that the risk of delayed radiation necrosis increases in proportion to the total radiation dose administered, and that dose schemes exceeding 6000 cGy over 30 fractions are to be considered unsafe. With conventional schemes of 5000 cGy in 25 fractions the risk of delayed necrosis is <5%. The most frequent complications of BCNU are delayed myelosuppression at 3-5 weeks (duration 1-3 weeks), pulmonary fibrosis in 20% of patients, and rarely, nephrotoxicity (Wilson, et al. 1982).

1.3 Immunobiology of Malignant Gliomas

1.3.1 Immune Priviledge

Traditional concepts of the brain as an immunologically priviledged site arose as a result of studies on tumor transplantation conducted by Shirai (1921), and Murphy and Sturm (1923) in an earlier part of this century. Their conclusions were upheld by Medawar (1948) who demonstrated that rabbit skin homograft transplants which normally underwent rapid rejection if

implanted subcutaneously, would grow indefinitely if transplanted into the brain. It is now known that as a result of breakdown of the blood-brain barrier in malignant gliomas, there is free mixing between the tumor compartment and the systemic circulation. Both humoral (Mahaley, 1972) and cellular (Ridley and Cavanagh, 1971) immune elements can gain access to CNS tumors, and the converse also appears to be true. Morley (1959) has reported the recovery of glioma cells from the venous sinuses of patients undergoing craniotomy. Once the brain has been intruded upon by a malignant neoplastic process, it behaves less as an 'immunologically priviledged' site.

1.3.2 General Immune Status

It is now well established that many patients with malignant gliomas are significantly immunosuppressed, especially with regards to cell-mediated functions. Ironically, few patients are cachectic. The finding of immunosuppression in the absence of significant systemic wasting would suggest that the former is due to tumor-intrinsic mechanisms, rather than a reflection of excessive tumor burden, as is usually the case. Humoral immune mechanisms in patients with malignant gliomas also appear to be depressed, albeit they remain intact.

In a study by Mahaley, et al. (1977) of 42 patients with glioblastoma multiforme and 17 patients with other

anaplastic gliomas, preoperative anergy to cutaneous recall antigens was commonly found, and appeared to correlate with the degree of anaplasia. In the same study, serial monitoring of immune functions in postoperative patients not given corticosteroids revealed reductions in a variety of immune parameters which coincided with declines in clinical status.

Many patients with glioblastomas presented with low peripheral blood lymphocyte counts (<1,000 cells/mm^3) and elevated serum IgM levels (>150 mg/100 ml) which declined progressively over the course of their disease. In a follow-up study (Brooks, et al. 1981), it was shown that isolated immunological determinants are not clinically useful in the prediction of tumor recurrence, although using a combination of *in vitro* lymphocyte indices, recurrence could be predicted prior to clinical deterioration.

The relative lymphocytopenia seen in some glioma patients appears to be due to a depletion of E-rosetting lymphocytes (Brooks, et al. 1977). In one study (Gerosa, et al. 1981), selective failure of T cell immunity was noted in 76 long-term patients with supratentorial gliomas not treated with corticosteroids, compared to normal healthy controls. This was manifest as a selective decrease in "E-active" rosette-forming cells, and a reduction in mitogen-induced blastogenesis. Similarly,

Young, et al. (1976) observed depressed blastogenesis in response to the mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) in 50% of patients with glioblastomas.

Finally, in a study of 24 preoperative glioma patients not given corticosteroids, Servadei, et al. (1983) showed a statistically significant impairment of non-specific antibody-dependent cellular cytotoxicity (ADCC) to Chang liver cells, and spontaneous lymphocyte-mediated cytotoxicity (SLMC) to the human erythroleukemic cell line K562, when compared to normal healthy controls, bladder, and renal carcinoma patients. Unfortunately, it was not clear whether the patients in the study by Servadei, et al. (1983) had received any anti-convulsant therapy or not.

Some authors (Neuwelt, et al. 1983; Kikuchi, et al. 1984) have drawn attention to the fact that the anti-convulsant agents phenytoin and phenobarbital, and the corticosteroid dexamethasone, are independantly capable of depressing lymphocyte responsiveness to Con A, PHA, and pokeweed mitogen (PWM). Since the former agents are routinely given to control seizures and reduce intracranial pressure in neurosurgical patients, great care must be taken when interpreting data from studies on 'natural' immunity to malignant gliomas.

1.3.3 Cell-Mediated Immunity

Against this background of generalized immunosuppression in patients with malignant gliomas, there exists some evidence for a tumor-specific cellular immune response. Perivascular lymphocytic infiltration has long been recognized as a favorable prognostic factor in glioblastomas (Brooks, et al. 1978; Palma, et al. 1978) as well as other forms of cancer. Using monoclonal antibodies (mAb) to surface T and B cell markers, Yasuda, et al. (1983) demonstrated that on average, 41% of infiltrating cells in 15 malignant gliomas possessed surface immunoglobulin, and that 21% possessed the common T cell marker (Lyt 3^+). In addition to B and T-lymphocytes, macrophages are also capable of infiltrating CNS tumors. Morantz, et al. (1979) studied the macrophage content of 11 glioblastomas using the IgGEAC rosette assay, which measures the number of cells bearing IgGFc receptors and/or C3 receptors. Their results indicated that the mean macrophage content in glioblastomas was 45%, although the range was from 8% to 78%.

More recent studies on the nature of tumor-infiltrating lymphocytes (TIL) were contributed by Miescher, et al. (1988) and Sawamura, et al. (1988). These investigators prepared single cell suspensions of fresh biopsy material, and isolated the lymphocytes using a Ficoll-Hypaque density gradient in much the same way as

described in the present study (see section 2.1.1). The TILs were then characterized using a panel of mAbs to lymphocyte differentiation antigens.

Miescher, et al. (1988) found the TILs to be small $T11^+$, $T3^+$ $(CD3)^+$ lymphocytes that did not express the DR antigen or the receptor for IL-2. In contrast, Sawamura, et al. (1988) observed that glioma-derived lymphocytes grown in the presence of recombinant IL-2 were 89% $CD3^+$, 86% HLA-DR^+, and had an increased proportion of the $CD8^+$ cytotoxic/suppressor phenotype (55%) as compared to PBLs. The important difference between these two studies is that, in the former, the TILs were characterized prior to expansion *in vitro*, whereas, in the latter, they were analyzed after some time in culture. Such studies have been used as circumstantial evidence for the presence of selectively cytolytic effector cells infiltrating malignant gliomas.

More specific evidence was contributed by Miescher, et al. (1988) who established proliferating clones of TILs from glioma patients with cytotoxic activity against either autologous or allogeneic tumor cell targets. Numerous clones with NK-like activity were obtained, although none appeared to be directed to glioma-specific antigens. Proof of monoclonality of any of the T-lymphocyte clones in this particular study was also lacking.

The standard method for studying the specificity of cell-mediated immunity is by means of various microcytotoxicity assays (Levy, et al. 1972; Woosley, et al. 1977; Levy, 1978a). One confounding aspect of these assays is the presence, both in patients and controls, of natural killer (NK) cells which are cytotoxic to a variety of neoplastic and non-neoplastic targets. In addition, many investigators have failed to characterize the biochemical nature of the non-autologous target antigens. For some targets, the presence of alloantigens or xenoantigens cannot be ruled out, so results with this type of assay have been quite variable, in part due to the multiplicity of types of effector cell-target cell interactions.

Others (Martin-Achard, et al. 1980) have reported the presence of cytotoxic antibodies capable of eliciting ADCC against glioma cell lines in the sera of 20 of 143 (14%) of glioma patients; however, the anti-glioma reactivity was absorbed by cells of unrelated tumors and by normal platelets. The authors concluded that their glioma patients did not possess a tumor-specific humoral response capable of participating in ADCC.

In contrast, using the leukocyte adherence inhibition assay (LAI), which focuses on T cell-mediated immunity, Sheikh, et al. (1979) were able to demonstrate a significant inhibition of autologous leukocytes in 21 of 26

(81%) patients with malignant gliomas to uncharacterized 3 *M* KCl extracts of glioma tissue, as compared to normal brain extracts. From these studies, it was concluded that patients with malignant gliomas manifested a cellular immune response to glioma-associated antigens which could be measured by the LAI assay.

1.3.4 Humoral Immunity

Methods of studying the specificity of the humoral immune response to autologous gliomas include complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), immunofluorescence (IF), and immune adherence (IA). General criticisms of these methods are similar to those which apply to the study of specific cell-mediated immunity, i.e. lack of sufficient biochemical characterization of antigen targets and the presence of alloantibodies in patient's sera.

Using a CDC assay, Wood, et al. (1979) reported their experience with sera from over 400 individuals with a variety of CNS tumors over a five year period. In an initial 'ideal' series (Kornblith, et al. 1974), sera from 36 of 45 (80%) preoperative and postoperative glioma patients demonstrated significant cytotoxicity against allogeneic astrocytoma cells, compared to 4 of 55 (7%) normal healthy controls. Over the next five years, assay results varied considerably, and attention was eventually

focused on such factors as the condition of the target cells, batch to batch variability of rabbit complement and human cord serum, and testing of large numbers of sera under uniform conditions. The authors later concluded (Kornblith, et al. 1979) that under carefully controlled conditions, their assay was capable of detecting the presence of cytotoxic antibodies directed against *allogeneic* neuroectodermal antigens in the sera of up to 82% of glioma patients.

More recently, the same group (Kornblith, et al. 1983) reported finding cytotoxic antibodies directed against *autologous* tumor cells in the sera of glioma patients whose activity could not be absorbed with autologous fibroblasts, clearly indicative of a non-HLA antigen reactivity. The same study also found a statistically significant inverse correlation between tumor grade and the presence of cytotoxic antibodies, with 15 of 20 (75%) grade I, II, and III astrocytoma patients seropositive, compared to 5 of 22 (23%) of grade IV astrocytoma patients ($p<0.000015$).

In contrast, Martin-Achard, et al. (1980) using a similar CDC assay and *allogeneic* targets, were able to detect cytotoxic antibodies in the sera of only 8 of 80 (10%) glioma patients harboring tumors of all grades. The same group also reported absence of a tumor-specific humoral immune response in glioma patients using an ADCC assay (see section 1.3.3).

By means of the immune adherence and anti-C3 immunofluorescence assays, Garson, et al. (1981) were able to show that 9 of 16 (56%) glioma patients possessed IgM and IgG antibodies to cultured autologous tumor cells. Earlier work by Coakham, et al. (1980) with a similar IA assay indicated the presence of antibodies to cultured autologous glioma cells in 50% of the sera from 25 patients. Using an extensive series of absorption experiments. the latter group were able to categorize the autologous responses of their patients into several antigenic groups ranging from 'highly glioma-restricted' to 'brain and fibroblast-associated oncodevelopmental' antigens.

Finally, Tabuchi and Kirsch, (1978) have reported the detection of IgG on the cell membranes of 3 out of 9 primary glioblastomas, using the direct immunoperoxidase technique. Likewise, Solheid, et al. (1976) tested sera from glioma patients for IgG antibodies to the cell surface of cultured glioblastoma, astrocytoma, and reactive glial cells using indirect immunofluorescence, and found reactivity in the sera of 10 out of 12 (83%) patients. In view of the study by Aarli, et al. (1975), which demonstrated by means of indirect immunofluorescence that normal human IgG will bind non-specifically via the Fc fragment to the basic proteins of myelin sheaths, glial cells, and nerve cell bodies, claims of anti-tumor

specificity in experiments such as these must await further characterization of the antibody-antigen interaction.

1.3.5 Immunosuppression and Evasion

There have been case reports of immunosupression preceding malignancy, as with renal transplant patients on long-term corticosteroids and azathioprine, who develop leukemias or lymphomas. These malignancies are derived from the immune system itself, and may not arise by the same mechanisms as tumors which occur spontaneously in healthy non-immunosuppressed individuals. In the case of spontaneously occuring malignant gliomas, however, there is some evidence that it is the tumor which causes the immunosuppression.

Brooks and Roszman (1980) have argued that immunosuppression may be responsible for the failure of brain-tumor patients to develop allergic encephalitis following breakdown of the blood-brain barrier and release of previously sequestered brain proteins. Coakham (1984) has postulated that the mechanism may involve a complex disturbance in immunoregulation due to shared brain and lymphoid antigens (Carrel, et al. 1982a). In addition, malignant gliomas may possess ways of evading immune detection through antigenic modulation, decreased immunogenicity, and production of solubilized antigens.

Specific humoral mechanisms of immunosuppression were studied by Brooks, et al. (1972) who described a 'heat-stable' factor in the sera of patients with intracranial tumors which blocked cell-mediated immunity and suppressed the blastogenic response of autologous and homologous lymphocytes to PHA. Washing the patient's leukocytes and replacing autologous plasma with normal plasma restored their reactivity. Analysis of the patient's serum IgG fraction showed that it contained strong immunosuppressive activity. Levy (1978b) was also able to demonstrate blocking of lymphocyte-mediated cytotoxicity with the sera of patients with gliomas, which was specific for allogeneic tumor cells.

In a study by Garson, et al. (1981) purified autologous IgG was shown to inhibit IgM complement-fixing activity against cultured autologous glioma cells. Wood and Morantz (1982) also observed depressed T-lymphocyte responses to PHA in glioma patients, which became even more depressed after treatment with anti-human IgG. The authors speculated that this occured through the stabilization of membrane-bound IgG on suppressor cells. Finally, Kikuchi and Neuwelt (1983) were able to isolate a non-dialyzable (high molecular weight) factor from the cyst fluid of glioma patients which significantly inhibited mitogen-induced proliferation of normal healthy lymphocytes, but

which could not be shown to be an IgG molecule based on absorption studies with protein A.

Recent work by de Martin, et al. (1987) on a glioblastoma-derived T-lymphocyte suppressive factor has suggested that a 97 kDa protein homologous to transforming growth factor-β_2 (TGF-β_2) may play a role in the suppression of lymphocyte reactivity *in vitro*. Others (Lauro, et al. 1986) have implicated prostaglandin E_2 (PGE_2) as a mediator of immunosuppression in cultured malignant gliomas, after it was shown that indomethacin was capable of blocking the suppression of lymphocytes exposed to factor(s) in cultured glioma supernatant.

Several investigators (Roszman, et al. 1982; Kril and Apuzzo, 1983; von Habwehr, et al. 1984; Roszman, et al. 1985, Elliott, et al. 1987; Bhondeley, et al. 1988), using various methods, have described alterations in the $CD4^+$ T-helper to $CD8^+$ T-cytotoxic/suppressor cell ratio in PBLs of patients with malignant gliomas. Most studies agree that there is neither a qualitative nor a quantitative change in the T-cytotoxic/ suppressor compartment, but that the number and efficiency of T-helper cells is reduced. This observation may also explain the defects in B-lymphocyte function regularly observed in these patients, e.g. decreased antibody production to tetanus and influenza booster immunization (Mahaley, et al. 1977).

Studies of T-lymphocytes from patients with primary intracranial tumors by Elliott, et al. (1984) have indicated a decreased response to PHA which could not be enhanced with lectin-free interleukin-2 (IL-2). Using ^{125}I-labelled leukoagglutinating PHA (L-PHA), Roszman and Brooks (1980) were able to quantitate the number of L-PHA binding sites on lymphocytes from patients with brain tumors, and discovered that the latter had on average, twice as many receptor sites per cell as did normal healthy controls. Earlier studies by Callard and Basten (1978) have shown that T-lymphocytes vary quantitatively in their ability to bind PHA on their cell membranes, and that those lymphocytes which bind a 'medium' amount of lectin are the ones which are responsible for PHA-induced blastogenesis. These results, therefore, appear consistent with a functional imbalance of T-lymphocyte subpopulations in patients with primary brain tumors, in which the cell distribution of T-lymphocytes are skewed to those with higher numbers of PHA receptors.

Others (Wood and Morantz, 1983) have focused on the role of monocytes as suppressor cells, after noting increased numbers of the latter in mononuclear cell suspensions from brain-tumor patients. Adherent cell depletion experiments were able to partially reconstitute T cell function; however, total reversal of the defect was

rare, and there was no relationship between monocytes and T cell dysfunction in some patients.

Two possible mechanisms of evasion from the immune system are decreased antigenicity, and the formation of solubilized antigens. Gately, et al. (1984) observed pericellular 'halos' in 8 of 12 glioma lines co-cultured with allogeneic peripheral blood mononuclear cells in a mixed lymphocyte-tumor culture. Analysis of these 'halos' revealed that they represented glycosaminoglycan coats which consisted mainly of hyaluronic acid, and afforded the tumor cells a degree of non-specific protection from cellular immune attack.

Martin-Achard, et al. (1979) and Lauro, et al. (1983) were able to detect circulating immune complexes associated with brain tumors, whose presence correlated with histological grade and prognosis. Unfortunately, the biochemical nature of the non-immunoglobulin components were never characterized, although it is possible that they represent tumor-associated antigens. It is also unclear whether the antibody components represent the same IgG-immunoreactive suppressor factors described earlier, or whether they are anti-idiotype antibodies capable of down-regulating both the cellular and humoral immune responses to autologous tumor antigens.

1.4 Immunotherapy of Malignant Gliomas

Immunotherapy of malignant gliomas has not yet achieved wide acceptance. This is because, with the possible exception of one study by Mahaley, et al (1983a), no single approach, has proven to be effective, and few have been studied in a randomized prospective manner.

Two main categories of immunotherapy can be distinguished: active and passive. Active immunotherapy may be broken down into 1) specific immunization (e.g. immunization with irradiated tumor cells mixed with Freund's adjuvant), and 2) non-specific immune enhancement (e.g. administration of BCG or levamisole). Passive immunotherapy may involve 1) adoptive cellular immunity (e.g. intratumoral infusion of autologous leukocytes), or 2) adoptive humoral immunity (e.g. monoclonal antibodies). The latter will be discussed separately (see section 6.3).

1.4.1 Active Specific Immunotherapy

Some early attempts at active specific immunotherapy were reported by Bloom, et al. (1960) and Grace, et al. (1961). These investigators implanted autologous glioma tissue subcutaneously into the thighs of their patients and observed local growth of the implants with metastases to regional lymph nodes, but no therapeutic benefit on the primary tumor. Later, Trouillas (1973) conducted a randomized trial to study the effects of post-operative

immunization with autologous tumor tissue in complete Freund's adjuvant in patients with malignant gliomas who were not receiving immunosuppressive therapy in the form of corticosteroids. In the group which received surgery and immunotherapy (n = 10), median survival was significantly better ($p<0.005$; 7.4 vs. 5.4 months) than among patients who were treated with surgery alone (n = 17). When the former group was compared to patients who were given radiotherapy alone (n = 20), median survival appeared to be the same (7.4 and 7.5 months, respectively). Survival in patients who received surgery, immunotherapy, and radiotherapy (n = 18) seemed better than radiotherapy alone (n = 20; 10.1 vs. 7.5 months); however, no statistical comparison was reported.

From the data quoted it is not possible to make such a calculation, and it must be assumed that the results were not significant at the $p<0.05$ level. More importantly, there was a deficiency in the design of this study because there was no treatment group given surgery and radiotherapy, but not immunotherapy. Such a 'standard' group would enable comparisons with the usual treatment protocol. That there was a difference between the surgery, radiotherapy, and immunotherapy vs. radiotherapy alone groups comes as no surprise since it is now well established that the addition of surgery alone has a beneficial effect on survival (Salcman, 1980).

All 28 patients who received immunotherapy developed delayed cutaneous hypersensitivity to autologous tumor extracts several months following immunization, whereas only 3 of the 28 reacted prior to immunization. Some patients' sera produced identifiable immunodiffusion precipitates to extracts of autologous tumor. Finally, one patient given immunotherapy developed a confusional and inflammatory episode reminiscent of experimental allergic encephalomyelitis (EAE), a syndrome characterized by the presence of antibody to myelin basic protein.

Bloom, et al. (1973) also conducted a randomized trial using irradiated autologous tumor cells. A total of 62 patients were studied, all of whom were treated with surgery and post-operative radiotherapy. Twenty seven patients received an additional immunization with irradiated autologous tumor cells, injected subcutaneously into the left thigh (10 received multiple inoculations). All 27 patients who received tumor cells were dead at 30 months, whereas 7 of the 35 controls were alive at this time. It was concluded that the post-operative administration of irradiated autologous cells was of no benefit to patients with malignant gliomas.

In a phase I study by Mahaley, et al. (1983a), 20 patients with malignant gliomas received 10^8 lethally irradiated cells from the human glioma lines D-54MG or U-251MG, mixed with 500 µg of BCG cell wall in the first

inoculum, and levamisole, 2.5 mg/kg for 3 days per week every other week. Radiotherapy and chemotherapy with BCNU were begun one month after the start of immunization. Further immunizations with irradiated cell lines alone were also given at this time, and at monthly intervals thereafter. In general, the procedure was well tolerated and there was no evidence of allergic encephalomyelitis. Nine patients who received the U-251MG line had a slightly longer survival time than 11 patients who received the D-54MG line (not statistically significant).

Comparison of the U-251MG group to 58 historical control patients given post-operative radiotherapy, chemotherapy, and levamisole (Mahaley, et al. 1981) showed a significant increase in survival ($p<0.02$) in the former group. Most patients developed antibodies to fetal calf serum (FCS) and histocompatability antigens (HLA); however, after extensive absorption of nongliomatous antibody activity, the serum from one patient continued to exhibit strong reactivity to the original cell line used for immunization (Mahaley, et al. 1983b). The report did not include any information concerning the biochemical nature of the antigen recognized in this particular case.

1.4.2 Active Non-specific Immunotherapy

Active non-specific enhancement of the immune response in patients with malignant gliomas has been attempted using

such agents as Bacille de Calmette-Guérin (BCG), *Corynibacterium parvum*, levamisole, and interferon. Early studies on small numbers of patients using the first two of these agents proved disappointing (Takakura, et al. 1972). In one initial study by Selker, et al. (1978), intravenous administration of *C. parvum* to patients with malignant gliomas was sometimes associated with a rise in intracranial pressure, which discouraged any further investigations.

A study by Mahaley, et al. (1981) examined the effects of levamisole on 100 patients with malignant gliomas in a randomized prospective manner. When given concurrently with post-operative radiotherapy and chemotherapy, levamisole had no effect on survival. In addition, levamisole did not enhance general immune competence and was not associated with any evidence of specific cellular or humoral immune reactivity in those patients receiving it.

In a phase I trial of partially purified human leukocyte α-interferon (Mahaley, et al. 1984a), nine patients with malignant gliomas were given progressively increasing intravenous doses of the drug and monitored clinically. The interferon was given 3 weeks after surgery, for the 3-week period prior to the start of radiotherapy. Febrile responses were common, but no untoward neurological effects were observed. In a

subsequent report (Mahaley, et al. 1984b), survival data for the 9 patients were analyzed and found to be no different than for a comparable group of patients treated with post-operative radiotherapy and BCNU.

The same investigators (Mahaley, et al. 1985) later reported on a larger series of patients (n = 17) with malignant gliomas treated with human lymphoblastoid interferon (HuIFN-αLy) in escalating doses up to 900 x 10^6 U/m^2 for 8 weeks, following conventional surgery and radiotherapy. Seven patients were determined by CT scan to be treatment responders at 12 weeks, and had a median survival time of 511 days compared to 10 non-responders (median survival time 147 days). Neurotoxicity consisting of somnolence, disorientation and memory loss was considered to be the most serious side effect, and was observed in 9 patients. Despite the apparently dramatic increase in median survival in the interferon-treated group, no statistical comparison was reported by the authors. For this reason the conclusion of Mahaley, et al. (1985) that "interferon appears to be efficacious in the treatment of recurrent anaplastic gliomas as defined by CT brain scan responses following therapy" can not be satisfactorily substantiated.

In a phase II trial using human fibroblast interferon (Duff, et al. 1986) (IFN-β), twelve patients with recurrent glioblastomas who had previously undergone craniotomy and

conventional radiotherapy were given combined intravenous (10×10^6 U/day) and intratumoral (10×10^6 U every other day) IFN-β over three 10-day cycles. The mean survival time of the 11 patients who died was 5.5 months following admission to the study, a figure no different from the expected historical survival time of patients receiving conventional therapy (surgery and radiotherapy). The one patient who remained alive at the completion of the study had only equivocal tumor foci on biopsy of his 'recurrence', and probably should not have been admitted to the study in the first place. The authors conclusions stressed only the importance of distinguishing between radiation necrosis and tumor recurrence.

Clinical complications of interferon therapy include fever, nausea and vomiting, diarrhea, fatigue, headache, and myalgias. Of these, neurotoxocity is the most serious, albeit transient problem. Hematological complications are usually temporary and involve transient depression of leukocytes, platelets, erythrocytes, hemoglobin, and hematocrit. Some patients will show a rise in serum SGOT during therapy. The most alarming complication described so far was the development of circulating IFN-neutralizing antibody in one glioblastoma patient given a total of $13,676 \times 10^6$ U IFN-α over 44 weeks (Otsuka, et al. 1984). The patient developed a reciprical dilution antibody titre of 483 at 6 weeks which rose to >12,000 at 40 weeks.

1.4.3 Adoptive Cellular Immunotherapy

Initial experience with direct infusion of autologous leukocytes into recurrent glioblastomas was reported by Takakura, et al. (1972) and Young, et al. (1977). In the latter study, autologous leukocytes were obtained by leukaphoresis from 17 patients previously treated with surgery, radiotherapy, and/or chemotherapy (BCNU), and injected intratumorally in quantities of up to 4×10^9 leukocytes in a maximum volume of 12 ml. Eight patients appeared to respond to the therapy, with one survivor living for 17 months after only a single infusion. Transient increase in intracranial pressure was the major side effect noted, with no evidence of hyperimmune encephalitis in 6 detailed autopsies. The results of this study, although encouraging, are rather anecdotal and incomplete.

Neuwelt and Doherty (1977) have also reported on the use of human (xenogeneic) and syngeneic lymphocytes infused intrathecally into normal rabbits. Toxicity was observed to be minimal, especially after syngeneic infusion. Lymphocyte concentration in the CSF rose to a maximum of 70,000 cells/mm^3 at 12 hours post-infusion, and decreased logarithmically thereafter, possibly due to escape from the subarachnoid space systemically. No migration of host cells into the subarachnoid space was noted in response to the infusion. In a later study (Neuwelt, et al. 1978), the

same group reported on the intrathecal infusion of between 1×10^6 and 5×10^9 autologous lymphocytes in four patients with malignant gliomas. No toxicity was observed; however, CSF glucose decreased transiently in 2 patients. An autopsy performed on one of the patients suggested that the lymphocytes had gained access to the tumor bed as well as to the rest of the subarachnoid space. This conclusion was based on the observation that the degree of perivascular lymphocytic infiltration in the tumor bed was greater than would be normally seen following surgery and irradiation. However, such an isolated observation requires further objective study to substantiate that conclusion.

Recently, Grimm, et al. (1982) reported that lymphocytes from cancer patients could be activated *in vitro* with interleukin-2 (IL-2), yielding lymphokine-activated killer (LAK) cells with the ability to lyse natural killer (NK) resistant autologous melanoma, sarcoma, and adenocarcinoma targets. Bosnes and Hirschberg (1988) were able to demonstrate that in spite of depressed immunological reactivity, glioma patients are capable of generating LAK cells which are just as cytotoxic against both NK-sensitive and NK-resistant targets as LAK cells derived from normal healthy controls.

The IL-2/LAK technique has been applied to malignant gliomas in phase I trials (Jacobs, et al. 1986a, Jacques, et al. 1987). Combinations of IL-2, LAK cells, and IL-2

plus LAK cells were attempted. In both studies, the IL-2 and LAK cells were administered intraoperatively by direct injection into the brain tissue surrounding the tumor resection cavity. No systemic toxicity or neurotoxicity was observed. Selective killing of tumor cells by the LAK cells was demonstrated *in vitro* by Jacobs, et al. (1986b) using a chromium release microcytotoxicity assay. Since these were only phase I trials, therapeutic effectiveness and survival data were not reported.

More recently, Shimizu, et al. (1987) have reported a phase II study using LAK cells in the treatment of meningeal carcinomatosis and gliomatosis. Two patients were treated with intracisternal injections of 1-2 x 10^8 LAK cells twice a week (total dose 2 x 10^9 cells), in combination with 500 U recombinant IL-2. In both cases, the adoptive transfer of LAK cells was considered very effective in alleviating the clinical symptoms and signs, and in eliminating malignant cells from the CSF.

At present, methods are being devised for the stereotaxic implantation and chronic infusion of LAK cells into brain tumors (Plunkett, et al. 1988). The rationale for this approach is twofold: LAK cells are unlikely to cross the blood-brain barrier and reach small tumor targets if administered by the intravenous route, and often the only means of reaching such targets is via a stereotaxic approach.

1.5 Glioma-Associated Antigens

1.5.1. Antigens Shared with Normal Adult Brain

Malignant gliomas possess many antigens in common with the normal cells from which they were derived. As a result, it is virtually impossible to prepare conventional heteroantisera to glioma tissue without encountering some degree of cross-reactivity with normal adult brain. This was first observed over fifty years ago by Siris (1936), who noted that: "The sera of rabbits immunized with alcoholic extract of glioblastoma...exhibited a high antibody titer, but gave no evidence of immunological specificity for the type of tumor used. Brain antigen effected greater fixation of complement with these antisera than did glioblastoma antigen".

The shared antigens may be either intracellular, membrane associated, or extracellular in nature. Among the former is glial fibrillary acidic protein (GFAP), an intermediate filament constituent originally isolated from multiple sclerosis plaques (Eng, et al. 1971), which is structurally related to, but antigenically distinct from other intermediate filament proteins: the cytokeratins, neurofilaments, vimentin, and desmin. GFAP is confined predominantly but not exclusively to astrocytic cells. In addition to being expressed in normal astrocytes, GFAP is also found on some ependymomas; it is a 50 kDa protein which combines with vimentin to form the so-called 'glial

filaments', and is the traditional histological marker of astrocytic differentiation.

Another histological marker with an even wider distribution than GFAP, is the S-100 protein. This protein is present on normal glial cells, melanomas, and also Schwann cells of the peripheral nervous system. Expression of GFAP and S-100 protein in primary malignant gliomas is often reduced compared to low grade tumors, and sometimes it is absent altogether (Jacque, et al. 1978; van der Meulen, et al. 1978). GFAP expression is also frequently lost after several passages of glioma cells in tissue culture (Diserens, et al. 1981).

The class I HLA antigens are examples of cell-membrane antigens shared between gliomas and normal brain, and are mentioned here because they are often a source of concern in studies of antibody specificity. As it turns out, normal brain is a very poor source of class I HLA antigens. Using a radioimmunoassay (RIA), Lampson and Hickey (1986) were able to demonstrate the presence of HLA-A, B, C and β_2 microglobulin in extracts of normal brain, but quantitative inhibition assays showed that brain levels of class I HLA antigens were 70 times less than in spleen. Microscopic analysis of monoclonal antibody-stained frozen sections of normal and reactive astrocytes, neurons, oligodendrocytes, myelin, and microglia revealed that class I expression was exclusively confined to blood vessel

walls. Only occasionally was class I activity noted on the cell bodies of astrocytoma cells. These results are in agreement with the earlier work of Hirschberg, et al. (1982) who identified class I expression in cultured astrocytomas also by means of a RIA. Expression of class II HLA antigens in malignant gliomas is discussed in section 1.5.3.

1.5.2 Antigens Shared with Other Neuroectodermal Tumors

Evidence for shared glioma-neuroectodermal tumor antigens based on studies of glioma patient's sera (Coakham, et al. 1980) was presented in the discussion of specific humoral immunity (section 1.3.4). Similar serological studies were carried out by Pfreundschuh, et al. (1978) who examined the sera of 30 astrocytoma patients for antibodies reactive to cell surface antigens of cultured astrocytoma cells by means of mixed hemabsorption assays, immune adherence and protein A assays, and anti-C3 mixed hemabsorption assays. Absorption studies defined three classes of antigens: 1) restricted to autologous astrocytoma cells, 2) present on astrocytomas, neuroblastomas, sarcoma, and most melanomas, and 3) widely distributed on cultured normal and malignant cells of both human and non-human origin.

Heteroantisera studies by Wahlström, et al. (1974), and Coakham and Lakshmi (1975), have also supported the existence of glioma-associated antigens. Both groups raised antibodies in rabbits to lyophilized glioblastoma multiforme tissue or cultured astrocytomas, respectively, and carried out extensive absorption experiments with brain and other normal and malignant cells. Unfortunately, reactivity with melanomas, neuroblastomas, or other neuroectodermal tumors was never assessed; therefore there is no way of ascertaining the neuroectodermal specificity of the antisera.

In a study by Schnegg, et al. (1981a), an antiglioma antiserum was produced by immunizing a rabbit with membrane-enriched preparations of the human malignant glioma cell line LN-18. After absorption with normal peripheral blood lymphocytes, a pool of cells from 4 different lymphoblastoid cell lines, an endometrial carcinoma, and a colon carcinoma, the serum reacted in a complement-dependent cytotoxic ^{51}Cr-release assay with cells from malignant gliomas, melanomas, and fetal brain. Subsequent absorption with cells from 2 different melanoma lines preserved reactivity with 7 malignant gliomas. Further absorption with normal adult brain abolished antiserum reactivity with all but 3 of the 7 gliomas. The authors concluded that the antiserum described could recognize 3 types of antigen: 1) common to cells of

neuroectodermal origin, 2) shared by malignant gliomas and adult brain, and 3) restricted to some gliomas.

Studies with murine mAbs raised against glioma cell lines were reported by Schnegg, et al. (1981b) and Bourdon, et al. (1983). Both groups immunized mice with cultured human glioma cell lines, and fused spleen cells with the mouse myeloma lines P3X63-Ag8 or P3X63-Ag8.653, respectively. The mAbs BF7, GE2, described by Schnegg et al. (1981b) appear to be relatively glioma-restricted, whereas antibody CG12 (de Tribolet, et al. 1984) reacts with a spectrum of gliomas, melanomas, and neuroblastomas. Antibody 81C6 was produced and characterized by Bourdon, et al. (1983) and found to react with a glioma-mesenchymal extracellular matrix (GMEM) antigen expressed on gliomas, neuroblastomas, melanomas, sarcomas, and cultured fibroblasts. In addition to the above, the GMEM antigen was also found in normal liver sinusoids, spleen red pulp sinusoids, kidney medullary tubule interstitium, and glomerular mesangium. Whereas absorption of CG12 with normal adult and fetal brain abolished binding activity, neither BF7, GE2, nor 81C6 were affected by the same treatment.

Finally, monoclonal antibodies raised against melanomas (Carrel, et al. 1982b; Herlyn, et al. 1980; Liao, et al. 1981), and a neuroblastoma (Kennett and Gilbert, 1979) have been shown to react with gliomas and

retinoblastomas, thus underscoring the existence of antigenic similarities between these different types of neuroectodermal tumors.

1.5.3 Antigens Shared with Lymphoid Tissues

Reif and Allen (1964) were the first to describe a murine thymic antigen, known as AKR, which was also present in high concentrations in nervous tissue. The antigen was later referred to as θ, or Thy-1, and was shown to be a 35 kDa glycoprotein homologous to the domain structures which make up the MHC class 1 and 2 antigens, and the immunoglobulin molecules. Studies with a monoclonal antibody against human Thy-1 antigen by Seeger, et al. (1982) demonstrated the presence of this lymphoid differentiation antigen on neuroblastoma, glioma, sarcoma, and teratoma cells. The presence of Thy-1 on normal glial cells was reported by Kemshed, et al. (1982).

Other lymphoid differentiation antigens, such as the common acute lymphoblastic leukemia antigen (CALLA) and class II HLA antigens (HLA-DR), have been found to be present on malignant gliomas (Carrel, et al. 1982a). Examination of frozen sections of human brain biopsies (Lampson and Hickey, 1986) ranging from 'histologically normal' to infiltrated with astrocytoma cells, revealed class II expression in occasional cell bodies in both white and grey matter. The affected cells had the morphological

appearance of microglia or reactive astrocytes. In addition, Budka, et al. (1985) screened a variety of neurogenic tumors with a battery of 24 monoclonal antibodies raised against human hemopoietic cells, and found some cross-reactivity with 12 of 24 antibodies.

As suggested earlier (section 1.3.5), the presence of cross-reacting antibodies to lymphoid antigens in the sera of glioma patients might have an effect on immune regulation, and account for the T and B cell immunosuppression frequently observed in these individuals. One may also speculate that the presence of HLA-DR antigens on reactive or transformed astrocytes could facilitate the development of a primary immune response against oncodevelopmental tumor-associated antigens.

1.5.4 Antigens Shared with Embryonic Tissues

Included in this category are the so-called oncodevelopmental antigens, stage-specific embryogenic markers that are not present in normal adult tissues, but are re-expressed as a result of a malignant transformation. The presence of these antigens in malignant gliomas was first suggested by Trouillas (1971), who immunized 14 glioma patients with a crude extract of autologous tumor in complete Freund's adjuvant, and observed the formation of precipitin bands when the patient's sera were reacted with fetal brain extracts. Later, Wikstrand and Bigner (1979)

were able to produce nonhuman primate antisera by hyperimmunization of monkeys (*Macaca fascicularis*) with human glioblastoma multiforme tissue or glioma cell lines. Absorption studies and cytotoxic antibody testing showed that 3 antisera contained antibodies directed against normal adult and fetal brain antigens.

Allan, et al. (1983) and Kemshead, et al. (1983) have reported the production of two monoclonal antibodies, UJ 13A, and UJ 127:11, both derived from the fusion of a murine myeloma line, P3X63-Ag8.653, with spleen cells from mice immunized with human fetal brain. Both antibodies recognize antigens present on a subset of adult and fetal tissues: UJ 13A appears to bind to a variety of neuroectodermal tissues; however, UJ 127:11 detects a 220-240 kDa glycoprotein restricted to cells of neural origin. Finally, Irie, et al. (1982) have established two long-term human B-lymphoblastoid cell lines, L55 and L72, that produce IgM kappa antibodies to the human tumor-associated fetal antigens, OFA-I-1 and OFA-I-2, respectively. The OFA-I antigen is a cell membrane antigen originally defined by serum antibodies from melanoma patients (Irie, et al. 1976), and is also found on fetal brain. OFA-I-2 is a related antigenic specificity found only on tumor cells of neuroectodermal origin (melanoma, glioma, and neuroblastoma), and was determined by Cahan, et al. (1982) to be the cell surface glycolipid, ganglioside GD2.

In summary, the existence of tumor-specific antigens on spontaneous malignant gliomas as defined by patient's autologous sera, conventional heteroantisera, and mAbs, is presently unsubstantiated. Only glioma-associated antigens have been reported thus far; and these appear to be interrelated sets of overlapping specificities (see Figure 1.4). Fortunately, this does not preclude the use of mAbs in a variety of clinical applications, such as radioimmunolocalization studies, and also humoral immunotherapy because of the concept of 'operational specificity'. In practical terms, there may be a sufficiently high receptor gradient on neoplastic versus normal cells to allow radiolabelled mAbs to be used for purposes of diagnostic imaging or to provide for a satisfactory therapeutic index.

1.6 Production of Human Monoclonal Antibodies

1.6.1 Epstein-Barr Virus Transformation of B-lymphocytes

Under standard culture conditions, normal human peripheral B-lymphocytes (PBLs) are capable of only limited proliferation *in vitro*. Infection of B-lymphocytes with EBV, a lymphotropic herpesvirus, will result in the production of lymphoblastoid cell lines. The method

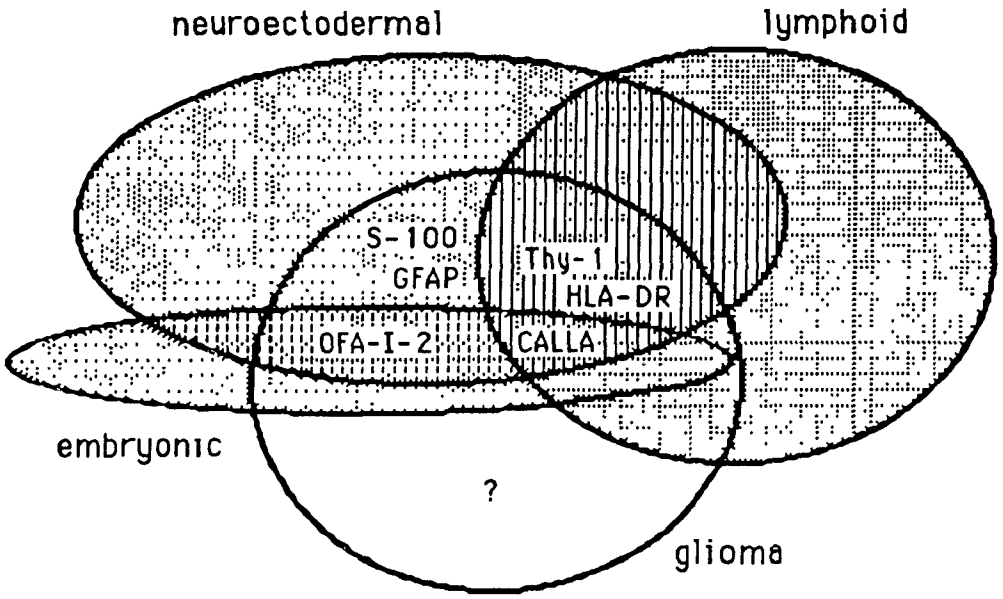


Figure 1.4: Glioma-associated antigens

Glioma-associated antigens are thought to exist as a series of overlapping specificities, represented by the above Venn diagram. Antigens may be shared between gliomas and lymphoid tissues, gliomas and other neuroectodrmal tissues, and gliomas and fetal or embryonic tissues. OFA-I-2 is a ganglioside found on gliomas, melanomas, and fetal brain (Irie, et al. 1976). S-100 protein and glial fibrillary acidic protein (GFAP) are classical markers for normal and differentiated astrocytes (Eng, et al. 1971). Thy-1 is a lymphoid differentiation antigen found on human T-lymphocytes, normal brain, and gliomas (Seeger, et al. 1982). Class II human leukocyte antigen (HLA-DR) is shared between lymphoid cells and human gliomas, but not normal astrocytes (Carrel, et al. 1982a; Lampson and Hickey, 1986). Common acute lymphoblastic leukemia antigen (CALLA) is distributed on lymphoid cells and human gliomas (Carrel, et al. 1982a), cultured melanoma cells (Carrel, et al. 1983), and an embryonic (germ cell) tumor (Brox, et al. 1986).

involves: 1) cultivation of lymphocytes in a mass cell culture system using conditioned media derived from cultures that produce EBV; 2) seeding the infected cells in 24- or 96-microwell plates at a density of 10^5-10^6 cells/well; and 3) cloning desired cells by limiting dilution (Olsson, 1985). Lymphoblastoid cells may retain many of the original characteristics of the B cells from which they were derived, including immunoglobulin production and secretion. Using a variation of the above method, Steinitz, et al. (1977), were the first to report the derivation of a B-lymphoblastoid cell line secreting HmAbs against the synthetic hapten, NNP (4-hydroxy-3,5-dinitrophenacetic acid). Others (Irie, et al. 1982) have derived long term lymphoblastoid cell lines secreting HmAbs against the melanoma-associated antigen, ganglioside GD2.

The major drawbacks of this technique are low levels of Ig secretion (0.5-5.0 $\mu g/10^6$ cells/24 hours), loss of Ig production over time (usually by 3 months), release of infectious viral material into culture, and difficulty with subcloning by the limiting dilution method (Cole, et al. 1984a). In addition, it has been shown that EBV infection of B-lymphocytes results in a predominance of IgM secreting clones (Brown and Miller, 1982; Stein, et al. 1983) due to the higher density of EBV receptors on B cells bearing surface IgM. Human IgM is biochemically more unstable than IgG, and may be undesirable for certain clinical

applications due to its high molecular weight. With these limitations in mind, investigators have used human-human hybridomas as an alternative method of producing HmAbs.

1.6.2 Human-Human B Cell Hybridomas

Three key elements are required for the successful production of either human or murine hybridomas: 1) immune B cells; 2) a suitable immortalizing partner; and 3) some means of bringing about their fusion. Because of certain limitations imposed by the nature of human experimental material, the production of human hybridomas often falls short of the ideal.

Experiments with immunized mice have shown that splenocytes are better fusion partners than PBLs. Unlike rodents, however, experiments in humans which involve inoculation with a desired antigen followed by splenectomy and harvesting of splenocytes, as in the original report of Olsson and Kaplan (1980), are considered rare. Only PBLs and, to a certain extent, lymph node lymphocytes are routinely available from cancer patients. Finally, the number of suitable human myeloma fusion partners is limited by the paucity of true myeloma cell lines. Investigators have therefore been forced to consider both lymphoblastoid and myeloma-like cell lines as potential fusion partners for human B cells (Tables 1.2 and 1.3).

Table 1.2: Human lymphoblastoid cell lines and their HGPRT[a] deficient derivatives

Parent and Derivative	*Ig*[b]	*Reference*
ARH-77[c]	γ, λ	Burk, et al. (1978)
LICR-LON-HMy-2[d]	γ, λ	Edwards, et al. (1982)
LICR-LON-HMy-2/CAM1	γ, λ	Sikora, et al. (1983)
GM1500	γ, κ	Croce, et al. (1980)
GM1500-6TG-A1-1	γ, κ	Croce, et al. (1980)
GM1500-6TG-A1-2	γ, κ	Croce, et al. (1980)
KR-4	γ, κ	Kozbor, et al (1982)
GM-4672	γ, κ	Shoenfeld, et al. (1982)
GK-5	γ, κ	Dwyer, et al. (1983)
WI-L2	μ, κ	Levy, et al. (1968)
UC 729-6	μ, κ	Glassy, et al. (1983)
UC 729-6-HF_2	- -	Abrams, et al. (1983)
LTR228	μ, κ	Larrick, et al. (1983)
H35.1.1	μ, κ	Chiorrazzi, et al. (1982)
PGLC 33H	μ, λ	Sato, et al. (1972)
0467.3	μ, λ	Chiorrazzi, et al. (1982)

Adapted from Cole, et al. (1984a)

[a]hypoxanthine-guanine phosphoribosyl transferase

[b]immunoglobulin secreted

[c]parent cell line

[d]HGPRT-deficient derivative

Table 1.3: Human myeloma cell lines and their HGPRT[a] deficient derivatives

Parent & Derivative	*Source*	*Ig*[b]	*Reference*
RPMI-8226[c]	peripheral blood	λ	Matsuoka, et al. (1967)
8226-8AzR[d]		λ	Abrams, et al. (1983)
U-266	peripheral blood	ε, λ	Nilsson, et al. (1970)
SKO-007 (U-266 AR_1)		ε, λ	Olsson and Kaplan, (1980)
U-266 ($8Az^R$)		ε, λ	Abrams, et al. (1983)
FU-266		ε, λ	Teng, et al. (1983)
KMM-1	plasmacytoma	λ	Togawa, et al. (1982)
Karpas-707	marrow and peripheral blood	λ	Karpas, et al. (1982)

Adapted from Cole, et al. (1984a)

[a]hypoxanthine-guanine phosphoribosyl transferase

[b]immunoglobulin secreted

[c]parent cell line

[d]HGPRT-deficient derivative

The fusion of B-lymphocytes with myeloma-like cells or lymphoblastoid cells can be achieved in a routine manner by the controlled addition of the fusogen polyethylene glycol (PEG). The cells are mixed in a predetermined ratio (lymphocytes usually predominating), the PEG is added, and then slowly diluted out. The resulting mixture will contain 5 types of cell: 1) unfused lymphocytes; 2) unfused myeloma cells; 3) fused lymphocytes; 4) fused myeloma cells; and 5) hybrid lymphocyte-myeloma cells. The problem now is to remove the former four, while retaining the lymphocyte-myeloma hybrids.

Spontaneous death of lymphocytes usually occurs after 7-10 days in culture; however, myeloma cells have the potential to proliferate indefinitely *in vitro*. Pre-selection of myeloma cells in specialized media containing 6-thioguanine or 8-azaguanine will result in the emergence of variant sublines deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase ($HGPRT^-$; EC 2.4.2.8). This enzyme plays a key role in the purine salvage pathway (Fig. 1.5), and is needed when the main pathway of purine biosynthesis has been blocked by the folate antagonist aminopterin, or the glutamine antagonist azaserine. Exposure of the fusion mixture to either of these two agents will result in the selective death of myeloma cells with sparing of the hybrid cells (Littlefield, 1964), since

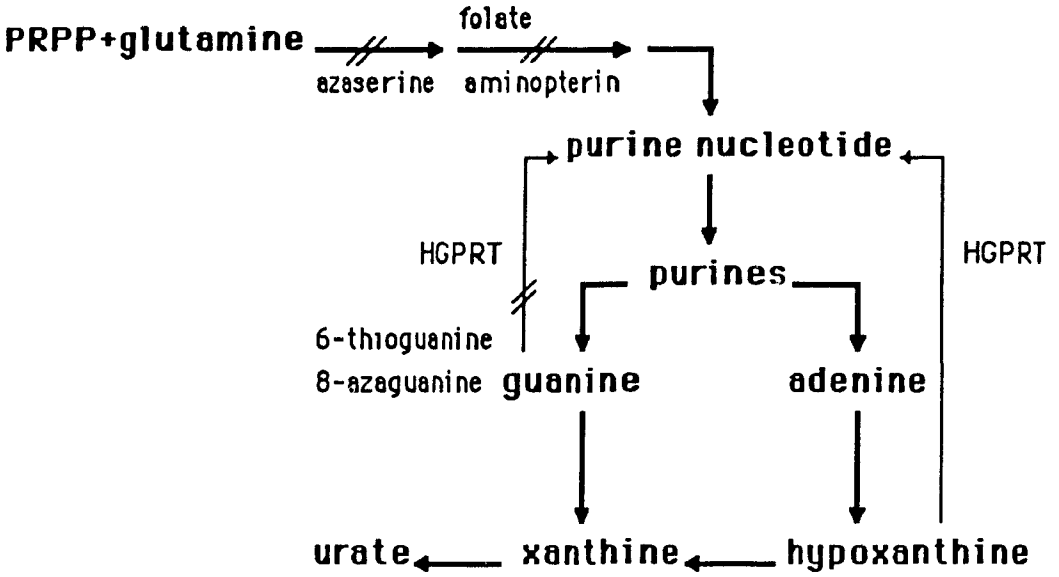


Figure 1.5: Principal biosynthetic and salvage pathways for purines

Growth of myeloma or lymphoblastoid cells in the presence of 6-thioguanine or 8-azaguanine will produce mutants deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), a necessary enzyme of the purine salvage pathway. After re-introduction of HGPRT by fusion of normal human lymphocytes to the mutant cells, the principal biosynthetic pathway is blocked with aminopterin or azaserine. The cells must now rely on HGPRT to re-cycle purines from xanthine and hypoxanthine. Unfused myeloma cells, which are deficient in HGPRT, are unable to do so and are killed, whereas unfused lymphocytes, which have only a limited lifespan *in vitro*, die off spontaneously. Only hybrid lymphocyte-myeloma cells are capable of surviving under these conditions. PRPP = 5-phosphoribosyl-1-pyrophosphate. Adapted from Zilva and Pannall (1979).

the latter have acquired a copy of the HGPRT gene from the lymphocyte parent.

Lymphoblastoid cell lines can also be made HGPRT deficient and fused to B-lymphocytes; however, some lymphoblastoid lines are known to secrete EBV in culture. In such a case, the EBV is independently capable of immortalizing B cells, and the hybrid nature of the immortalized cells must be proven. This can be accomplished, to some extent, with karyotypic analysis. Most lymphoblastoid cell lines are diploid or pseudodiploid. Myeloma lines are typically aneuploid (Table 1.4). Hybrid cells usually start off as tetraploid (4*n*), but lose chromosomes after prolonged culture *in vitro* to usually stabilize at between 3*n* and 4*n*.

The loss of chromosomes appears to be a random process, except for mouse-human heterohybridomas, in which case human chromosomes are preferentially lost. Of prime importance, however, is the fact that the human immunoglobulin genes are located on 3 separate chromosomes. Heavy chain genes are located on chromosome 14 (Croce, et al. 1979), κ light chain genes are located on chromosome 2 (Malcolm, et al. 1982), and the λ light chain genes are located on chromosome 22 (Erikson, et al. 1981). Immediately after fusion, a hybridoma will possess a duplicate set of these Ig genes, potentially capable of independent expression. But with random loss of

Table 1.4: Distinguishing characteristics of myeloma and lymphoblastoid cells

Characteristic	*Myeloma*	*Lymphoblastoid*
growth in culture	single cells or loose clumps	clumps
electron microscopy	prominent rough endoplasmic reticulum and Golgi apparatus	poorly developed endoplasmic reticulum, free polyribosomes
EBNA[a]	negative	positive
karyotype	aneuploid	usually diploid
growth rate	slow	fast
sIg[b]	±	++
rate of secretion	high	low
cell morphology	round, regular	irregular, elongated with prominent pseudopodia

Adapted from Kozbor, et al. (1986)

[a]Epstein-Barr Nuclear Antigen
[b]surface immunoglobulin

chromosomes, even functional Ig genes may be deleted, resulting in the emergence of non-producing clones. With periodical subcloning of the hybridomas by the limiting dilution method, it is possible to maintain stable Ig production over prolonged periods of time.

1.7 Purpose of the Present Study

The principal objective of this study was to prepare human monoclonal antibodies from patients with malignant gliomas and other neurological tumors which would be of potential value in tumor immunodiagnosis and immunotherapy. Based on the information discussed in the previous sections, it was postulated that patients with neurological tumors would posess circulating B-lymphocytes with anti-glioma specificities. In addition, it was also postulated that tumor-reactive antibodies derived from glioma patients would recognize common, non-allelic determinants on human gliomas which might differ in specificities and distribution from those recognized by xenogeneic systems.

A number of ways of immortalizing human B-lymphocytes were considered. The human-human hybridoma method was chosen because of 1) familiarity with the technique, 2) the availability of a suitable fusion partner, the human myeloma-like line TM-H2-SP2, and 3) certain other potential advantages over the EBV transformation method, involving

differences in selection of immunoglobulin class and Ig production levels.

Figure 1.6 is a flow chart representation of the strategy which was followed. Beginning at the top of the chart, a sample of peripheral blood was obtained from glioma patients prior to the administration of corticosteroids. Lymphocytes were separated by Ficoll-Hypaque density centrifugation, and fused to TM-H2-SP2 to yield hybridomas. If the patients went to surgery, a sample of autologous tumor was also obtained, and used to prepare crude whole cell extracts. The latter were used in an ELISA assay to screen for anti-tumor activity in the hybridoma supernatants. At a subsequent stage, promising hybridomas were screened for reactivity with glioma cell surface antigens using the technique of flow cytometry.

In studying the B-lymphocyte specificities derived in the initial phase of this research, it became clear that an equally important issue was that of tumor heterogeneity. Five stable human-human IgM secreting hybridomas were produced, none of which reacted with the totality of tumor cells in any of the cell lines studied. This observation confirmed the work of others regarding tumor heterogeneity, and prompted a search for the biological significance, both *in vitro* and *in vivo*, of the subpopulation of tumor cells recognized by the monoclonals.

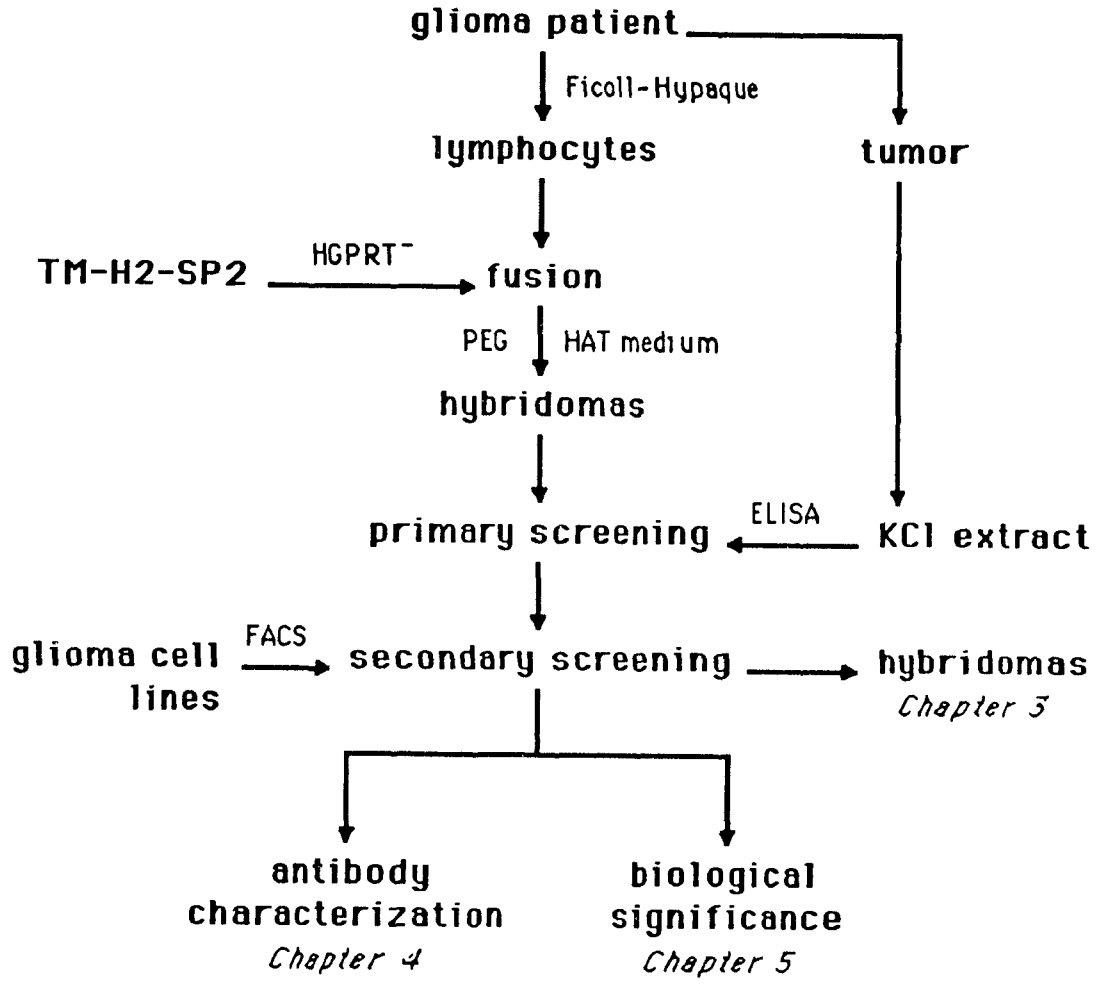


Figure 1.6: Outline of the present study

See section 1.7 for details.

The results of the cell fusions, hybridoma outgrowth, primary, and secondary screening are given in chapter 3. Chapter 4 describes in some detail five HmAbs which recognize glioma cells and the biological distribution of HmAb reactivity; the surface antigens are also discussed. In chapter 5, the problem of glioma cellular heterogeneity is addressed, and the biological roles of the cellular compartments recognized by the monoclonals are discussed.

2 MATERIAL AND METHODS

2.1 Derivation of Human Anti-Glioma Monoclonal Antibodies from Patients with Neurological Tumors

2.1.1 Clinical Material

Patients who were considered eligible for this study were suspected of having a brain tumor on the basis of history, physical examination, and CT scan. All individuals were admitted to a McGill University teaching hospital between 1 July, 1985 and 30 June, 1987. After obtaining the patient's verbal informed consent, in accordance with the guidelines approved by the respective teaching hospital's ethics committees, approximately 40 ml of peripheral venous blood was withdrawn into four heparinized glass tubes (Vacutainer), and stored at room temperature until further use.

Within 2 hrs. of sampling, the lymphocytes were separated on a Ficoll-Hypaque density gradient (SG 1.077) by carefully layering two 20 ml aliquots of whole blood onto 20 ml of 5% (w/v) Ficoll-Hypaque and centrifuging at room temperature for 25 minutes at 1000 G. The lymphocytes were harvested from the interface, washed once with sterile phosphate-buffered saline (PBS), and re-suspended in 10 ml alpha minimum essential medium (αMEM) containing 10% (v/v)

fetal calf serum (FCS; Flow Laboratories, Rockville, MD), 292 mg/L L-glutamine, 44 mg/L L-asparagine, 100 μU/mL penicillin, and 100 μg/mL streptomycin.

The lymphocytes were then counted in a hemacytometer after diluting 0.1 ml of the mixture with 0.9 ml of 2% glacial acetic acid, in order to lyse any remaining red blood cells. If TM-H2-SP2 cells in logarithmic growth phase were available, a fusion procedure would be carried out immediately (see section 2.1.4); otherwise the lymphocyte mixture was stored at 4 °C if it was felt that the TM-H2-SP2 line would be ready for fusion within the next 24 hrs. If no fusion procedure was to be carried out, the lymphocyte mixture was re-suspended in 1 ml αMEM + 10% FCS, and stored at -70 °C in a cryotube (Nunclon, Denmark) containing 0.1 ml sterile dimethyl sulfoxide (DMSO) as a preservative.

At operation, a portion of autologous tumor (1-5 gm) was removed and placed in sterile Elliott's solution (Abbott Laboratories Inc.) for preparation of KCl extracts. After mincing with fine scissors, fresh tumor was placed in a conical test tube and agitated overnight at 4 °C with 3 *M* KCl (1 ml/gm wet tissue). The crude cell lysate was diluted 1:10 with ddH_2O, and clarified by high speed centrifugation at 12,000 G for 20 min. at 4 °C. The extract was then dialyzed and equilibrated to PBS over the next 6 hrs, and then filter-sterilized with a 0.22 μm

Millipore filter. The protein concentration of the tumor extract was determined using the method of Lowry, et al. (1951), and the necessary dilutions made with PBS to bring the final protein concentration to 100 µg/ml. Aliquots were stored at -20 °C until ready for use in the screening assay (see section 2.1.6).

2.1.2 TM-H2-SP2 Cell Line

The human myeloma-like cell line TM-H2-SP2 used in these studies is the immunoglobulin non-secreting subline of the parent line, TM-H2, described by Sullivan, et al. (1982). TM-H2 is a hypoxanthine guanine phosphoribosyl transferase (EC 2.4.2.8) deficient ($HGPRT^-$) derivative of an unknown human myeloma-like line, which was selected in 0.8% (w/w) methylcellulose for resistance to 6-thioguanine (6 µg/ml) and failure to grow in hypoxanthine-aminopterin-thymidine medium (Littlefield, 1964). TM-H2 produces and secretes up to 3 µg/ml of IgG(κ) in supernatant culture fluid when grown to a concentration of 5×10^5 cells/ml in αMEM containing 10% FCS. The subline TM-H2-SP2 produces but does not secrete immunoglobulin, as determined by quantitative ELISA. TM-H2-SP2 has a 46, XX karyotype, and was grown continually in the presence of 6-thioguanine (6 µg/ml) to prevent HGPRT revertants. The line is negative for Epstein-Barr nuclear antigen ($EBNA^-$), and therefore

does not appear to be an EBV-immortalized derivative of lymphoid cells.

2.1.3 Human Glioma Cell Lines

The human glioma cell lines used in this study were contributed by the following individuals: 1) Dr. L. Panasci, The Lady Davis Research Institute, Montreal, Quebec (SK-MG-1, SKI-1), 2) Dr. N. de Tribolet, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland (LN-215, LN-340), and 3) Dr. J.G. Cairncross, London Regional Cancer Centre, 391 South Street, London, Ontario, N6A 4G5 (SK-MG-13, U-178, U-373). The glioma lines designated as "SK" originated at the Memorial Sloan-Kettering Cancer Centre, New York, NY, 10021 (Pfreudschuh, et al. 1978). The "LN" glioma lines were developed by Dr. de Tribolet and co-workers in Lausanne (Schnegg, et al. 1981b), and the "U" glioma lines by Westermark, et al. (1973) at the University of Uppsala, Uppsala, Sweden.

All cell lines were grown in αMEM containing 10% fetal calf serum (FCS; Flow Laboratories), 292 mg/L L-glutamine, 44 mg/L L-asparagine (anhydrous), 100 μU/mL penicillin, and 100 μg/mL streptomycin in the presence of 5% CO_2 atmosphere at 37 °C. The cells were grown to confluence in standard tissue culture flasks (Nunclon, Denmark), and passaged in a split ratio of 1:2 using citrated PBS (Gibco, Grand Island, NY) containing 0.1%

trypsin (Difco Laboratories, Detroit, MI) to remove the cells, and FCS to stop the action of the enzyme on the cells.

2.1.4 Fusion Procedure

The method used was a variation of the original method of Köhler and Milstein (1975). Twenty-four hours prior to fusion, the TM-H2-SP2 cell line culture in mid- to late-logarithmic phase was diluted 1:1 with fresh αMEM containing 10% FCS and 6-thioguanine (6 μg/ml). This was done to ensure a majority of cells were cycling in growth phase. At the time of fusion, an aliquot of TM-H2-SP2 cells were stained with an equal volume of 0.16% trypan blue, and the viable cell count was determined in a hemacytometer by the dye exclusion method (Gorer and O'Gorman, 1956). If the viable cell count was below 80% the cells were not used on that day.

The TM-H2-SP2 cells were then combined directly with the peripheral lymphocytes in a lymphocyte to myeloma cell ratio of 4:1, and pelleted together by centrifugation at 500 G for 5 min. The supernatant was discarded, and the cells were fused under serum-free conditions by the gradual addition of 1 ml of 50% polyethylene glycol MW 1450 (Sigma, St. Louis, MO) diluted 1:1 in serum-free αMEM over 1 minute (Pontevorco, 1976). This was followed by dilution over the next 4-5 minutes with 10 ml of serum-free αMEM, of which 2

ml were added with gentle stirring over the first 2 minutes.

The fusion mixture was then pelleted by centrifugation as before, and washed twice with 10 ml of hypoxanthine-aminopterin-thymidine (HAT) medium containing 10% FCS, 10^{-4} M hypoxanthine, 4 x 10^{-7} *M* aminopterin, 1.6 x 10^{-5} *M* thymidine, 292 mg/L L-glutamine, 44 mg/L L-asparagine, 100 µU/mL penicillin, and 100 µg/mL streptomycin. A viable cell count on 0.1 ml of the fusion mixture was performed as before, and the cells diluted to a concentration of either 1 x 10^5 or 2.5 x 10^5 viable myeloma cells/ml by addition of fresh HAT medium. The cells were then dispensed in 200 µl aliquots into 96-microwell trays (Linbro Laboratories Inc., McLean, VA; 0.28 cm^2/well), and incubated at 37 °C in 5% CO_2 atmosphere.

2.1.5 Culture Conditions

The hybridomas were maintained in HAT medium for 7 days post-fusion, with exchange of half the culture media on day 5, followed by re-feeding with fresh HAT medium. On day 7, 50% of the culture media was again removed, and the hybridomas re-fed with hypoxanthine-thymidine (HT) medium (HAT medium minus aminopterin). This process of exchange feeding with HT medium was continued every 5 days until the hybridomas became microscopically visible (approximately 4-6 weeks post-fusion), at which point they were ready for

testing by ELISA. Selected hybridomas were continuously propagated up to transfer into 25 cm^2 culture flasks (Nunclon, Denmark) in a volume of 2 ml via 24 well trays (Linbro Laboratories Inc., McLean, VA), then frozen down (see section 2.1.10). All hybridomas were maintained continually in HT medium.

2.1.6 Tumor Extract ELISA

The screening method used is a modification of Douillard and Hoffman (1983). Briefly, 96 well polyvinyl chloride Microtiter ELISA plates (Dynatech, Alexandria, VA) were incubated for 12-18 hours at 4 °C with 200 µl/well of 3 *M* KCl autologous tumor extract (see section 2.1.1) at a concentration of 5 µg protein/ml (1 µg/well) in carbonate-bicarbonate buffer, pH 9.6 (0.8 gm Na_2CO_3, 1.47 gm $NaHCO_3$, 0.1 gm NaN_3 made up to 500 ml with ddH_2O). The plates were washed 3 times with PBS containing 0.05% (v/v) Tween-20® (PBS-Tween; Sigma, St. Louis, MO). Then 50 µl of the hybridoma supernatant to be tested was added to 50 µl PBS per well.

Culture supernatant from the parental myeloma line, TII-H2, containing IgG(κ), was used as a nonspecific immunoglobulin control for the initial 15 fusions, whereas culture supernatant from one of the hybridomas (BT27/2D2) containing 1-2 µg/ml IgM was used as a control in subsequent screening assays.

After 2 hours incubation at room temperature, the plates were washed 3 times with PBS-Tween, and 200 µl/well alkaline phosphatase (ALP)-conjugated goat anti-human κ plus goat anti-human λ Ig light chain (TAGO, Burlingame, CA) were added at 1:2000 dilution in 1% bovine serum albumin (BSA; Fraction V, Sigma, St. Louis, MO) in PBS. After a further 2 hours incubation at room temperature, the plates were again washed 3 times with PBS-Tween, and developed with freshly prepared Sigma 104 phosphatase substrate (*p*-nitrophenyl phosphate disodium; Sigma, St. Louis, MO), 1 mg/ml in diethanolamine buffer, pH 9.8 (97 ml diethanolamine, 100 mg $MgCl_2.6H_2O$, 0.2 gm NaN_3, ddH_2O added to bring volume to 1L). The plates were left at room temperature for 20-30 minutes, then phosphatase activity was determined as absorbance at 405 nm using a Dynatech Microelisa autoreader (Dynatech, Alexandria, VA). A hybridoma supernatant was considered to be 'positive' if phosphatase activity exceeded the mean background level of wells with control culture supernatants by greater than 2 standard deviations.

2.1.7 Fixed Cells ELISA

The method used was adapted from Suter, et al. (1980). Flexible polyvinyl chloride Microtiter ELISA plates were coated with 100 µl/well freshly prepared poly-L-lysine (50 µg/ml in PBS), and allowed to stand at room temperature for

40 min. The outer rows and columns were not used because they tended to give false positive absorbance values for this particular ELISA assay. The poly-L-lysine was then removed, and the plates washed once with PBS. Freshly harvested human glioma cells, which had grown to confluence in αMEM in a 175 cm^2 tissue culture flask, were removed in PBS with a rubber policeman and seeded into microwells at 10^5 cells per well in 100 µl PBS. The plates were allowed to incubate for 45 minutes at room temperature, then gently rinsed 3 times with PBS.

The cells were fixed by the addition of 100 µl/well of 0.1% glutaraldehyde in PBS for 3 minutes, then gently rinsed 3 times with PBS. To block any residual gluteraldehyde activity, 100 µl/well of PBS containing 0.2% gelatin 275 Bloom (Fisher, Montreal, QC) with 0.2% NaN_3 (PBS-gelatin) was added, and the plates allowed to incubate at 4 °C for at least 12 hours before proceeding further with the assay. In this condition, the plates could be stored at 4 °C for up to 6 months.

On the day of the assay, the PBS-gelatin was removed by inversion of the plate, and 100 µl of hybridoma supernatant was added directly to each microwell without prior washing. Control supernatants for this assay were the same as for the autologous tumor extract assay described in section 2.1.6. The plates were incubated for 30 minutes at 37 °C, then cooled for 15 minutes at room

temperature. They were washed 5 times with freshly prepared PBS containing 0.05% (v/v) Tween-20® and 0.1% (v/v) gelatin (PBS-Tween-gelatin), and the microwells filled with 100 µl of ALP-conjugated goat anti-human κ and goat anti-human γ diluted 1:1,000 each in PBS-Tween-gelatin.

After 30 minutes incubation at 37 °C and 15 minutes cooling at room temperature, the plates were washed 3 times with PBS-Tween-gelatin. Phosphatase activity was developed with the addition of 200 µl/well Sigma 104 phosphatase substrate solution (1 mg/ml) freshly made in diethanolamine buffer, and assessed as absorbance at 405 nm using an automated Dynatek Microelisa reader. Hybridoma microwells with phosphatase activity which exceeded mean background levels of the control supernatant wells by greater than 3 standard deviations were considered 'positive' in this assay.

2.1.8 ELISA Reactivity and Immunoglobulin Concentration

In order to test whether reactivity in the screening ELISA was merely a function of Ig concentration in the hybridoma supernatants, the following simple experiment was carried out: Polyvinyl chloride Microtiter ELISA plates were coated with goat anti-human polyvalent Ig (IgG + IgA + IgM) antiserum (Cappel Laboratories, Cochranville, PA) at a

concentration of 1 µg/ml in carbonate-bicarbonate buffer, and incubated overnight at 4 °C. Hybridoma supernatants from 31 microwells, which were determined to be 'positive' in the BT-20 tumor extract ELISA, were added in the usual manner. Alkaline phosphatase-conjugated goat anti-human κ and goat anti-human γ light chain antibodies were added as in the screening assay, and the plates developed with Sigma 104 phosphatase substrate. Absorbance at 405 nm for individual microwells in this assay was then assigned a *Z* score (Johnson, 1976) based on the calculated mean and standard deviation of a sample of TM-H2 control serum (containing 10 replicates), and the results plotted in a simple scattergram. The *Z* score reflects the probability that a given data point differs significantly from the mean. In general, *Z* scores >1.96 or <-1.96 are considered significant.

2.1.9 Flow Cytometry

Confluent phase cultures of the cell lines were gently detached from tissues culture flasks with a rubber policeman and collected in ice cold PBS containing 1% FCS (In general, all cell preparations were scraped. In some experiments, however, the cells were recovered by trypsinization, which did not result in any differences in labelling). Approximately 1 x 10^6 cells per sample were aliquoted into 10 ml test tubes, and pelleted by

centrifugation for 5 min at 500 G. The PBS-1% FCS was poured off and 300 µl of spent hybridoma supernatant was added to each sample. After resuspending the pellet, the cells were placed on ice for 60 min. Following 3 washes with 5 ml cold PBS-1% FCS, 100 µl of FITC-conjugated goat anti-human IgM (IgG fraction, µ chain specific; Cappel Laboratories, Cochranville, PA), diluted 1:10 in sterile PBS, was added to each sample. The cells were then placed on ice for 60 min, and washed 3 times with PBS-1% FCS as before. After the final wash, the cell pellet was resuspended in 500 µl of PBS-1% FCS, and transported to the flow cytometer on ice.

The apparatus used in these experiments was a FACS-III fluorescence-activated cell sorter (Beckton-Dickinson FACS Systems, Mountain View, CA) linked to a custom-built microprocessor-based system capable of performing realtime correlated acquisition, storage, and display of multiparameter (3-parameter) data (Stewart and Price, 1986). Prior to each experiment, the FACS-III was calibrated and standardized for small angle light scatter and fluorescence intensity with polystyrene latex microspheres and "Fluoresbrite" carboxylated microspheres (Polysciences Inc., Warrington PA). Fluorescence intensity between 530 and 560 nm was measured on all samples using an excitation wavelength of 488 nm. Logarithmic small angle scatter, fluorescence, and 90° scatter data were collected

on a minimum of 2 x 10^4 gated cells from each sample, and the information stored on flexible diskettes. Fluorescein-labelled preparations are excited at 488 nm, and fluorescence is measured between 530 nm and 560 nm using a suitable combination of optical filters. Rhodamine-labelled preparations were excited at 514 nm, and fluorescence was measured above 620 nm.

2.1.10 Freezing and Thawing Procedures

All cells (gliomas, hybridomas, etc.) were frozen in essentially the same manner. Approximately 10^7-10^8 cells were pelleted by centrifugation at 500 G for 5 minutes, and the supernatants were discarded. The cells were re-suspended in 1 ml of the usual tissue culture media, and pipetted into a 38 x 12 mm cryotube (Nunclon, Denmark). Sterile DMSO (0.1 ml) was added to the cryotube, and the cells placed directly into the -70 °C freezer. After 24 hours, the cells could be removed to liquid nitrogen for long term storage.

Cells were thawed by removing a cryotube from the -70 °C freezer and placing it directly in a 50 °C water bath for 70 seconds. Before the ice pellet was fully dissolved, the contents of the cryotube were emptied into a screw-cap conical tube (Sarstedt, W. Germany) containing 50 ml of tissue culture medium, and pelleted by

centrifugation at 500 G for 5 minutes. The supernatant was then discarded, and the cells re-suspended in fresh media and plated or placed in suspension culture according to the cells' requirements.

2.1.11 Karyotyping of Hybridomas

Metaphase chromosome spreads were prepared by first culturing ≈1 x 10^6 hybridoma cells overnight in 3 ml of HT medium containing 10% FCS and 0 06 µg/ml colcemid (Fisher). The next day, the cells were washed in PBS and pelleted before resuspension in 3 ml 0.07 *M* KCl for 20 min at room temperature. Then 10 ml of methanol:acetic acid (3:1) was added, and the cells were pelleted at 1700 rpm. The cells were washed twice again with methanol:acetic acid before re-suspension in 1 ml of methanol:acetic acid. The mixture was dropped from a height of ≈2 m onto pre-warmed glass slides in order to burst the cells and spread their chromosomes. The cells were stained for 3 min with Giemsa (Fisher, Montreal, QC), followed by mounting with Permount (Fisher, Montreal, QC). Fifteen to 20 metaphase spreads were counted to establish the chromosome number distribution.

2.1.12 Subcloning

Hybridomas were subcloned by the limiting dilution method using 96-well flat bottom tissue culture plates

(Linbro Laboratories Inc., McLean, VA; 0.28 cm^2/well). After performing viable cell counts on logarithmic phase cultures by the dye exclusion (0.16% Trypan blue) method (Gorer and O'Gorman, 1956), the appropriate dilutions were made to ensure a concentration of 1, 10, 100, and 1000 cells per microwell. The hybridomas were seeded in 200 µl of HT medium containing 10% FCS and grown under standard conditions. They were exchange-fed a volume of 100 µl fresh media every 5 days until ready for testing.

Once growth began to appear, the supernatants were tested for immunoglobulin production and reactivity with tumor extract using the ELISA procedures outlines in section 2.1.6. Colonies arising from plates in which the proportion of microwells with cell growth less than 30% and which seemed to arise from a single focal source were deemed as derived from a single cell, i.e. a clone (Coller and Coller, 1983).

2.1.13 Southern Blotting

Genomic DNA was isolated from TM-H2-SP2, BT27/1A2, BT27/2A3, BT32/A6.5 (a subclone of BT32/A6), and BT27/2D2 cells according to Maniatis, et al. (1982). Ten µg of DNA were digested overnight at 37 °C with *Bam* HI and *Hind* III restriction enzymes (≥3 units/µg DNA; Boehinger-Mannheim, West Germany) according to the manufacturer's specified

conditions. After digestion, the DNA was subjected to electrophoresis on a 0.8% (w/v) agarose gel (Maniatis, et al. 1982), and transferred to a Genescreen Plus membrane (NEN, Boston, MA) by the usual method. Following prehybridization with herring sperm DNA (Boehinger-Mannheim, West Germany), the Southern blot was hybridized (Kaufmann, et al. 1985) overnight at 65 °C with a ^{32}P-labelled J_H probe (Oncor Inc., Gaithersburg, MD), specific activity of 5-6 x 10^8/µg. The probe includes the entire human J_H region, and totals 5.6 kbp (Ravetch, et al. 1981). Following washing, the blot was exposed to Kodak X-ray film with intensifying screens at -70 °C.

2.2 Characterization of Human Monoclonal Antibodies to Glioma Cell Surface Antigens

2.2.1 Determination of Immunoglobulin Isotype and Concentration

Immunoglobulin chain class was determined by coating four sets of 96-well microtiter ELISA plates with goat anti-human Ig (IgA + IgG + IgM) (Cappel Laboratories, Cochranville, PA) at 1:4500 dilution, as outlined in section 2.1.6. Four replicates of the supernatants to be tested were added to each of the plates, and the latter incubated at room temperature for the usual 2 hrs. Spent culture media from the parental line, TM-H2, served as a source of κ and γ chains, and supernatant from the human

B-lymphocyte line, RPMI 1788 (Huang and Moore, 1969), as a source for λ and μ chains. One plate was then labelled with ALP-conjugated goat anti-human κ chains, another with ALP-conjugated anti-human λ chains, the third with ALP-conjugated anti-human γ chains, and the last plate with ALP-conjugated anti-human μ chains. [All ALP-conjugated antisera (Tago Inc., Burlingame, CA) were used at a dilution of 1:2000 in PBS and 1% BSA.]. The ELISA plates were developed with Sigma 104 phosphatase substrate in the usual manner, and O.D. measurements performed at 450 nm.

Measurement of human IgM concentrations in spent hybridoma culture media from the 3 hybridomas was performed by quantitative ELISA some 10 months post-fusion for BT27/1A2 and BT27/2A3, and 9 months after the fusion date for BT32/A6. From the time of fusion to the date the ELISA was performed, the hybridomas were grown continuously *in vitro* for a total of 4-6 months.

On the day the ELISA was performed, the hybridomas were growing in logarithmic phase at a concentration of $\approx 5 \times 10^5$ cells/ml, in culture media which had been completely replenished 5 days previously. Concentrations of Ig were determined by quantitative ELISA.

For each hybridoma supernatant to be tested, one 96-well ELISA plate was coated with goat anti-human Ig (IgA + IgG + IgM) (Cappel Laboratories, Cochranville, PA) at 1:4500 dilution, as outlined in section 2.1.6. Affinity-

purified human IgM (Cappel Laboratories, Cochranville, PA) at a concentration of 5.5 mg/ml was diluted in PBS to a final concentration of 500 µg/ml. From the latter solution, dilutions in PBS were prepared with concentrations of 50 µg/ml, 5 µg/ml, 0.5 µg/ml, and 0.05 µg/ml.

The IgM standards were then applied to the ELISA plates, along with the supernatants to be tested. ALP-conjugated goat anti-human IgM (µ chain specific; Tago, Inc., Burlingame, CA) was added as a second antibody, and the plates developed with Sigma 104 phosphatase substrate in the usual manner. A standard curve of IgM concentration versus O.D. was plotted using a 4-cycle, semi-logarithmic scale, and the mean O.D. values for the hybridoma supernatants converted to IgM concentration.

2.2.2 Antibody Dilution Curves

Spent culture supernatants from each of the hybridomas were diluted in non-specific control IgM (BT27/2D2) and reacted in an ELISA assay with the 3 *M* KCl extract of BT-27 (high grade astrocytoma), as outlined in section 2.1.6. Undiluted control supernatant from BT27/2D2 was also included in each ELISA plate for purposes of comparison.

For each ELISA plate, the mean background (BT27/2D2) O.D. value was subtracted from the mean O.D. value for each dilution, and the difference (Δ O.D.) plotted as a function

of dilution. Mean O.D. values which were greater than background were compared with the latter using Student's *t*-test for unpaired data.

2.2.3 Antibody Affinity ELISA

The method used is a modification of De Bernardo and Davies (1987). Briefly, a 96-well ELISA plate was coated with the 3 *M* KCl extract of BT-27 (high grade glioma), as outlined in section 2.1.6. The plate was washed 3 times with PBS containing 0.05% Tween-20® (PBS-Tween), and the wells filled with 50 µl of PBS. Fifty µl of spent hybridoma culture supernatant from each of the 5 HmAbs (BT27/1A2, BT27/2A3, BT32/A6, BT34/A5, BT54/B8), as well as control IgM (BT27/2D2) were added to half of the wells. Fifty µl of PBS was added to the remaining wells.

After 14 hrs incubation at 4 °C, 50 µl of fluid was withdrawn from the PBS-containing wells, and hybridoma supernatants added back. The ELISA plate was then incubated for a further 4 hrs at 4 °C, after which it was emptied, and washed 3 times with PBS-Tween. ALP-conjugated goat anti-human IgM (µ chain specific; Tago, Inc., Burlingame, CA) diluted 1:2000 in 1% BSA-PBS was then added to the wells, and the incubation continued for a further 2 hrs at room temperature. The plate was developed with Sigma 104 phosphatase substrate in the usual manner, and

mean O.D. values for each supernatant and incubation time were calculated.

Statistical comparisons between different supernatants incubated for the same length of time were carried out using Student's *t*-test, or the Mann-Whitney *U*-test [where two variances differed significantly according to the *F*-test ($p<0.05$)]. Comparisons of the same supernatant incubated for different lengths of time were also made. The results were plotted on a bar graph as mean differences ± standard errors.

2.2.4 Antigen Expression and Glioma Culture Density

The human glioma cell line, SK-MG-1, was grown to confluence in T25 flasks (Nunc, Denmark) under standard conditions, and removed with trypsin as outlined in section 2.1.3. The cells were diluted in αMEM containing 10% FCS, and re-seeded into T25 flasks at variable split ratios ranging from 1:2 to 1:8. After 72 hrs in culture without any exchanges of media, the old culture media was poured off, and ice cold PBS containing 1% FCS was added to the flasks. The cells were then gently scraped off with the aid of a rubber policeman, and labelled with either BT27/2A3, or spent culture supernatant from the parental myeloma line, TM-H2, as outlined in section 2.1.9.

Rhodamine isothiocyanate (RITC)-conjugated goat anti-human IgM [$F(ab')_2$ fraction, μ chain specific; Cappel

Laboratories, Cochranville, PA] was used as a second antibody for the BT27/2A3-labelled cells, and RITC-conjugated goat anti-human IgG [$F(ab')_2$ fraction, γ chain specific; Cappel Laboratories, Cochranville, PA) was used for the TM-H2-labelled cells. Flow cytometric analysis was then performed as described in section 2.1.9.

2.2.5 Primary Tumor Explant Cultures

Sterile surgical biopsy specimens of primary neuroectodermal tumors were collected in ice cold αMEM containing 20% FCS. Approximately 0.5-1 gm of specimen was finely minced with sterile scissors, and 5-10 pieces measuring $\approx$1 mm^3 were placed in T25 flasks containing 10 ml of culture media. After 7 days of incubation under standard conditions, the media was replenished, and the cultures returned to the incubator. In 2-3 weeks, the explants formed confluent monolayers which could be passaged and analyzed by FCM.

Four such explants were labelled with BT27/2A3 and RITC-conjugated goat anti-human IgM (μ chain specific; Cappel Laboratories, Cochranville, PA). The percentage of reactive cells was estimated by overlapping the BT27/2A3 and control (BT27/2D2) fluorescence distributions (plotted to peak height), and determining the channel number where the two distributions crossed. The percentage of cells lying to the right of this point for the control

distribution was then subtracted from the BT27/2A3 distribution, to give a minimal estimate of the number of cells detected as significantly labelled by the HmAb.

2.2.6 Immunofluorescence Microscopy

Human cell lines which had been labelled for FCM (see section 2.1.9) were also used to prepare cytocentrifuge preparations for examination under the fluorescence microscope. The FITC-labelled cells were spun down at 500 G for 5 min onto glass slides, air dried, mounted in PBS containing 10% (v/v) glycerol, and examined with a Leitz fluorescence microscope apparatus. Photography was performed with Fujichrome film (400 ASA).

2.2.7 Immunohistochemistry

Freshly trypsinized glioma cells were seeded into Lab-Tek tissue culture chamber/slides (Miles Scientific, Napierville, IL) in 1 ml of αMEM containing 10% FCS, and grown under standard conditions until confluent (≈72 hrs), without exchanging the media. The chamber/slides were then placed directly on ice for 20 min, after which the culture media was poured off, and the cells incubated with ice cold PBS containing 0.1 % (w/v) sodium azide for an additional 30 min. All subsequent solutions and incubations were carried out at 4 °C, unless stated otherwise.

After a quick rinse with PBS, spent hybridoma culture supernatants were applied directly to the chamber slides, and incubated for 30 min. The slides were then washed with PBS, and incubated with PBS containing 0.1% (w/v) gelatin for 20 min. Following 2 quick rinses with PBS, the cells were fixed with ice cold methanol for 15 min. This was followed by 2 more washes with PBS, an incubation with PBS containing 0.1% gelatin for 20 min, and 3 washes with PBS.

Peroxidase-conjugated goat anti-human IgM (μ chain specific; Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:40 in PBS was then added for a 30 min incubation, followed by a quick wash with PBS. The cells were then incubated with PBS containing 1% (v/v) Normal Goat Serum (Gibco, Grand Island, NY) and 0.1% (w/v) NaN_3 for 20 min, and rinsed twice with PBS at room temperature.

AEC (3-amino-9-ethylcarbazol) stock solution was prepared by dissolving 20 mg of AEC (Aldrich Cemical Co., Milwaukee, WI) in 5 ml of N,N-dimethyl formamide (Fisher, Montreal, QC). A working solution was prepared by adding 100 μl of stock solution to 2 ml of 0.1 *M* acetate buffer, pH 5.1, and 100 μl of 0.3% (v/v) H_2O_2.

The cells were developed with the AEC working solution for 40 min, and counterstained with Mayer's hematoxylin (Fisher, Montreal, QC) for 30 min, both at room temperature. After washing with lukewarm tap water, they

were mounted in PBS containing 30% (v/v) glycerol, 15% (v/v) dH_2O, and 0.05% (w/v) NaN_3.

2.2.8 Reactivity with Allogeneic Tumors Extracts

Reactivity with 3 *M* KCl tumor extracts from 12 different patients, all with primary neuroectodermal tumors, was analyzed by two different methods. Each ELISA plate was first coated with 3 *M* KCl tumor extract, then divided into 4 sectors (15 wells each) and exposed to culture supernatants of BT27/1A2, BT27/2A3, BT32/A6, and BT27/2D2 (non-specific IgM control) as outlined in section 2.1.6. For each plate, mean O.D. values of each of the 3 HmAbs were compared to background (BT27/2D2) using Student's *t*-test, and a *t*-statistic was calculated.

In the first method, *t*-statistics for each HmAb were ranked with respect to tumor extract, and compared using Spearman's rank correlation coefficient. The second method involved calculating the increase in O.D. above background for each HmAb and tumor extract, plotting the results as a series of peaks and valleys, and superimposing the distributions. Using this method, each HmAb had a unique 'signature' which could be compared visually with the other 2 HmAbs.

2.2.9 Western Blotting

Cell membrane vesicles were prepared as follows: For each cell line to be studied, 1 medium sized (850 cm^2) roller bottle (Fisher, Montreal, QC) containing approximately 1 x 10^8 cells and grown to confluence was harvested in PBS-citrate (50 ml) and then washed 3 times with ice cold PBS containing 5 m*M* EDTA (PBS-EDTA). After re-suspending the cells in 8 ml of PBS-EDTA, 40 µl of 200 m*M* PMSF was added to a final concentration of 1 µ*M*, and the mixture placed in the nitrogen cavitation apparatus at 400 atmospheres for 20 min. at 4 °C. The cell lysate was then subjected to the following centrifugations: 1) 800 G for 10 min at room temperature (Table top centrifuge), 2) 12,000 G for 15 min at 4 °C (Sorval High Speed Centrifuge), and 3) 100,000 G for 1 hr at 4 °C (Beckman L8-70 Ultracentrifuge). The resulting proteinaceous pellet was then dissolved in 300 µl of sample buffer (10 m*M* Tris-HCl, containing 2% SDS, 10% glycerol, and 0.02% bromphenol blue, pH 6.8), and stored at -20 °C in a tightly-sealed cryotube (Nunclon, Denmark).

Electrophoresis and electroblotting were performed according to Laemmli (1970) and Towbin, et al. (1979). Using a 12 cm long, water-cooled gel apparatus (Bio-Rad, Richmond, CA), a 7.5% (w/v) polyacrylamide resolving gel (pH 8.8) was first poured (9 cm), followed by a 3% stacking gel (pH 6.8; 3 cm). The electrode buffer used contained

25 m*M* Tris-HCl, 0.192 *M* glycine, and 0.1% SDS, pH 8.3. Ten microliters of sample with 20 µl of sample buffer were individually heated to 95 °C for 5 min (Canlab block heater, Montreal, QC), and quickly added to each lane. Ten microliters of prestained protein molecular weight standards (BRL, Gaithersburg, MD) in the range of 14.3-200 kDa were mixed with 20 µl of sample buffer without SDS, heated to 95 °C, and added to the gel.

Electrophoresis was carried out at 150 V for a duration of 3-4 hrs. A portion of the gel containing replicates of the samples to be blotted was set aside and stained with Coomassie Blue (41% methanol, 7% glacial acetic acid, 0.0125% Coomassie Blue) to reveal the individual protein bands in each lane. Transfer of the remaining gel onto nitrocellulose paper (Bio-Rad, Richmond, CA) was carried out overnight at 30 V, in buffer containing 20% methanol, 25 m*M* Tris-HCl, 0.192 *M* glycine, and 0.01% SDS using a Bio-Rad apparatus.

Following transfer, the blots were blocked for 1 hr at room temperature with PBS containing 10% Donor Horse Serum (Flow, Rockville, MD), then washed 5 times with PBS containing 0.1% Tween-20® (PBST), and reacted with hybridoma supernatant for 2 hrs at room temperature. After 5 more washes in PBST, the blots were reacted for 1 hr at room temperature with Miles-Yeda (Rehovot, Israel) peroxidase-conjugated goat anti-human IgM, diluted 1: 7,500

in PBS containing 1% BSA. The blots were then washed 3 times in PBST, and once in 0.05 *M* Tris-HCl, pH 7.5. The peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) dissolved in 0.05 *M* Tris-HCl (pH 7.5) at a final concentration of 0.5 mg/ml, in the presence of 0.05% H_2O_2. After rinsing with ddH_2O, the blots were dried between 2 sheets of filter paper and photographed promptly.

2.2.10 Lipid Dot Blots and Immunochromatography

Total lipid extracts of cell lines to be analyzed were prepared according to Svennerholm and Fredman (1980). Briefly, approximately 5 x 10^8 cells were grown to confluence in standard 850 cm^2 tissue culture roller bottles, scraped off in PBS, and homogenized in 3 ml of ddH_2O at 4 °C. The homogenate was then combined with 10.8 ml of methanol, and 5.4 ml of chloroform, which was added dropwise with constant stirring. After mixing for 30 minutes at room temperature, the remaining unlysed cells and debris were spun down at 10,000 RPM for 10 min. The remaining supernatant was placed in a 37 °C water bath and evaporated to dryness under a constant stream of N_2. The residue was dissolved in 200 µl of chloroform:methanol (2:1), clarified by centrifugation in an Eppendorf microcentrifuge for 10 min, and stored at -20 °C in a tightly capped freezer vial (Nunclon, Denmark).

Dot blots were prepared by spotting 2 µl of each cell line to be tested onto Merck HPTLC aluminum backed silica gel 60 plates (Applied Analytical Industries, Wilmington, NC). The plates were then dipped in a solution of 0.05% polyisobutyl-methylmethacrylate (Polyscience, Inc., Warrington, PA) in hexane for 30-60 seconds, and air dried for 15 min. After blocking with 1% BSA in Tris-buffered saline (20 m*M* Tris-HCl, 0.5 *M* Nacl, pH 7.5; TBS) for 2 hrs. at 4 °C, the blots were exposed to hybridoma supernatants for an additional 2 hrs. at 4 °C, then washed with TBS 6 times. Peroxidase conjugated goat anti-human IgM (µ chain specific; Kirkegaard and Perry, Gaithersburg, MD) diluted 1:400 in 1% BSA-TBS was then added for a further 2 hrs. at 4 °C, and the plates washed once again in TBS. The peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) dissolved in 0.05 *M* Tris-HCl (pH 7.5) at a final concentration of 1 mg/ml, in the presence of 0.05% H_2O_2. After rinsing with ddH_2O, the plates were dried with a hair dryer and photographed promptly.

For thin layer immunochromatograms, 4 µl of lipid extract was spotted onto each lane of the HPTLC plates, and dried under vacuum for at least 4 hrs. The plates were then chromatographed in chloroform:methanol:water (60:35:8) and dried under vacuum overnight, at least 16 hrs. The plates were then coated with polyisobutylmethylmethacrylate

as described above. The plates were blocked with 5% BSA in Tris-buffered saline at 4 °C for 2 hrs. The chromatograms were then exposed to hybridoma supernatants for an additional 2 hrs. at 4 °C and washed as described above. Peroxidase conjugated goat anti-human IgG (H+L chain specific)(BIO/CAN, Toronto, ON) diluted 1:2000 or peroxidase conjugated goat anti-human IgM (Fc5 specific)(BIO/CAN, Toronto, ON) diluted 1:2000 in 1% BSA-TBS was added for a further 2 hrs. at 4 °C. After washing again, the plates were developed as described for the lipid dot blots.

2.2.11 Labelling of Normal Human Astrocytes

Cultured normal human astrocytes were obtained as a gift from the laboratory of Dr. Jack Antel, Montreal Neurological Institute, Montreal, QC. The cells were derived from patients undergoing craniotomy for epilepsy, and were grown on glass coverslips. They were labelled on the coverslips using the same general method (reagents and time) as outlined in section 2.1.9, with the exception that all washes were carried out on coverslips using PBS containing 1% FCS and 0.02% (w/v) NaN_3. The coverslips were wet-mounted using PBS with FCS and azide, and viewed with a Leitz fluorescence microscope.

2.3 Biological Significance of a Glioma Cell Subpopulation Identified by Human Monoclonal Antibodies

2.3.1 Generation of Random Clones from SK-MG-1

Random clones were generated by first dissociating an established culture of SK-MG-1 with trypsin, resuspending the cells in αMEM containing 10% FCS, and plating out the cells into flat-bottom 96-microwell plates (Falcon) at a concentration of 1 viable cell/well, estimated by the dye exclusion method (Gorer and O'Gorman, 1956). As colonies appeared (1-2 weeks), the plates were scanned, and only those microwells containing single colonies were selected for further study.

Once the bottoms of the microwells were completely covered, the cells were removed with trypsin and gentle scraping, and passaged into 24-well plates (Linbro; 2.01 cm^2/well) in a volume of 1 ml of αMEM + 10% FCS. After a further passage in T25 flasks (Falcon), 10 clones were chosen for further study, and assigned numbers (SK-MG-1.1 to SK-MG-1.10). The clones were then tested for reactivity with BT27/2A3 by FCM as outlined in section 2.1.9.

2.3.2 Fetal Calf Serum Concentration and Subpopulation Size

The effect of varying concentrations of FCS on the growth and proportion of small (S) and large (L) cell

populations in SK-MG-1 was assessed. Confluent cells from a single T175 flask (Nunc, Denmark) were removed with trypsin in the usual manner, and washed twice in HB104 medium (Gibco, Grand Island, NY), a serum free complete tissue culture medium. After resuspending in 8 ml of HB104, the cells were counted in a hemacytometer and divided into eight T80 flasks (Nunc, Denmark) containing $\approx 8 \times 10^5$ cells each. Duplicate flasks were then incubated for 4 days at 37 °C in 5% CO_2 in αMEM containing FCS at the following concentrations: 1%, 2%, 5%, and 10%.

On day 4, the cells were scraped off with a rubber policeman, and the total number of viable cells per flask estimated by the dye exclusion method (see section 2.1.1). Each flask was then divided into 2 aliquots, and the duplicate samples labelled with antibody BT27/1A2 and control antibody BT27/2D2 as outlined in section 2.1.9.

The cells were then transported to the FACS-III apparatus on ice. Using the usual parameters, reactivity with BT27/1A2 was confirmed for each duplicate sample. The BT27/1A2 samples were then run in triplicate (5×10^4 cells/run), and the total number of viable S- and L-cells counted by setting the appropriate small angle scatter and fluorescence gates. Six replicates were thus obtained for the number of S- and L-cells at each of the 4 different FCS concentrations. The percentage of S- cells was calculated as follows: % S-cells = # S-cells / (# S-cells + # L-

cells). The total number of S-cells at a given FCS concentration was then estimated by multiplying the total number of viable cells times the percentage of small cells.

2.3.3 Cell Sorting

An established culture of SK-MG-1 (98th passage) was scraped with a sterile rubber policeman, resuspended in 1 ml of αMEM + 10% FCS, and transported to the FCM on ice. After the usual calibration of the FCM, simultaneous fluorescence and small angle scatter data were collected on 5 x 10^4 cells in order to establish 2-dimensional gates for the purpose of sorting. Each area was chosen so as to correspond to either S- or L-cells. A total of 2 x 10^4 cells from each area were sorted and collected into 1 ml of αMEM + 10% FCS, and placed on ice.

Each sample of sorted cells was then diluted with fresh αMEM + 10% FCS to a volume of 40 ml, and distributed into two 96-microwell plates, approximately 200 µl per well. The cells were fed every 5 days by exchanging half the old supernatant for fresh media. On the 10th day following the sort, the total number of colonies containing >25 cells were counted in each of the 4 plates. An individual microwell might contain anywhere from one to six individually distinguishable colonies.

Ten S-cell-derived, and 10 L-cell-derived clones from wells with a single colony were then selected and passaged

further as outlined in section 2.3.1. The S-cell clones were assigned the numbers S101-S110, and the L-cell clones L101-L110. The clones were later tested for reactivity by FCM as described in section 2.1.9.

2.3.4 Cell Cycle Analysis

Confluent cultures of the human glioma cell line, SK-MG-1, were detached from tissues culture flasks with a rubber policeman and fixed in ice cold 70% (v/v) ethanol for 30-60 min. Approximately 1 x 10^6 cells per sample were aliquoted into 10 ml test tubes, pelleted by centrifugation for 5 min at 500 G, and washed in PBS. Twenty µl of RNase (Boehringer Mannheim, West Germany; 1 mg/ml in 10 m*M* Tris-HCl, 5 m*M* NaCl, pH 7.5) was added to each sample to digest RNA, along with 1 ml of propidium iodide (50 µg/ml in 10 m*M* Tris-HCl, 5 m*M* $MgCl_2$, pH 7.4). After incubating for 60 min at 37 °C, the cells were placed on ice for an additional 30 min, then transported over to the FACS-III apparatus on ice.

After calibrating for linear small angle scatter, red fluorescence, and DNA content using the human pseudodiploid cell line, HL-60, data was collected on a minimum of 2 x 10^4 gated cells, and stored on flexible disks for subsequent analysis.

2.3.5 Cell Morphology

After labelling SK-MG-1 for FCM with BT27/2A3 and BT27/2D2 in the usual manner (see section 2.1.9), small angle scatter vs. fluorescence data were collected on 5 x 10^4 cells in order to establish 2-dimensional windows for purposes of sorting. The windows were chosen in order to define the morphology of 1) S-cells which increase in fluorescence after labelling with BT27/2A3; 2) S-cells which remained unchanged in terms of fluorescence after labelling with BT27/2A3; and 3) L-cells.

Approximately 1 x 10^4 cells were sorted from each window, and collected in ice cold αMEM + 10% FCS. Samples from each sorted area, as well as total unsorted cells, were then centrifuged onto glass slides, air dried, and stained with Wright's stain (Fisher, Montreal, QC). The cytocentrifuge preparations were then viewed through a Leitz microscope, and photographed with Fujicolor HR film (ASA 400).

2.3.6 Karyotyping of SK-MG-1 Sublines

Metaphase chromosome spreads were prepared in a manner similar to that outlined in section 2.1.11. For each cell line to be studied, one confluent T25 flask (Nunc, Denmark) was incubated overnight in 3 ml of αMEM + 10% FCS and 0.06 μg/ml colcemid (Gibco, Grand Island, NY). The remainder of

the procedure was followed exactly as outlined in section 2.1.11.

2.3.7 Use of TM-H2 (IgG) versus BT27/2D2 (IgM) as Control Antibody

In some experiments TM-H2 (IgG) antibody was used as a negative control instead of BT27/2D2 (IgM). It was a consistent and general observation that the background labelling, whether in ELISA or quantitative fluorescence flow cytometry, were quantitatively equivalent or higher with TM-H2 (IgG).

3 DERIVATION OF HUMAN ANTI-GLIOMA MONOCLONAL ANTIBODIES FROM PATIENTS WITH NEUROLOGICAL TUMORS

3.1 Patient Clinical and Laboratory Profiles

Fusions were performed with PBL obtained from a total of 18 individual patients at 4 separate McGill University teaching hospitals. The clinical and laboratory profiles of each patient are summarized in Tables 3.1 and 3.2, respectively. As each patient was entered into the study, he or she was assigned a number preceded by the letters 'BT' for 'brain tumor'. All clinical material originating from the same individual (e.g. lymphocytes, primary tumor samples) was assigned the same BT number.

Over 60 patients contributed clinical material to this study; however, not all patients were able to provide both lymphocytes and autologous tumor material. Some fusions had to be excluded due to contamination, change of diagnosis after pathological examination, or overgrowth of cultures by adherent cells. For the 18 patients included in this report, every effort was made to obtain both PBL and primary tumor.

Patient ages ranged from 2 to 85 years (mean = 42.0). Eleven were male (61%), and 7 female (39%). Seizures were the most common presenting symptom (35%), followed by headache (20%), speech or cognitive changes (20%), motor

Table 3.1: Patient clinical profiles

Fusion	*Age*	*Sex*	*Initial Symptoms*	*Location*	*Histology*
BT-1	62	F	L facial numbness	L temporal	metastatic ca
BT-2	37	M	seizures x 1 year	R temp-par	oligodendroglioma
BT-3	66	F	dysphasia x 10 mos	L pterional	meningioma
BT-4	66	F	confusion x 1 month	R frontal	glioblastoma
BT-6	46	M	memory loss x 6 mos	L frontal	oligoastro II
BT-11	68	M	confusion x 4 months	L temporal	glioblastoma
BT-15	85	M	seizures x 6 weeks	L par-occ	glioblastoma
BT-17	70	F	left hemiparesis	R frontal	glioblastoma
BT-20	21	M	seizures x 1.5 years	R par-occ	astrocytoma II-III
BT-21	2	F	gait difficulty, 6 mos	post fossa	pilocytic astro
BT-24	5	M	ataxia, seizures	R frontal	PNET
BT-27	23	M	seizures x 1.5 years	R frontal	astrocytoma III
BT-32	14	M	IVH	R lat ventr	fibrillary astro
BT-34	11	F	headache x 8 years	post fossa	fibrillary astro
BT-38	53	M	severe H/A x 3 weeks	R frontal	glioblastoma
BT-39	22	M	seizures x 2 years	R frontal	oligoastrocytoma
BT-54	53	M	single seizure	L frontal	glioblastoma
BT-55	52	F	H/A, ↓ vision x 10 d	R parietal	glioblastoma

Abbreviations used: F-female, M-male, L-left, R-right, mos-months, d-days, temp-temporal, par-parietal, occ-occipital, ca-carcinoma, II-grade II, III-grade III, post-posterior, PNET-primitive neuroectodermal tumor, IVH-intraventricular hemorrhage, lat ventr-lateral ventricle, astro-astrocytoma, H/A-headache, ↓-decreased.

Table 3.2: Patient laboratory profiles

Fusion	*Blood Group*	*Leukocytes*	*Medications*[a]
BT-1	A positive	7.2 x 10^3/mm^3	Synthroid, Empracet-30
BT-2	O positive	18.4 x 10^3/mm^3	Dilantin, Tegretol, Fiorinal
BT-3	O positive	11.9 x 10^3/mm^3	Cimetidine
BT-4	O negative	25.6 x 10^3/mm^3	Dyazide, Slow-K
BT-6	A positive	19.7 x 10^3/mm^3	no medications
BT-11	A positive	4.1 x 10^3/mm^3	Inderal, Isordil, NTG[b]
BT-15	A positive	13.3 x 10^3/mm^3	Dyazide
BT-17	O positive	5.6 x 10^3/mm^3	Aldomet, Dyazide
BT-20	A negative	26.5 x 10^3/mm^3	no medications
BT-21	A positive	6.4 x 10^3/mm^3	no medications
BT-24	O positive	21.9 x 10^3/mm^3	no medications
BT-27	O negative	5.1 x 10^3/mm^3	Dilantin, phenobarbital
BT-32	O positive	8.6 x 10^3/mm^3	no medications
BT-34	B positive	10.7 x 10^3/mm^3	no medications
BT-38	A positive	11.0 x 10^3/mm^3	Aspirin with codeine
BT-39	O positive	10.8 x 10^3/mm^3	Dilantin, codeine
BT-54	A positive	5.8 x 10^3/mm^3	no medications
BT-55	A positive	10.4 x 10^3/mm^3	Aspirin

[a]on admission
[b]nitroglycerin

impairment (15%), and sensory changes (including vision) 10%. Ten tumors (56%) were located on the right side, 6 (33%) on the left, and 2 (11%) in the posterior fossa. Astrocytomas and glioblastomas accounted for 67% of all tumors, with the rest consisting of other neuroectodermal tumors, and one poorly differentiated carcinoma. The latter originated in the maxillary sinus and grew by extension into the middle cranial fossa.

Nine patients (50%) were blood group O, 8 (44%) belonged to group A, 1 (6%) was blood group B, and none were type AB. Average leukocyte count on admission was 12.4 x $10^3/mm^3$, with a range of 4.1-26.5 x $10^3/mm^3$. Seven patients (39%) were not taking any medications at the time of admission, and only 3 (17%) were taking some kind of anticonvulsant drug. None of the patients were receiving corticosteroids at the time of collection of PBL.

3.2 Hybridoma Growth

Two series of fusions were performed. The first (Table 3.3), consisting of BT-1, 2, 3, 4, 6, 11, 15, 17, 20, and 21, failed to yield any hybridomas capable of growth beyond the 96 microwell stage. This situation was corrected in the second series by using fresh tissue culture media and fetal calf serum (FCS), supplemented with glutamine (292 mg/L) and asparagine (44 mg/L). Results

Table 3.3: First fusion series

Fusion	*PBL*[a]	*Plating Density*[b]	*Wells*[c]
BT-1	5.5×10^7	2.5×10^5	290
BT-2	5.0×10^7	2.5×10^5	131
BT-3	3.3×10^7	2.5×10^5	107
BT-4	6.0×10^6	2.5×10^5	26
BT-6	9.8×10^6	2.5×10^5	80
BT-11	9.8×10^6	1.0×10^5	74
BT-15	9.4×10^6	1.0×10^5	72
BT-17	2.4×10^7	1.0×10^5	180
BT-20	4.4×10^7	1.0×10^5	304
BT-21	4.5×10^7	1.0×10^5	285

[a]number of peripheral blood lymphocytes obtained from each patient

[b]number of viable myeloma cells per ml of fusion mixture, seeded at 200 µl/well

[c]number of microwells seeded

from the two series of fusions are therefore presented separately due to differences in tissue culture conditions.

In the second series (BT-24, 27, 32, 34, 38, 39, 54, and 55) (Table 3.4), hybridoma outgrowth ranged from 0 to 19.5%. Outgrowth is defined as the number of microwells containing macroscopic colonies of >50-100 cells (visible to the naked eye), divided by the total number seeded, expressed as a percentage.

The number of PBL in all 18 samples available for fusion varied from 6.0×10^6 to 5.8×10^7. Although the ratio of myeloma cells to lymphocytes remained constant at 1:4 for each fusion, the plating density that was used ranged from 1.0×10^5 to 2.5×10^5 myeloma cells per ml of fusion mixture. The lower plating density was chosen in some fusions in attempts to better encourage initial monoclonality, whereas the higher plating density seemed to favor improved hybridoma outgrowth. The concentration of PEG was 50% (v/v) in all 18 fusions.

Typically, discernable hybridoma growth appeared from 4 to 6 weeks after a fusion, and new growth in microwells often continued to be observed for several weeks thereafter. In general, those colonies of hybridomas which appeared earliest tended to be the most stable. Not all macroscopic colonies visible at the end of the 96 microwell (0.28 cm^2/well) culture period could be successfully propagated to amounts suitable for growth in 24 well dishes

Table 3.4: Second fusion series

Fusion	*PBL*[a]	*Plating Density* [b]	*Wells*[c]	*Growth*[d]
BT-24	7.8×10^6	1.0×10^5	64	1 (1.6%)
BT-27	1.9×10^7	1.0×10^5	136	10 (7.4%)
BT-32	1.7×10^7	1.8×10^5	82	12 (14.6%)
BT-34	2.0×10^7	2.5×10^5	82	16 (19.5%)
BT-38	5.8×10^7	2.5×10^5	185	0
BT-39	1.5×10^7	2.5×10^5	53	9 (17.0%)
BT-54	1.2×10^7	2.5×10^5	43	7 (16.3%)
BT-55	3.2×10^7	2.5×10^5	122	4 (3.3%)

[a]number of peripheral blood lymphocytes obtained from patients

[b]number of viable myeloma cells per ml of fusion mixture seeded at 200 µl/well

[c]number of microwells seeded

[d]macroscopic colonies of >50-100 cells and capable of sustained growth *in vitro;* number in parentheses is percentage of wells with sustained growth

(2.01 cm^2/well). In fusion BT-54, for example, of the 7 colonies which originally grew in 96 microwells, only one was capable of prolonged growth *in vitro*. Instances of hybridoma growth failure occuring later than three months post-fusion were not observed.

Hybridomas were assigned alpha-numeric codes as follows: the first group of numbers and letters indicates the fusion from which the hybridoma was derived (e.g. BT-27). This was followed by a diagonal line (/), then a number, letter, and number. The first number following the slash mark corresponds to the 96 microwell plate where the hybridoma originated, and the final letter-number combination, the individual microwell. For example, BT27/2A3 is a hybridoma derived from fusion BT-27, plate number 2, position A3.

3.3 Hybridoma Screening

Although macroscopic colonies did not appear in any of the fusions from the first series, this did not preclude testing for the presence of human immunoglobulin and reactivity with 3 *M* KCl extracts of autologous tumors, or glutaraldehyde-fixed glioma cell lines.

Of the 1,553 microwells screened in the first fusion series, 246 (15.8%) reacted with either extracts of autologous tumor or glutaraldehyde-fixed human glioma cell

lines in an ELISA assay. Reactivity in this assay is defined as an optical density at 405 nm greater than 2 S.D. above background reactivity for the parental myeloma supernatant (TM-H2). Screening was carried out around 4 weeks post-fusion, following at least 4 exchanges of culture media to ensure that any immunoglobulin secreted by unfused lymphocytes in the original fusion mixture would be diluted at least 16 fold. Table 3.5 lists the ELISA data from this first series, and indicates not only the number of microwells which tested positive in each fusion, but also the number that exceeded the mean background binding to antigen (obtained with TM-H2) by 2, 3, and 4 S.D.

In some of the earlier fusions, ELISA microtiter plates were coated with goat anti-human Ig (IgA + IgG + IgM), and reacted with hybridoma supernatants as described in section 2.1.6 to detect the presence of human immunoglobulin. Using this method, virtually 100% of the microwells were found to contain human immunoglobulin (results not shown). Thus, by 4 weeks post-fusion all microwells contained immunoglobulin-secreting hybridomas; however, only 15.8% reacted with tumor extracts or glioma cell lines.

In the second fusion series (Table 3.6), a total of 1,121 microwells were screened, of which 162 (14.5%) were found to react with tumor extracts, or glioma cell lines. Two fusions (BT-27 and BT-32) were screened for reactivity

Table 3.5: ELISA screening data from first fusion series

					Level of Positivity[e]			
Fusion	*Wells*[a]	*Screening*[b]	*Control*[c]	*2nd Ab*[d]	*2SD*	*3SD*	*>4SD*	*%Total*[f]
BT-1	290	autologous	TM-H2	κ+λ	4	2	31	12.8%
BT-2	135	autologous	TM-H2	κ+λ	5	7	23	26.7%
BT-3	107	autologous	TM-H2	κ+λ	0	0	0	0%
BT-4	26	autologous	TM-H2	κ+λ	0	2	22	92.3%
BT-6	80	autologous	TM-H2	κ+λ	19	7	3	36.%
BT-11	74	LN-340	TM-H2	κ+λ	4	3	1	10.8%
BT-15	72	SKI-1	TM-H2	κ+λ	2	3	10	20.8%
BT-17	180	autologous	TM-H2	κ+λ	1	0	1	1.1%
BT-20	304	autologous	TM-H2	κ+λ	13	9	9	10.2%
BT-21	285	autologous	TM-H2	κ+λ	24	24	17	22.8%

[a]number of wells assayed

[b]antigens used in screening: autologous-3 *M* KCl extract of autologous tumor, LN-340 and SKI-1 -0.1% glutaraldehyde-fixed cells (see section 2.1.7)

[c]culture supernatant fluid of the parental myeloma line, containing κIgG, was used to establish a background level of nonspecific binding of Ig

[d]a mixture of alkaline phosphatase conjugated goat anti-human κ+λ Ig light chain was used as an indicator of antigen-bound Ig

[e]number of microwells with a given S.D. above mean background level

[f]proportion of microwells reacting >2 S.D. above mean background level

Table 3.6: ELISA screening data from second fusion series

					Level of Positivity[e]			
Fusion	*Wells*[a]	*Screening*[b]	*Control*[c]	*2nd Ab*[d]	*2SD*	*3SD*	*≥4SD*	*%Total*[f]
BT-24	64	autologous	TMH2	κ+λ	6	9	39	84.4%
BT-27	136	autologous	TMH2	κ+λ	4	1	0	3.7%
		SK-MG-1	TMH2	κ+λ	2	1	2	3.7%
		LN-340	TMH2	κ+λ	1	0	3	2.9%
BT-32	82	SK-MG-1	TMH2	κ+λ	6	4	12	26.8%
		LN-340	TMH2	κ+λ	10	3	8	25.6%
BT-34	82	autologous	TMH2	κ+λ	17	9	4	36.6%
BT-38	185	autologous	TMH2	κ+λ	2	2	0	2.2%
BT-39	53	SK-MG-1	TMH2	κ+λ	1	0	0	1.9%
BT-54	43	BT-38	BT27/2D2	μ	1	0	3	9.3%
BT-55	122	BT-38	BT27/2D2	μ	3	3	6	9.8%

[a] see Table 3.5

[b] see Table 3.5; SK-MG-1 was fixed in 0.1% glutaraldehyde, BT38 is a 3 *M* KCl allogeneic tumor extract

[c] see Table 3.5; BT27/2D2 supernatant was used as a source of nonspecific μ chains

[d] see Table 3.5; in some experiments alkaline phosphatase conjugated goat anti-human IgM (μ chain specific) was used as second antibody

[e] see Table 3.5

[f] Ibid.

with several glioma cell lines, and instances were found of individual microwells which tested positive in multiple ELISA assays.

In the last two fusions of this series, the ELISA assay was modified to detect only those microwells which contained reactive IgM species. This was accomplished by substituting culture supernatant from hybridoma BT27/2D2 for TM-H2 as a control, and adding alkaline phosphatase conjugated goat anti-human IgM as a second antibody. The reason for this was that analysis of immunoglobulin chains present in 34 hybridomas from 4 fusions (BT-24, 27, 32, and 34) indicated that all 34 contained IgM. Careful study of a few of these hybridomas indicated that the IgM alone was responsible for anti-tumor activity. This was accomplished by using γ-chain and κ-chain specific antisera, and screening for reactivity by FCM (results not shown). Hybridoma BT27/2D2, which was consistently negative in various ELISA tests of immune reactivity, was chosen as a non-specific IgM control (1-4 μg/ml).

3.4 Assessment of Non-Specific Reactivity

Initially, there was some concern that the apparently significant binding to tumor extract by immunoglobulin from hybridoma supernatants could be falsely positive due to increased non-specific binding related to increased immunoglobulin concentrations in test relative to control

supernatants. A comparison of immunoglobulin concentration versus ELISA reactivity was therefore made. Figure 3.1 is a scatter plot representation of the data for 31 individual microwells. The calculated correlation coefficient for this distribution was $r = -0.284$, with 29 degrees of freedom, indicating no statistically significant correlation between Ig concentration and reactivity in the tumor extract ELISA.

3.5 Screening by Flow Cytometry

A total of 9 high immunoglobulin-producing hybridomas, all from the second fusion series, were screened for reactivity with the human glioma line, SK-MG-1, using the FACS-III Flow Cytometer. Five supernatants, designated as BT27/1A2, BT27/2A3, BT32/A6, BT34/A5, and BT54/B8 were found to label this particular glioma cell line. All five contained tumor-reactive IgM species. Thus, from 8 fusions and a total of 59 hybridomas which were macroscopically visible at 6 weeks, only 5 (8.4%) reacted with the cell surface of a glioma cell line, and were capable of sustained growth in culture.

Figure 3.2 is a composite representation of FCM screening data for 3 of the 5 HmAbs. Screen (A) is a 2-dimensional representation of small angle scatter (abscissa) and fluorescence (ordinate) data for control-labelled SK-MG-1. Two cell populations are evident, which

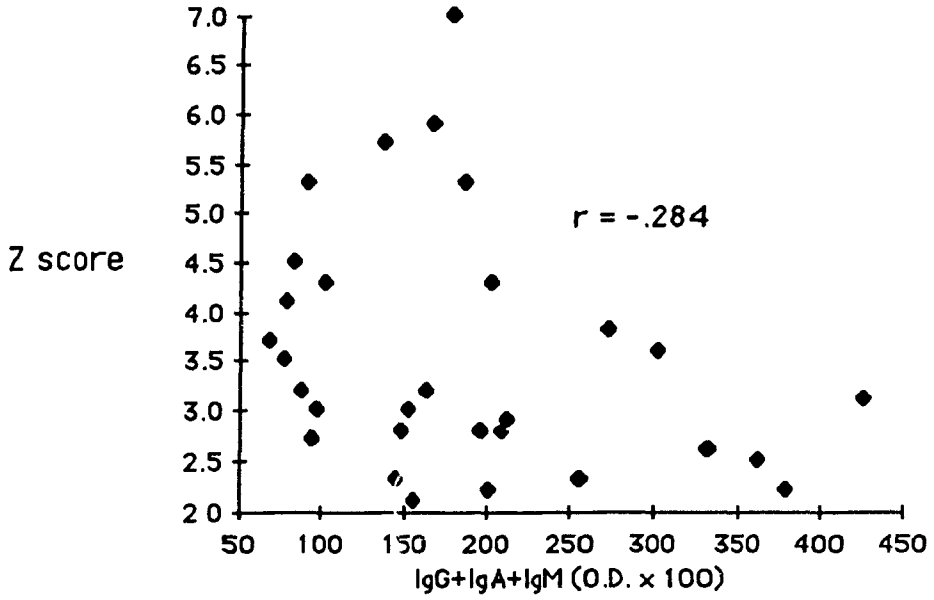


Figure 3.1: ELISA reactivity and Ig concentration

Thirty one hybridoma supernatants which tested positive against 3 *M* KCl autologous (BT-20) tumor extract in an ELISA assay (*Z* scores represented on the ordinate), were screened against microELISA plates coated with goat anti-human Igs (IgG+IgA+IgM) to obtain a relative estimate of total Ig present in each hybridoma supernatant (represented on the abscissa as O.D. at 405 nm). The lack of any significant correlation between Ig concentration and tumor ELISA reactivity suggests that for a few hybridomas, specific interactions were taking place.

Figure 3.2: Flow cytometric analysis of three human monoclonal antibodies

SK-MG-1 cells were labelled with control non-specific IgM, BT27/2D2 (A), and each of 3 HmAbs: BT27/1A2 (B), BT27/2A3 (C), BT32/A6 (D), as outlined in section 2.1.9. The above results are 2-dimensional flow cytometric data, plotted as $\log_2$ small angle scatter on the abscissa, and $\log_2$ green fluorescence on the ordinate. Relative cell number is given by the color legend to the right of the diagram. These results indicate that SK-MG-1 consists of two cell subpopulations, with 'small' cells outlined by a red square, and 'large' cells by a yellow square. Each of the 3 HmAbs appears to label only the 'small' cells.

A
B
C
D
Log2 Fluorescence Intensity
Log2 Small-Angle Light-Scatter
1
4
16
64
256
1K
4K

differ in size and fluorescence intensity. To facilitate comparisons with the antibody labelled cells, a red box has been superimposed on the small cell population, and a yellow box around the larger cells. Figures (B), (C), and (D) represent FCM data for SK-MG-1 labelled with BT27/1A2, BT27/2A3, and BT32/A6, respectively. In each case there is selective labelling of only the small cell population.

Antibodies BT34/A5 and BT54/B8 also exhibited selective labelling of only small cells. Figures 3.3 and 3.4 represent data for human glioma line U-373 labelled with these two HmAbs.

3.6 Hybridoma Karyotypes

Chromosomal content for each of the hybridomas is summarized in Table 3.7. The parental cell line, TM-H2-SP2, was also studied, and found to have a mean and modal number of 46 chromosomes, as previously reported (Sullivan, et al. 1982). All three hybridomas were pseudodiploid, with BT27/1A2 and BT32/A6 having 2-3*n* number of chromosomes, and BT27/2A3 slightly less than 2*n*. The latter observation was confirmed by DNA flow cytometry (results not shown).

3.7 Subcloning

Numerous attempts at subcloning the hybridomas by the limiting dilution method at dilutions of less than 1000

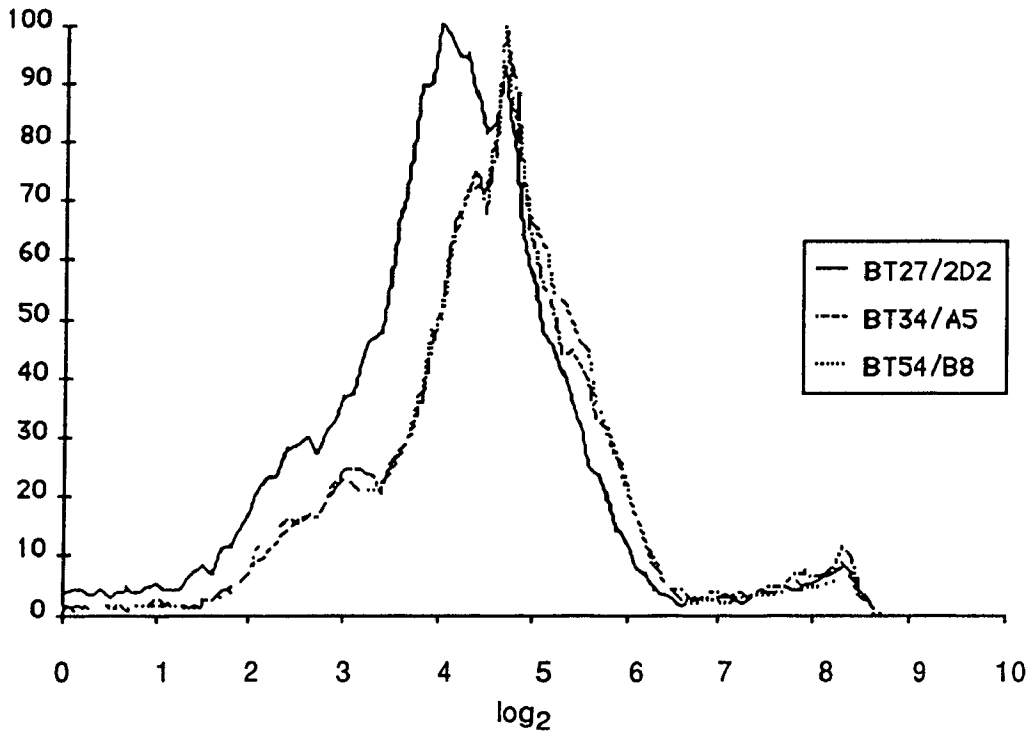


Figure 3.3: FCM screening of HmAbs BT34/A5 and BT54/B8

Cells from the human glioma line U-373 were labelled with either BT34/A5 or BT54/B8, and analyzed in the FACS-III flow cytometer. Supernatant from BT27/2D2 served as a source for control IgM. The resulting histograms are plotted to peak height to facilitate comparison. The abscissa represents $\log_2$ fluorescence in arbitrary units. The ordinate is the relative cell number. Both BT34/A5 and BT54/B8 produced a shift in the mean fluorescence of the small cell population of U-373, which might be better represented on a 2-dimensional plot incorporating small angle scatter data, but can be appreciated in this diagram by the shift from the left hand to right hand side of the distribution.

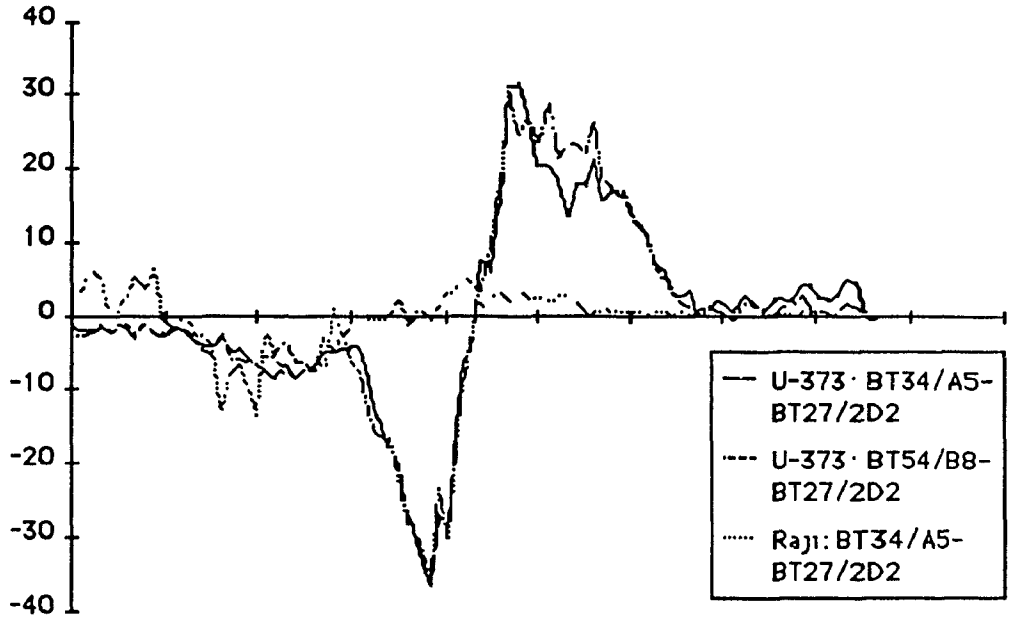


Figure 3.4: Subtraction histograms for BT34/A5 and BT54/B8

Human glioma line U-373 and human B cell line Raji were labelled with either BT34/A5 or BT54/B8, and analyzed in the FACS-III flow cytometer. The percentage of labelled cells per channel was subtracted from control IgM antibody (BT27/2D2), to yield a 'percentage labelling difference', which is represented on the ordinate. The abscissa is $\log_2$ green fluorescence, in arbitrary units. There is a significant difference for glioma line U-373 when labelled with each of the two antibodies. This difference in 'percentage labelling' is initially negative, but subsequently approaches and bypasses zero at the point where the two distributions overlap. Such a difference was not observed for Raji, which was not labelled by either of the 2 HmAbs (data for BT54/B8 not represented).

Table 3.7: Karyotypes of three human hybridomas

Hybridoma	*no. metaphases*	*mean ± S.D.*	*mode*	*range*[a]
BT27/1A2	20	61 ± 15	66	42 - 88
BT27/2A3	17	41 ± 2	40	37 - 46
BT32/A6	20	53 ± 8	56	43 - 69
TM-H2-SP2[b]	19	46 ± 2	46	42 - 49

[a]range represents the lowest to highest chromosome number counted

[b]parental cell line

cells/well were unsuccessful. Of the 5 hybridomas, only BT32/A6 could be cloned at 1000 cells/well. From two 96-well flat-bottomed tissue culture plates, 12 stable clones were derived after 8 weeks in culture. These were tested in a double ELISA experiment for immunoglobulin concentration and reactivity with the 3 *M* KCl extract of a non-autologous glioblastoma (BT-37). Three subclones (25%) were found to react more strongly with the tumor extract, and contained higher concentrations of IgM than the spent culture supernatant of the original parental hybridoma.

Figure 3.5 is a representation of double-ELISA data for the 12 subclones of BT32/A6, and indicates their relative reactivities and IgM concentrations. The hybridoma subclone selected for subsequent studies, BT32/A6.5, is represented by the solid diamond at the extreme upper end of the diagram. Attempts at subcloning hybridomas in soft agar were also unsuccessful.

3.8 Determination of Monoclonality

Southern blot analysis (Fig.3.6) revealed that hybridomas BT27/1A2, BT27/2A3, and BT32/A6.5, each possess two rearranged bands bearing homology to the J_H gene region. Hybridoma BT27/2D2 appears to possess three such bands. The B cell fusion partner used in these experiments, TM-H2-SP2, has only one band and an apparent

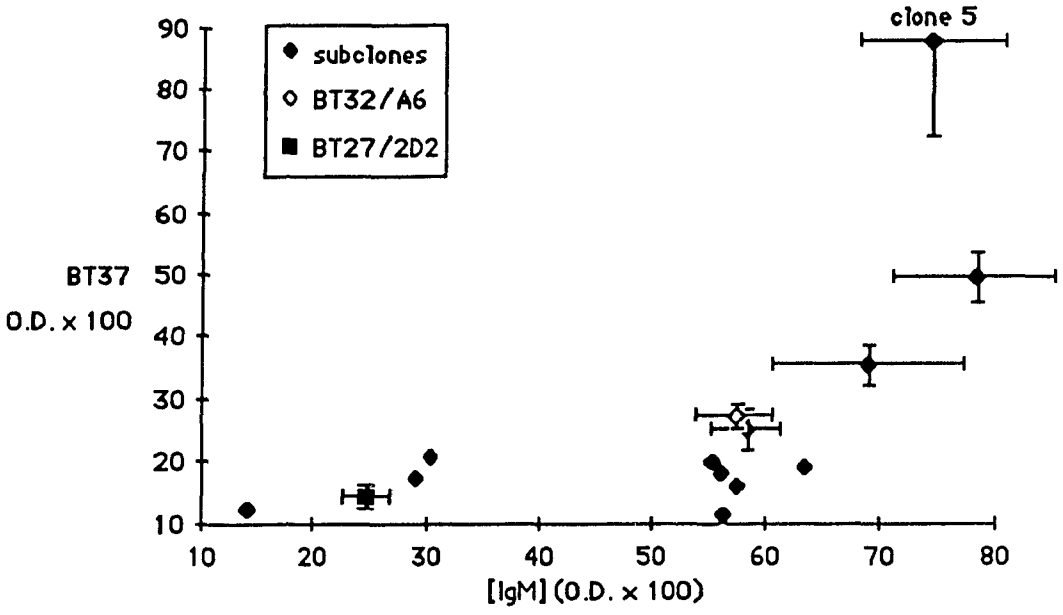
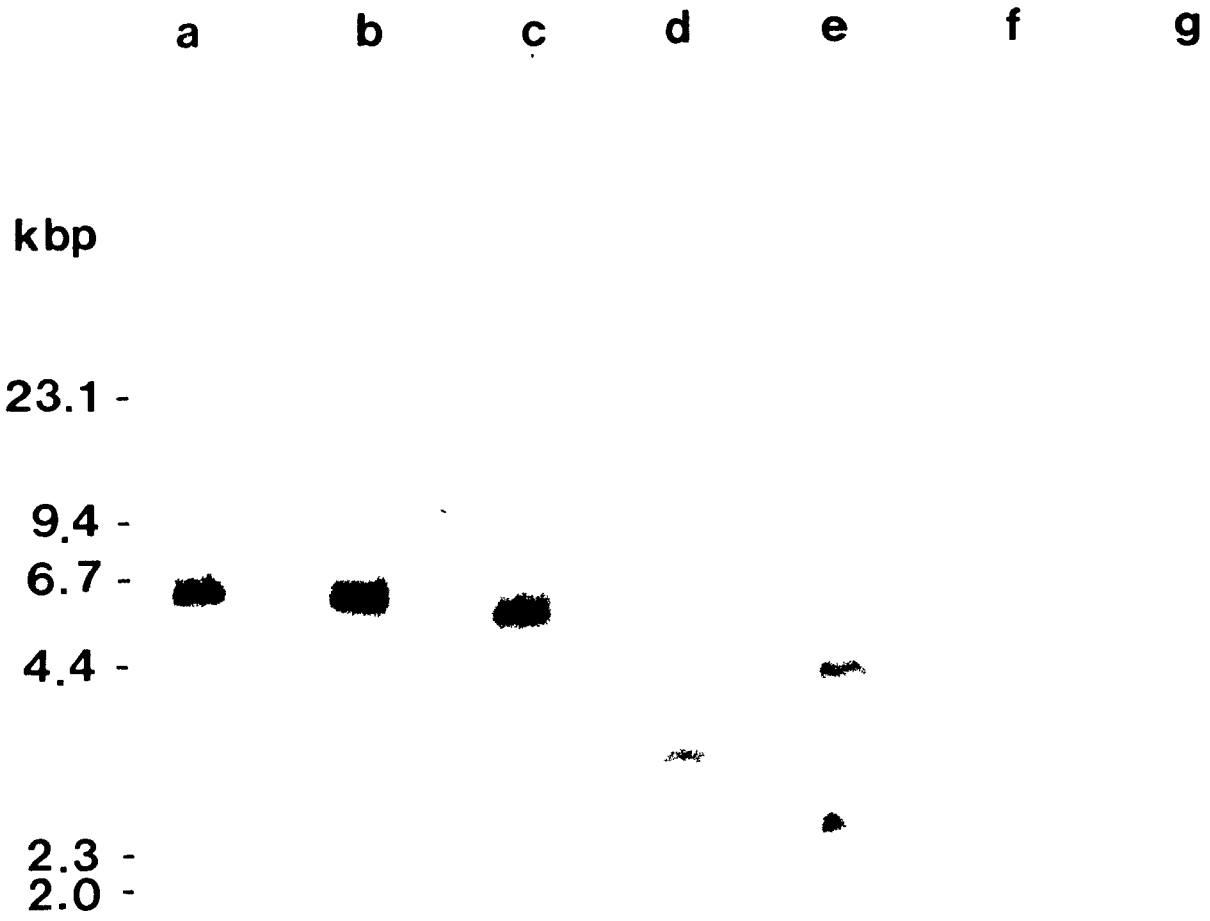


Figure 3.5: Subclones of BT32/A6
Hybridoma BT32/A6 was successfully subcloned at 1,000 cells/well. Twelve stable clones were derived from two 96-microwell plates (0.28 cm^2/well), and are represented as solid diamonds in this double-ELISA experiment (see section 2.1.12). Culture supernatant from the uncloned hybridoma BT32/A6 is represented by an open diamond with standard error bars. Control nonspecific IgM supernatant from hybridoma BT27/2D2 is represented by a solid square with standard error bars. The ordinate is ELISA binding reactivity to 3 *M* KCl extract of tumor BT37, expressed as O.D. x 100 at 405 nm. The abscissa is relative IgM concentration expressed as O.D. x 100 at 405 nm. It can be appreciated that the supernatant from uncloned BT32/A6 contained more IgM and reacted more strongly with autologous tumor extract than did BT27/2D2. Four subclones which either contained significantly more tumor-reactive IgM, or reacted more strongly than uncloned BT32/A6, were given standard error bars. One of them, BT32/A6.5, was eventually shown to be monoclonal using Southern blot analysis (see section 3.8).

Figure 3.6: Southern blot analysis of hybridoma DNA

Genomic DNA was isolated from hybridoma cells and digested with *Bam* HI and *Hind* III restriction enzymes, then electrophoresed on a 0.8% agarose gel, blotted and labelled with a J_H region probe (Oncor, Inc., Gaithersburg, MD), as described in section 2.1.13. Lane (a) represents placental DNA, Lane (b) is DNA from normal peripheral blood lymphocytes, Lane (c) represents TM-H2 (the parental line), Lanes (d), (e), and (f) are BT32/A6.5, BT27/1A2, and BT27/2A3, respectively, and Lane (g) is the non-specific control IgM hybridoma, BT27/2D2. These results indicate the presence of two J_H rearrangements in each of the three hybridomas, consistent with monoclonality.


a
b
c
d
e
f
g
kbp
23.1 -
9.4 -
6.7 -
4.4 -
2.3 -
2.0 -

deletion. Furthermore, there is no evidence for a TM-H2-SP2 type of rearrangement of the J_H region in any of the 'hybridomas'. As a control, normal PBL DNA, which is composed of 60-70% T cell derived genetic material, was found to yield a blot profile identical to placental (germline) DNA. There is also a common low MW band containing an unrelated homologous sequence present in each of the lanes.

3.9 Discussion

Following initial reports by Croce, et al. (1980), and Olsson and Kaplan (1980) of human hybridomas secreting mAbs to measles virus and 2,4-dinitrochlorobenzene (DNCB) respectively, numerous publications have appeared detailing the production of HmAbs to tumor-associated antigens. Human mAbs have been generated which are reactive with mammary carcinoma cells (Schlom, et al. 1980), human mammary tumors (Teramoto, et al. 1982), human melanoma cell lines (Irie, et al. 1982; Warenius, et al. 1983), autologous cervical carcinoma tissue sections (Hagiwara and Sato, 1983), various human malignant cell lines (Sikora, et al. 1983), lung tumor cell lines (Cole, et al. 1984b), leukemia cells (Olsson, et al. 1984), and colonic cancer cell lines and tissue sections (Haspel, et al. 1985; Borup-Christensen, et al. 1986; Sugiyama, et al. 1986).

A number of B cell fusion partners have been proposed, including human myeloma and lymphoblastoid cells (Abrams, et al. 19^6), as well as murine myelomas (Butler, et al. 1983), and even mouse x human hybrids (Foung, et al. 1986; Ostberg, 1986). The fusion partner chosen for the present study, TM-H2-SP2, has yielded stable B cell hybridomas secreting IgM antibodies which reacted with autologous tumor extracts and glioma cell lines. The growth difficulties encountered in the first 10 fusions were overcome by optimizing culture conditions in the subsequent 9 experiments. Optimization was achieved by supplementation with additional quantities of the amino acids L-glutamine and L-asparagine.

Comparisons of hybridoma outgrowth between investigators is complicated by the use of different plating densities, serum concentrations, and the presence or absence of feeder cells. Abrams, et al. (1986) fused PBL from a patient with chronic lymphocytic leukemia (CLL) with a human lymphoblastoid cell line, UC729-6, in a lymphocyte to cell line ratio of 10:1, and seeded the cells into 96 microwell plates at a concentration of 2.5 x 105 cells/well in the absence of feeder cells. Hybridoma outgrowth was 51%, and 43% of the growing hybridomas secreted immunoglobulin. These results must be viewed with the understanding that CLL lymphocytes are transformed

cells, and therefore may not be completely adequate as models of normal lymphocyte fusion partners.

In support of this argument, Glassy, et al. (1983) observed a hybridoma outgrowth of between 0.45 to 2.9 wells per 10^6 lymphocytes fused (with a plating density of 10^5 cells per well), using the human lymphoblastoid cell line, UC729-6, as a fusion partner for normal B-lymphocytes. Shoenfeld, et al. (1982) observed hybridoma growth in only 108 of 4,254 microwells (2.5%) when PBL from patients with autoimmune disease were fused to the human lymphoblastoid cell line, GM 4672. At present, no single human myeloma or lymphoblastoid cell line appears clearly superior as a fusion partner for normal PBL.

A comparison of various sources of lymphocytes as fusion partners for two human cell lines was made by Cote, et al. (1983) who obtained lymphocytes from lymph nodes, peripheral blood, spleen, and tumor specimens, and carried out fusions with the human cell lines LICR-LON-HMy2 (LICR-2) or SKO-007. Fusions between the lymphoblastoid line, LICR-2, and PBL were found to produce the highest percentage of microwells with growing clones which also contained immunoglobulin (74%).

Several investigators (Olsson and Kaplan, 1983; Warenius, et al. 1983) have carried out *in vitro* pre-stimulation of lymphocytes using pokeweed mitogen (PWM) prior to performing a fusion. This was done in order to

increase the overall yield of hybridomas, since activated B-lymphocytes were thought to be more suitable for productive fusions than quiescent ones. Unfortunately, PWM-stimulated PBL from cancer patients resulted in poor hybridoma yields, possibly as a result of impaired T cell function in these individuals (see section 1.3.2). Attempts at influencing the proportion of IgG secreting lymphocytes by *in vitro* antigen priming have generally been unsuccessful (Olsson and Kaplan, 1983).

The specificity of HmAbs from cancer patients has been investigated by Houghton, et al. (1983), and Cote, et al. (1986). The latter noted that 1) a significant proportion (305/4350; 7.0%) of hybridomas from tumor-bearing individuals reacted with cellular antigens, but 2) only a minority (5/111; 4.5%) of these antigens had a cell surface location. In the present study, a significant proportion (15.5%) of all microwells reacted with either autologous tumor extract or glutaraldehyde-fixed glioma cell lines, but only 3 of 59 (5.1%) hybridomas studied by FCM were found to recognize cell surface antigens present on the human glioma line, SK-MG-1, which was used as our primary indicator of glioma reactivity.

Some investigators (Lange, et al. 1976; Pesce, et al. 1978) have encountered difficulties with non-specific binding of Ig to plastic ELISA plates. With the proportion of 'positive' hybridomas per ELISA ranging from 0 to 92.4%

(the latter in the case of BT-4), it is quite possible that with a cutoff arbitrarily set at 2 S.D. above the mean, a number of hybridomas will be selected purely on the basis of non-specific reactivity. In order to determine whether non-specific binding of Ig to the ELISA plates was a factor, a 'double ELISA' experiment was conducted in which hybridoma supernatant was reacted both with anti-Ig and tumor extract-coated plates. The lack of any significant correlation between the two assays indicated that reactivity in the tumor extract ELISA was not solely a determinant of immunoglobulin concentration.

Proof of monoclonality was approached at the genotypic rather than phenotypic level. Rather than determine the number and class of different Igs secreted by each hybridoma, attention was focused on the Ig genes themselves. The Ig heavy chain gene is located on chromosome 14 in the human, and is composed of four distinct elements known as V_H (variable), D_H (diversity), J_H (joining), and C_H (constant) regions. In germline DNA these regions are separated by intervening sequences which are spliced out once the cell becomes committed to B cell differentiation.

Originally it was thought that only one chromosome underwent rearrangement while the other remained in the germline configuration (the principle of allelic exclusion). In the mouse heavy chain gene, nowever, the

second chromosome is essentially always rearranged (Nottenburg and Weissman, 1981). It was therefore proposed that for a given cell, the probability of a functional rearrangement had to be very low (Coleclough, et al. 1981). One would thus encounter the situation of two non-functional, or one functional and one non-functional, but very rarely two functional heavy chain rearrangements per cell. These observations are valid for the mouse, and also seems to be valid for higher mammalian species as well (Korsmeyer, et al. 1983).

The heavy chain probe used in these experiments spans the entire human germline J_H region. In situations where there has been rearrangement of germline DNA, digestion with the restriction enzymes *Bam* HI and *Hind* III will produce DNA fragments that differ in length from the 5.6 kbp J_H probe (Fig. 3.7). The finding of two such fragments in each of the hybridoma lanes indicates that two heavy chain rearrangements are detected for each hybridoma. This is consistent with a monoclonal origin for each of the three hybridomas.

Using a J_H probe similar to the one used in these experiments, Ford, et al. (1983) found two rearrangement bands among each of four monoclonal B-lineage cell lines: RPMI 1788, Bristol 7, REH, and NALM 6. Korsmeyer, et al. (1983) also found examples of heavy chain double rearrangements among non-T, non-B leukemias, indicating

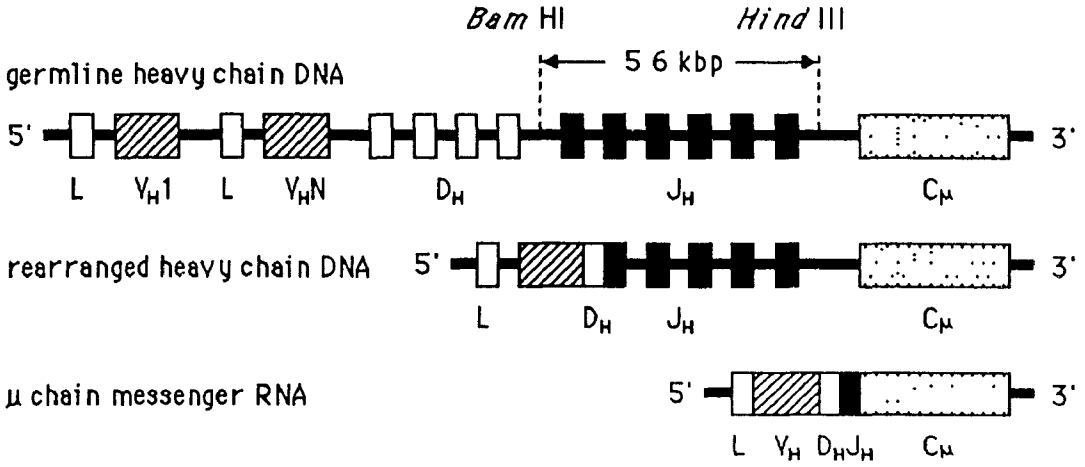


Figure 3.7: Organization and assembly of heavy chain genes

The human immunoglobulin heavy chain gene is composed of four distinct elements: V_H (variable), D_H (diversity), J_H (joining), and C_H (constant) gene regions. Once the cell becomes committed to B-lineage differentiation, gene rearrangement occurs and intervening sequences are spliced out. The heavy chain probe used in these experiments is 5.6 kbp in length, and spans the entire J_H gene region of germline DNA, bounded on either side by *Bam* HI and *Hind* III digestion sites. When rearranged heavy chain DNA is digested with these two enzymes, the length of the fragment containing the rearranged J_H region will vary. These differences can be detected by agarose gel electrophoresis and hybridization analysis (Southern blotting). Adapted from Korsmeyer, et al. (1983). L = leader sequence, 5' and 3' refer to the orientation of the DNA chain

that these cells are actually precursor cells already committed to B cell differentiation at the Ig gene level. Rudders, et al. (1988) also analyzed 52 tumors from patients with B cell-derived lymphomas for potential oligoclonality, using *Bgl* II, *Bam* HI, and *Eco* RI DNA digests probed with the J_H gene. In 72% of all cases, rearrangements of both J_H alleles could be detected following digestion with multiple restriction enzymes, suggesting that the process of H chain rearrangement in human B cell tumors is relatively inefficient, often requiring 2 attempts before rearrangement is productive.

Failure to find a TM-H2-SP2 heavy chain rearrangement in any of the three hybridomas would suggest that none of the latter are carrying the number 14 chromosome from TM-H2-SP2 with the heavy chain rearrangement, or else that a deletion on that chromosome has taken place involving the heavy chain alleles. Shortly after fusion, human-human hybridomas are thought to lose chromosomes in an exponential manner (Buck, et al. 1984). Karyotyping of BT27/1A2, BT27/2A3, and BT32/A6 suggests that this process has been occurring, since two hybridomas were found to be pseudodiploid (2-3*n*), and one hypodiploid (<2-2*n*).

4 CHARACTERIZATION OF HUMAN MONOCLONAL ANTIBODIES TO GLIOMA CELL SURFACE ANTIGENS

4.1 Background

The first three HmAbs to be derived were from hybridomas BT27/1A2, BT27/2A3 (both of which originated from the same fusion), and BT32/A6. These antibodies were characterized in some detail, especially from BT37/2A3. Only BT32/A6 has been subcloned, although all three hybridomas seem to be monoclonal (see section 3.7). Hybridomas and antibodies BT34/A5 and BT54/B8 were derived much later, and have been less well characterized. Many of the conclusions derived from the study of the first three HmAbs apply to the latter two antibodies.

The patient from whom BT27/1A2 and BT27/2A3 were derived was a 23 year old male electrician who first presented with seizures 1.5 years prior to this study. A CT scan done at that time revealed a right frontal mass which was interpreted as being a low grade astrocytoma. He was followed conservatively, and the seizures were controlled with diphenylhydantoin and phenobarbital. A routine follow-up CT scan performed 3 weeks before admission showed an increase in size of the mass. The patient underwent a right frontal craniotomy and excisional

biopsy of the mass, which was determined to be a malignant glioma.

BT32/A6 was derived from a 14 year old male who was diagnosed as having von Recklinghausen's neurofibromatosis at age 2. A routine CT scan done in 1978 demonstrated a right intraventricular mass. The patient was followed conservatively, and was eventually lost to follow-up in 1983. In October 1985, he presented to the Emergency Department with a history of sudden severe headache and nausea. A CT scan performed on admission revealed fresh intraventricular and subarachnoid blood, consistent with an intratumoral hemorrhage. The patient recovered without any neurological deficits, and subsequently underwent elective removal of the tumor. The tumor was a low grade, fibrillary astrocytoma. Peripheral blood lymphocytes were collected from this patient around the time of his surgery.

The patient who contributed BT34/A5 was an 11 year old girl with a benign fibrillary astrocytoma. She presented intitially at age 3 with episodes of headache and vomiting, and at age 5 was referred to a neurologist who recommended a CT scan of the head. The family failed to keep the appointment until the child was 10 years old and experiencing daily vertex headaches with nausea and vomiting upon awakening, ataxia, and dizziness. On examination she had obvious macrocrania with severe papilledema, a wide-based ataxic gait, dysmetria which was

more pronounced on the left, and hyperactive reflexes on the right with a positive Babinski sign on the right. A skull X-ray showed bony changes typical of chronic raised intracranial pressure, and a calcification in the posterior fossa. The CT scan revealed there was a large calcified midline cystic tumor of the cerebellum, which extended up to and through the tentorial hiatus. The patient eventually went on to have a subtotal resection of her tumor followed by local radiotherapy and a shunting procedure.

BT54/B8 was derived from a 53 year old male alcoholic who presented with a two week history of progressive right hemiparesis and dysphasia. A CT scan revealed a large left frontal contrast-enhancing mass which exerted significant compression on local structures, causing a 2.1 cm shift of the midline. At operation the tumor had all the gross morphological features of a malignant glioma, with focal necrosis, arterialized veins, and thrombosed vessels. Final pathology proved it to be a glioblastoma multiforme.

4.2 Immunoglobulin Isotypes and Production Levels

All five hybridoma supernatants were found to contain μ heavy chains. Only BT34/A5 contained λ light chains, the other four HmAbs contained κ light chains. Each HmAb was shown to label the cell surface of human glioma line SK-MG-1 using FCM and FITC-conjugated goat anti-human IgM

(IgG fraction, μ chain specific; Cappel Laboratories, Cochranville, PA), indicating that the IgM molecules were binding to the tumor cell surface membrane (data not shown). No γ chains were detected in any of the hybridoma supernatants.

After approximately six months of maintenance in culture, the estimated IgM concentration for BT27/1A2 was 5.0 μg/ml; for BT27/2A3, it was 44 μg/ml, for BT32/A6 3.5 μg/ml, for BT34/A5 2.4 μg/ml, and for BT54/B8 22.4 μg/ml. After a further 6 months in continuous culture, IgM production levels were found to be comparable.

4.3 ELISA Dilution Curves

Dilution curves for 3 of the 5 HmAbs are shown in Figure 4.1. At the initial concentration of supernatant fluid which was tested, the order of reactivity was BT27/2A3 > BT27/1A2 > BT32/A6, which is the same as the order of their respective IgM concentrations (see section 4.2). The final titre which gave O.D. readings significantly higher than control IgM baseline was 1:16 for BT27/2A3, 1:4 for BT27/1A2, and 1:2 for BT32/A6. Since this assay involves the addition of 50 μl of hybridoma supernatant to 50 μl of PBS in the initial step, the initial dilution tested in the ELISA was 1:2. There was no evidence of an initial plateau phase in any of the dilution

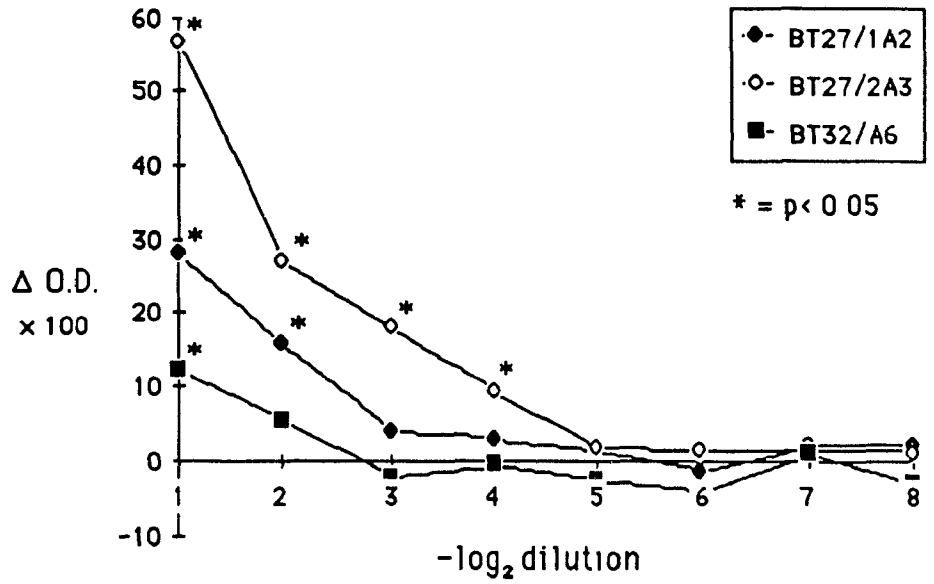


Figure 4.1: Antibody dilution curves

Serial dilutions of hybridoma supernatants were reacted in an ELISA assay with allogeneic glioblastoma multiforme extract. Dilution is represented on the abscissa, and O.D. x 100 (405 nm) above backround nonspecific IgM (BT27/2D2), is represented on the ordinate. An asterisk indicates $p<0.05$ by Student's *t* test.

curves, indicating that under the ELISA conditions which were used, the quantity of tumor extract was not a limiting factor.

4.4 Relative Antibody Affinity

In order to get some indication of relative antibody affinity, a modification of the method of De Bernado and Davies (1987) was used. The results of ELISA testing of the five HmAbs with tumor extract from BT-37 (glioblastoma multiforme) after either a long (18 hr) or a short (4 hr) incubation at 4 °C, are illustrated in Figures 4.2 and 4.3. Comparison of the O.D. readings for each HmAb at the two timepoints indicates that there is a statistically significant ($p<0.05$) increase in O.D. for BT32/A6 and BT54/B8 with the longer incubation. None of the other 3 HmAbs (BT27/1A2, BT27/2A3, and BT34/A5) showed a significant increase in reactivity after a longer incubation at 4 °C. These observations would suggest that BT32/A6 and BT54/B8 behave as a low affinity antibodies at low temperatures.

After the long incubation period (18 hrs at 4 °C), all five HmAbs confirmed positive ($p<0.05$) by ELISA when compared to the control. After the short incubation time (4 hrs at 4 °C), however, only BT27/1A2, BT27/2A3, and BT34/A5 were significantly positive; BT32/A6 and BT54/B8

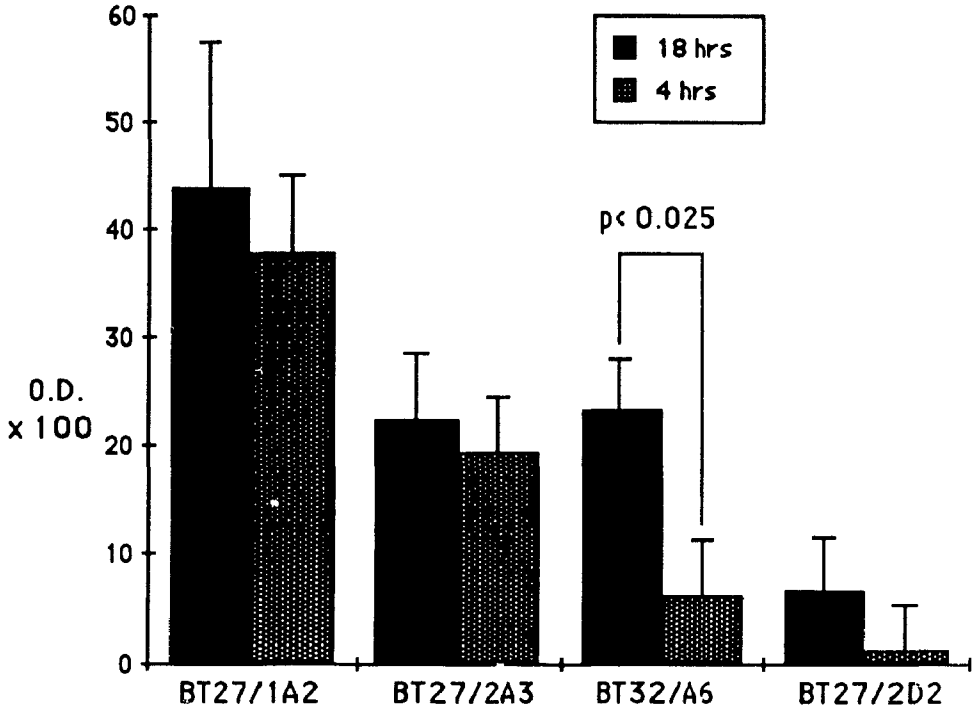


Figure 4.2: Relative affinities of three HmAbs
Culture supernatants from hybridomas BT27/1A2, BT27/2A3, and BT32/A6 were incubated at 4 ^{o}C with allogeneic glioblastoma multiforme extract for 4 and 18 hrs, and tested in an ELISA assay. Supernatant from hybridoma BT27/2D2 was used as a source of control IgM. With the long (18 hr) incubation, all three hybridomas were significantly positive compared to BT27/2D2. This was not the case with the short (4hr) incubation: BT32/A6 reacted significantly less ($p<0.025$) under these conditions than with a long incubation. None of the other hybridomas behaved in this manner. These results suggest that BT32/A6 behaves as a low affinity antibody at 4 ^{o}C. Bars indicate standard error of the mean; 5 replicates per sample.

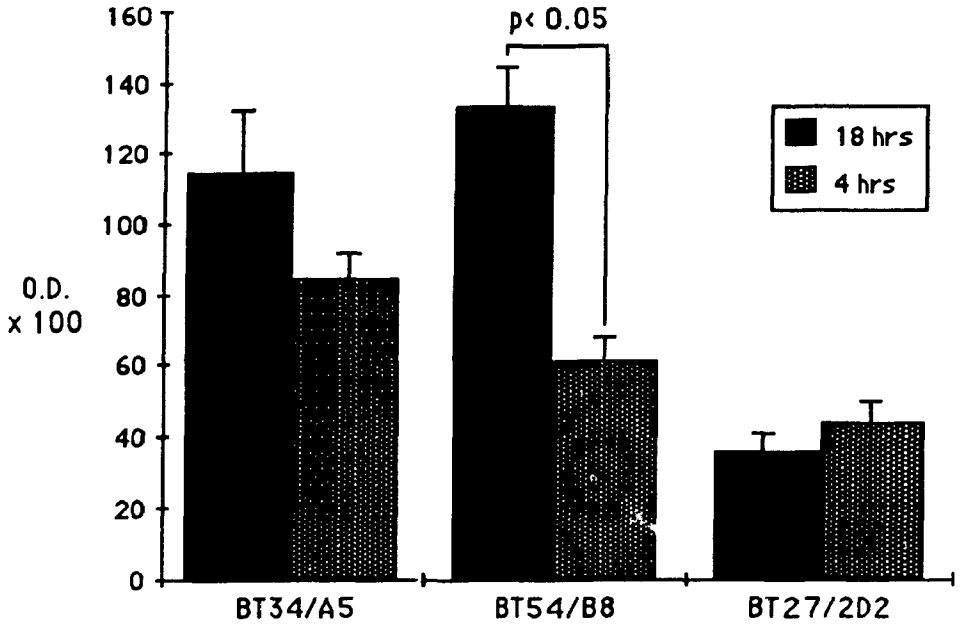


Figure 4.3: Relative affinities of HmAbs BT34/A5 and BT54/B8

Culture supernatants from hybridomas BT34/A5 and BT54/B8 were incubated at 4 °C with allogeneic glioblastoma multiforme extract for 4 and 18 hrs, and tested in an ELISA assay. Supernatant from hybridoma BT27/2D2 was used as a source of control IgM. With the long (18 hr) incubation, both hybridomas were significantly positive compared to BT27/2D2. This was not the case with the short (4hr) incubation: BT54/B8 reacted significantly less ($p<0.05$) under these conditions than with a long incubation. The affinity of BT34/A5 did not change significantly after the short incubation. These results suggest that BT54/B8 behaves as a low affinity antibody at 4 °C. Bars indicate standard error of the mean; 5 replicates per sample.

did not react significantly, possibly because of their low affinity nature.

4.5 Glioma Culture Density and Immunofluorescent Labelling

Initial FCM experiences with BT27/2A3 labelling of human glioma line SK-MG-1 indicated that reactivity could be consistently improved if the glioma cells were maintained in confluent cultures after feeding with fresh medium for at least 24 hrs prior to testing. Figure 4.4 shown the results of varying the cell plating density (by varying the passage split ratio), which affected the duration of time the cells are confluent before being tested.

Small angle scatter data are shown on the left hand side of the figure (A), and fluorescence data on the right (B). The shaded area indicates the scatter gating used to generate the fluorescence distribution. These results confirm an increase in fluorescence labelling using a culture passage split ratio of 1:2, compared to a ratio of 1:8. Cells passaged at intermediate ratios (e.g. 1:4, 1:6, etc.) were found to have intermediate fluorescence values (data not shown). It may be concluded that BT27/2A3 labelling of SK-MG-1 is enhanced if the latter is maintained at a high cell density prior to testing.

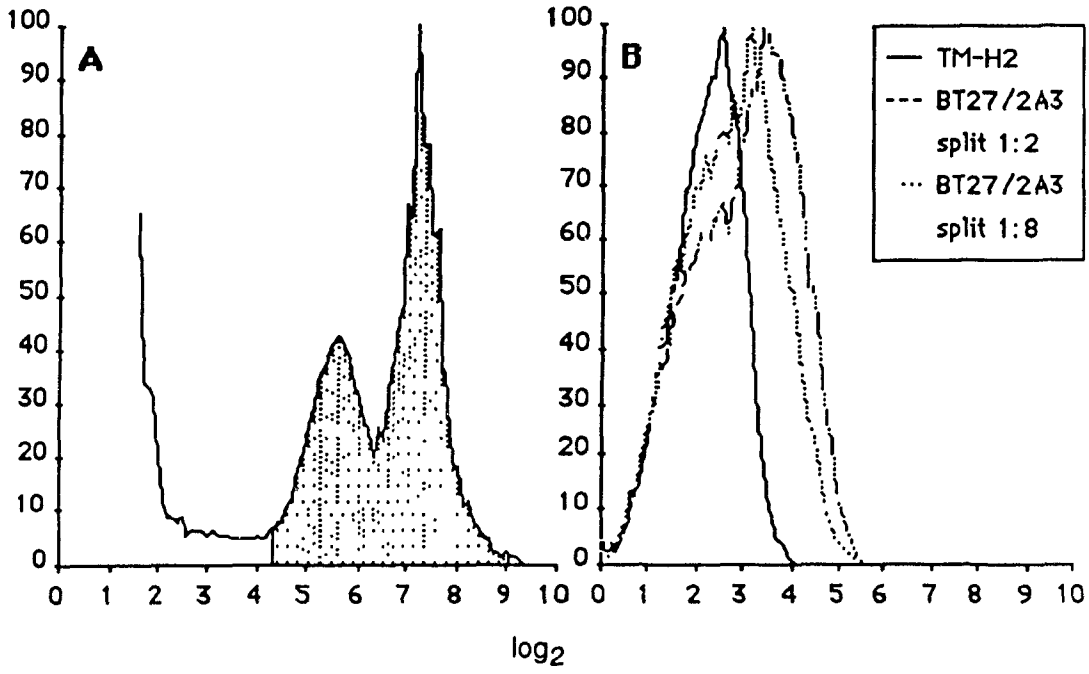


Figure 4.4: Antigen expression and glioma culture density

Human glioma line SK-MG-1 was passaged with split ratios of 1:2 and 1:8, grown in culture for 4 days, and reacted with BT27/2A3. Control nonspecific antibody (KIgG) was derived from culture supernatant of the parental myeloma line, TM-H2. Flow cytometry was performed, and $\log_2$ small angle scatter data in arbitrary units is represented on the abscissa in (A). The shaded portion corresponds to the gating used to genera'e the fluorescence histograms (B; also $\log_2$ scale in arbitrary units). Histograms were plotted to peak height, with relative cell number represented on the ordinate. Approximately 5 x 10^4 cells were analyzed for each sample. Antibody reactivity is significantly greater with the 1:2 split ratio, i.e. at higher cell densities.

4.6 Flow Cytometric Analysis of Human Tumor Cell Lines

Flow cytometric analysis of cultured human cell lines and strains was performed with the cells grown to confluence and maintained in that state with exchanges of fresh media at least 24 hrs prior to labelling. Suspension culture cell lines were maintained and used from high cell density cultures, i.e. near saturation. Screening was carried out according to the method outlined in section 2.1.9, in parallel on two or more separate occasions with established positive cell lines to verify maintenance of immune reactivity of the HmAbs.

Results of FCM screening with human cell lines are summarized in Tables 4.1 and 4.2. Several different classes of neuroectodermal, and non-neuroectodermal tumors and tissues were tested. All five HmAbs demonstrated a similar pattern of reactivity for the 30 cell lines which were studied, with few notable exceptions. For example, the human epithelial cervical carcinoma cell line, ME180, reacted with BT27/1A2 and BT27/2A3, but not BT32/A6. Antibodies BT34/A5 and BT54/B8 both failed to react with the glioma cell line SKI-1, but labelled melanoma line M-4.

Only HmAb BT32/A6 had a singular pattern of reactivity. Antibodies BT27/1A2 and BT27/2A3 exhibited one pattern of reactivity, and antibodies BT34/A5 and BT54/B8 had another pattern of reactivity, which differed slightly

Table 4.1: Reactivity of three HmAbs

Tumor Type	*Cell Line*	*BT27/1A2*[a]	*BT27/2A3*	*BT32/A6*
Glioma	SK-MG-1	positive[b]	positive	positive
	SK-MG-13	positive	positive	positive
	SKI-1	positive	positive	positive
	LN-215	negative	negative	negative
	LN-340	negative	negative	negative
	U-178	positive	positive	positive
	U-373	positive	positive	positive
Melanoma	M-4	negative	negative	negative
	IGR-37	negative	negative	negative
	IGR-39	negative	negative	negative
Neuroblastoma	IMR-32	negative	negative	negative
	SK-N-MC	negative	negative	negative
Retinoblastoma	Y-76	negative	negative	negative
Hematological	CEM-T[c]	negative	negative	negative
	K-562[d]	negative	negative	negative
	HL-60[e]	negative	negative	negative
Other Tumors	HeLa[f]	positive	positive	positive
	ME180[f]	positive	positive	negative
	C-33A[f]	negative	negative	negative
	SW1166[g]	negative	negative	negative
	SW1417[g]	negative	negative	negative
	SW948[g]	negative	negative	negative
	HT-29[g]	negative	negative	negative
	J82[h]	negative	negative	negative
Embryonic Fibroblast	HEF	negative	negative	negative
	WI-38	negative	negative	negative

Footnotes: [a]hybridomas, [b]cells were detected to have bound HmAb above the background level of labelling with control antibody, [c]T-cell leukemia, [d]chronic myelocytic leukemia, [e]acute promyelocytic leukemia, [f]epithelial cervical carcinoma, [g]colonic adenocarcimoma, [h]transitional bladder cell carcinoma.

Table 4.2: Reactivity of HmAbs BT34/A5 and BT54/B8

Tumor Type	*Cell Line*	*BT34/A5*[a]	*BT54/B8*	*BT27/2A3*
Glioma	SK-MG-1	positive[b]	positive	positive
	SKI-1	negative	negative	positive
	U-373	positive	positive	positive
Melanoma	M-4	positive	positive	negative
Hematological	CCRF-CEM[c]	negative	negative	negative
	K-562[d]	negative	negative	negative
	HL-60[e]	negative	negative	negative
	U-937[f]	negative	negative	N.D.
	Raji[g]	negative	negative	N.D.
Other Tumors	HeLa[h]	negative	negative	positive
	ME180[h]	negative	negative	negative
	C-33A[h]	negative	negative	negative
	SW1417[i]	negative	negative	negative
	SW1463[i]	negative	negative	negative
	J82[j]	negative	negative	negative
Embryonic Fibroblast	IMR-90	positive	positive	N.D.
	WI-38	negative	negative	negative

Footnotes: [a]hybridomas, [b]see Table 4.1, [c]T cell leukemia, [d]chronic myelocytic leukemia, [e]acute promyelocytic leukemia, [f]histiocytic lymphoma, monocyte-like, [g]B cell lymphoma, [h]epithelial cervical carcinoma, [i]colonic adenocarcimoma, [j]transitional bladder cell carcinoma, N.D.: not done.

from each other. None of the HmAbs reacted with any of the hematological cells lines which were tested.

4.7 Flow Cytometric Analysis of Primary Tumors Explant Cultures

Four tumor explants were adapted to tissue culture and studied by FCM after brief passages *in vitro*. The results are shown in Figure 4.5 as a composite illustration. Clinical data on each patient is summarized beside each figure. The greatest apparent proportion of labelling occured with a recurrent meningioma (46%), whereas the lowest proportion of labelling (7%) was found in a low grade astrocytoma. These results indicate that BT27/2A3 is capable of reacting with primary glial and non-glial neurological tumors in the early stages of adaptation to tissue culture.

4.8 Immunofluorescence Studies and Immunohistochemistry

Figure 4.6 is a composite picture of cytocentrifuge preparations of fluorescently labelled SK-MG-1. Antibodies BT27/1A2 and BT27/2A3 were found to label the cell surface in a contiguous fashion [(A) and (B)], whereas HmAb BT32/A6 appears in a punctate manner on the cell surface (C). Linear areas of increased labelling may be observed in (A)

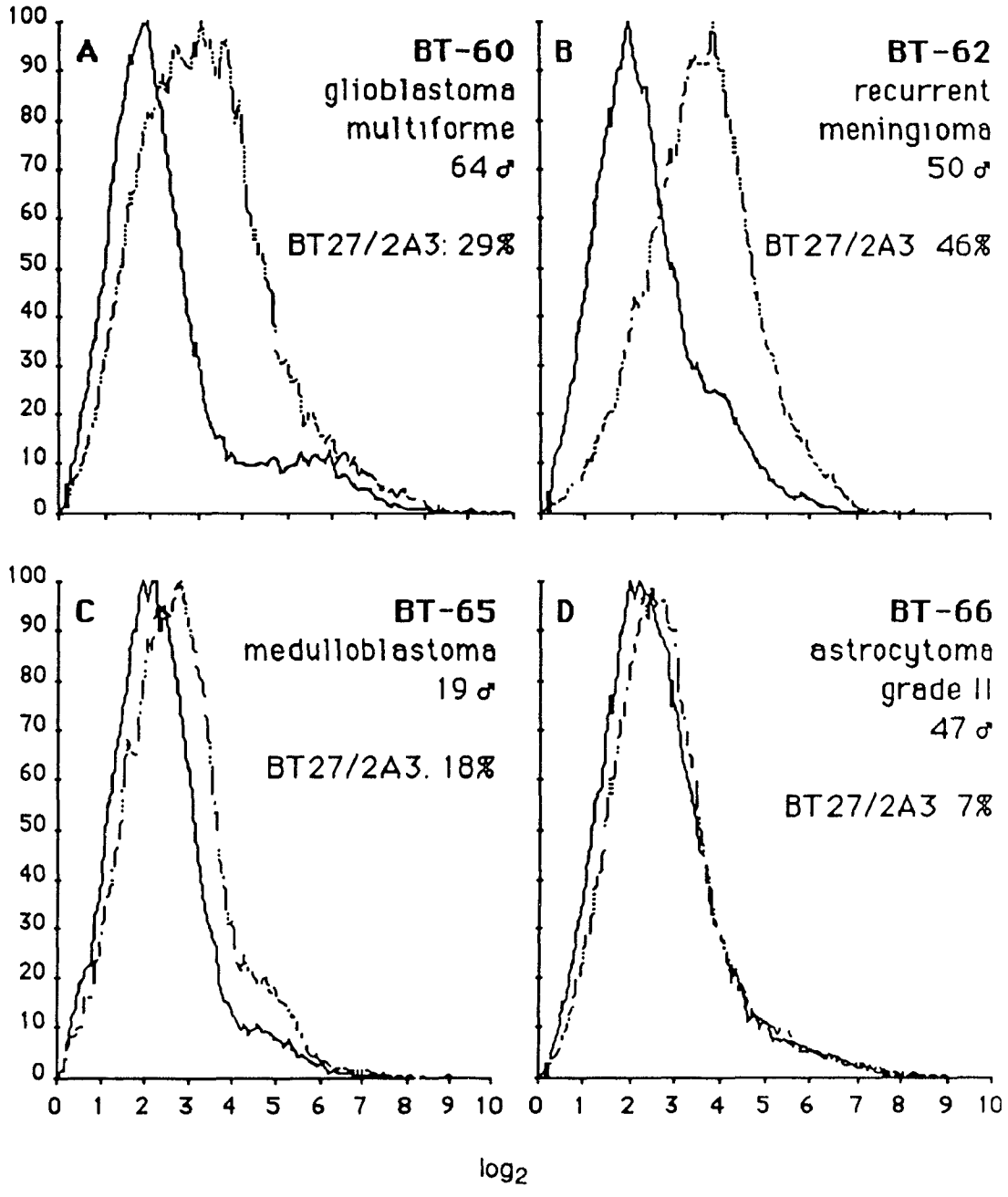
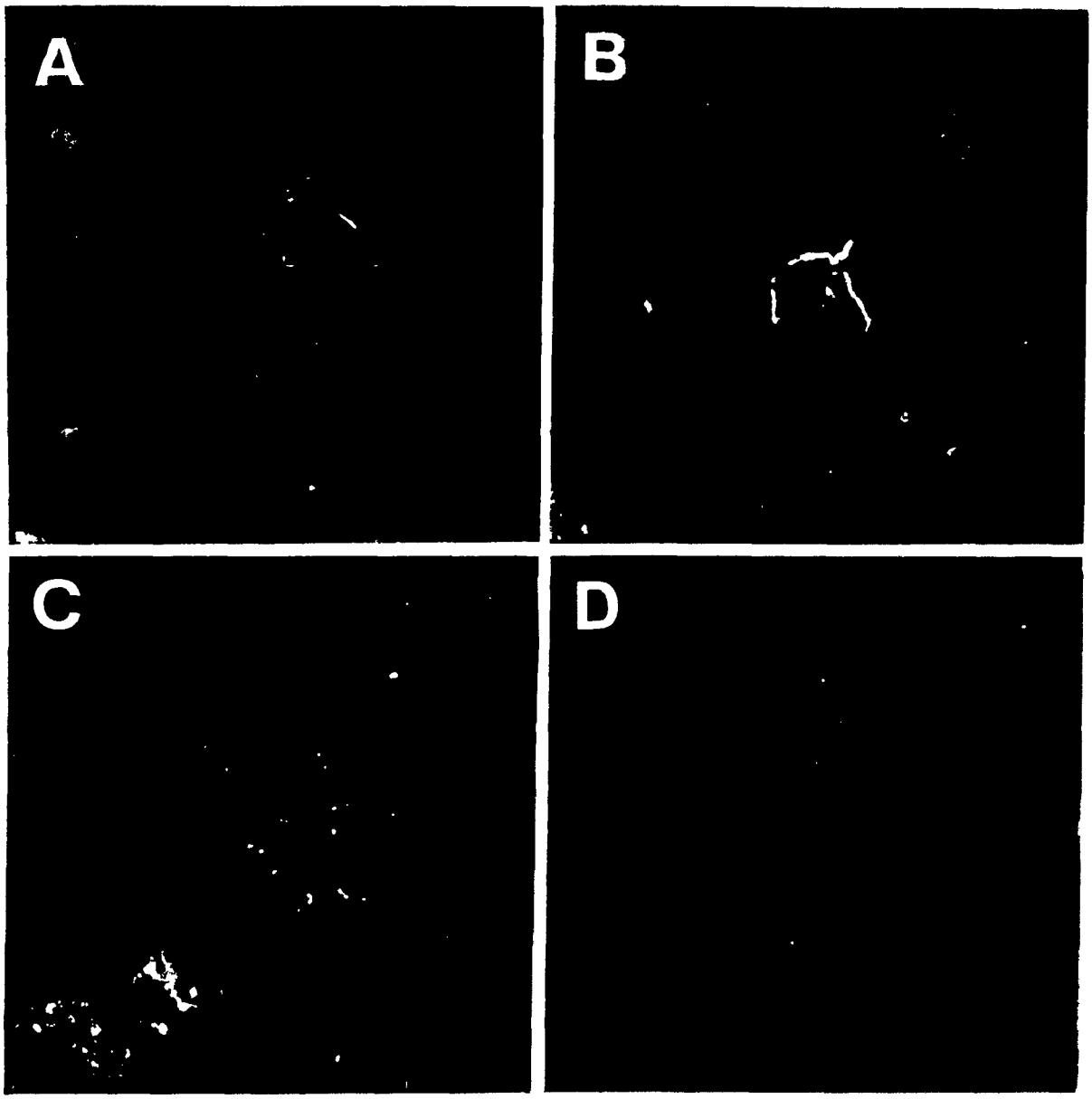


Figure 4.5: BT27/2A3 labelling of tumor explants

Flow cytometric data on four cultured tumor explants. Abscissa is $\log_2$ fluorescence in arbitrary units. Histograms are plotted to peak height, with relative cell number on the ordinate. Solid line is background labelling with BT27/2D2. See section 4.6 for details.

Figure 4.6: Fluorescence labelling of SK-MG-1 cells

SK-MG-1 cells were labelled for flow cytometry, as outlined in section 2.1.9, centrifuged onto glass slides, and examined under the fluorescence microscope. Panel A shows labelling with HmAb BT27/1A2, Panel B is BT27/2A3, Panel C is BT32/A6, and Panel D is control IgM, BT27/2D2. All exposure times for fluorescence photography were the same.


A
B
C
D

and (B). These represent folds in the cell membrane, and are likely a result of the method of cell preparation.

Immunohistochemical staining of SK-MG-1 is represented in Figure 4.7. Compared with the immunofluorescence studies, this method allows for an easier assessment of cell morphology. Antibodies BT27/1A2 and BT27/2A3 clearly outline the cell membrane; using this immunohistochemical method, BT32/A6 can also be seen to produce an apparently different labelling pattern.

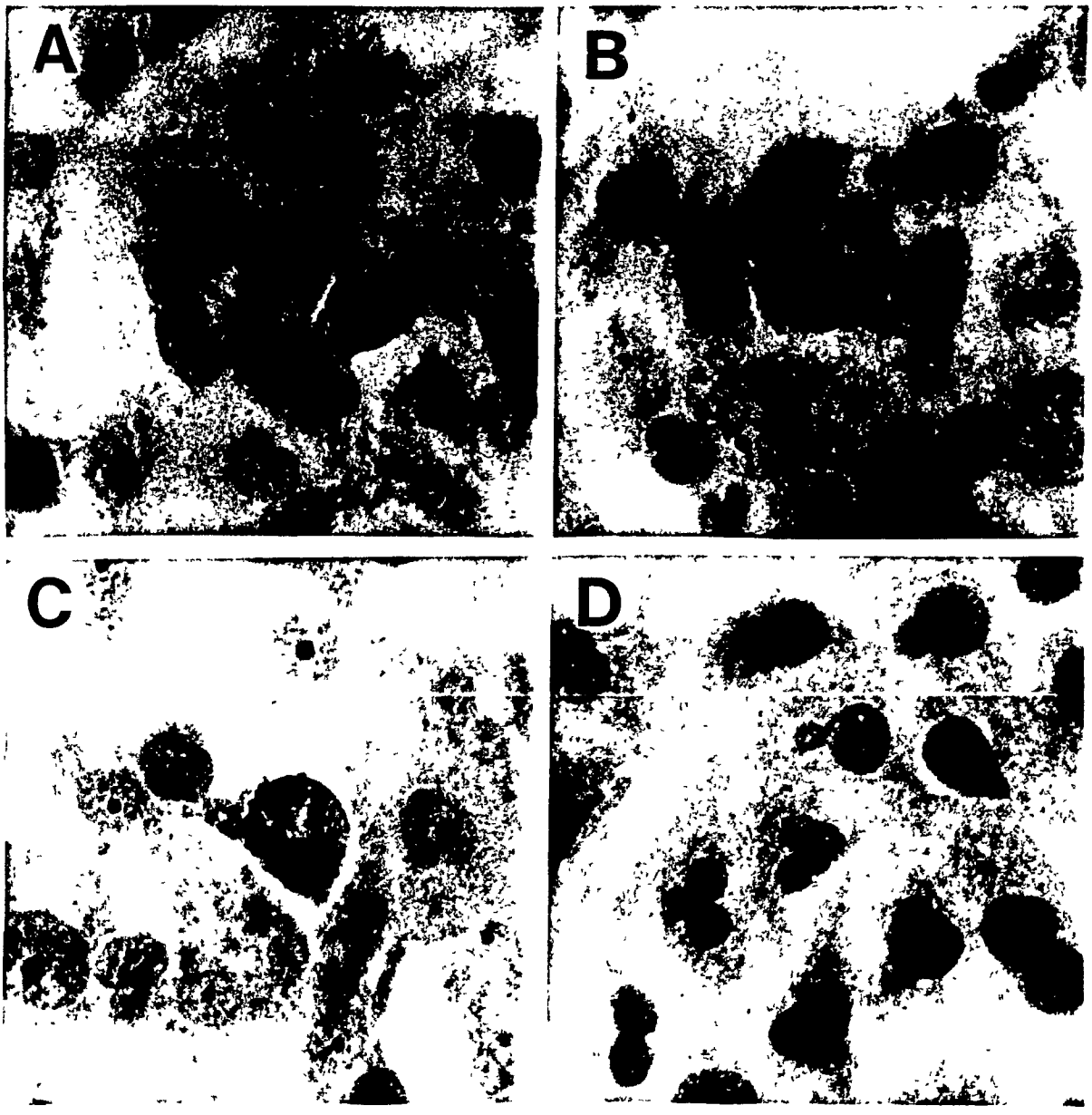
4.9 Analysis of Reactivity with Allogeneic Tumor Extracts

Reactivity of 3 of the 5 HmAbs with 12 tumor extracts was evaluated by ELISA in order to determine their potential relationship to each other regarding antigen targets. Analysis of the ranked *t*-statistics using Spearman's rank-correlation coefficient gave values of r_s = 0.65 for BT27/1A2 vs. BT27/2A3 ($p<0.05$), r_s = 0.00 for BT27/2A3 vs. BT32/A6 ($p>0.10$), and r_s = 0.18 for BT27/1A2 vs. BT32/A6 ($p>0.10$). BT27/1A2 and BT27/2A3 were significantly correlated in reactivity to the 12 tumor extracts; BT32/A6 was not correlated in reactivity to either BT27/1A2 or BT27/2A3.

Another way of analyzing the ELISA data for the 12 tumor extracts is to calculate increase in O.D. above backround (Δ O.D.), and graphically compare the

Figure 4.7: Immunohistochemical staining of SK-MG-1 cells

SK-MG-1 cells were grown on chamber/slides under standard conditions until confluent, and stained as outlined in section 2.2.7. Panel A shows labelling with HmAb BT27/1A2, Panel B is BT27/2A3, Panel C is BT32/A6, and Panel D is control IgM, BT27/2D2.


A
B
C
D

quantitative level of reactivities of the mAbs among the various extracts. Figure 4.8, part (A), illustrates the O.D. plots for BT27/1A2 and BT27/2A3. The images appear to be almost identical, with each series rising and falling in conjunction with the other. In contrast, part (B) shows the data for BT27/1A2 and BT32/A6. As one series of points rises, the other tends to fall in a mirror-like fashion.

These results suggest that antibodies BT27/1A2 and BT27/2A3 are recognizing the same epitope or different epitopes with similar distributions in tumors and cell lines. The results of immunofluorescence and immunohistochemical staining also indicate a similar labelling pattern for BT27/1A2 and BT27/2A3; however, antibody BT32/A6 appears to recognize a different epitope than BT27/1A2 or BT27/2A3.

4.10 Preliminary Antigen Characterization

Numerous attempts at establishing hybridoma reactivity with Western blots of cell membrane vessicles from cultured neuroectodermal tumor cell lines were unsuccessful. Figure 4.9 illustrates one such example. Antibody BT27/2A3 is on the left hand side of the figure, and control antibody, BT27/2D2, on the right. No appreciable quantitative or qualitative difference in banding pattern between the two antibodies is evident. Only non-specific association with

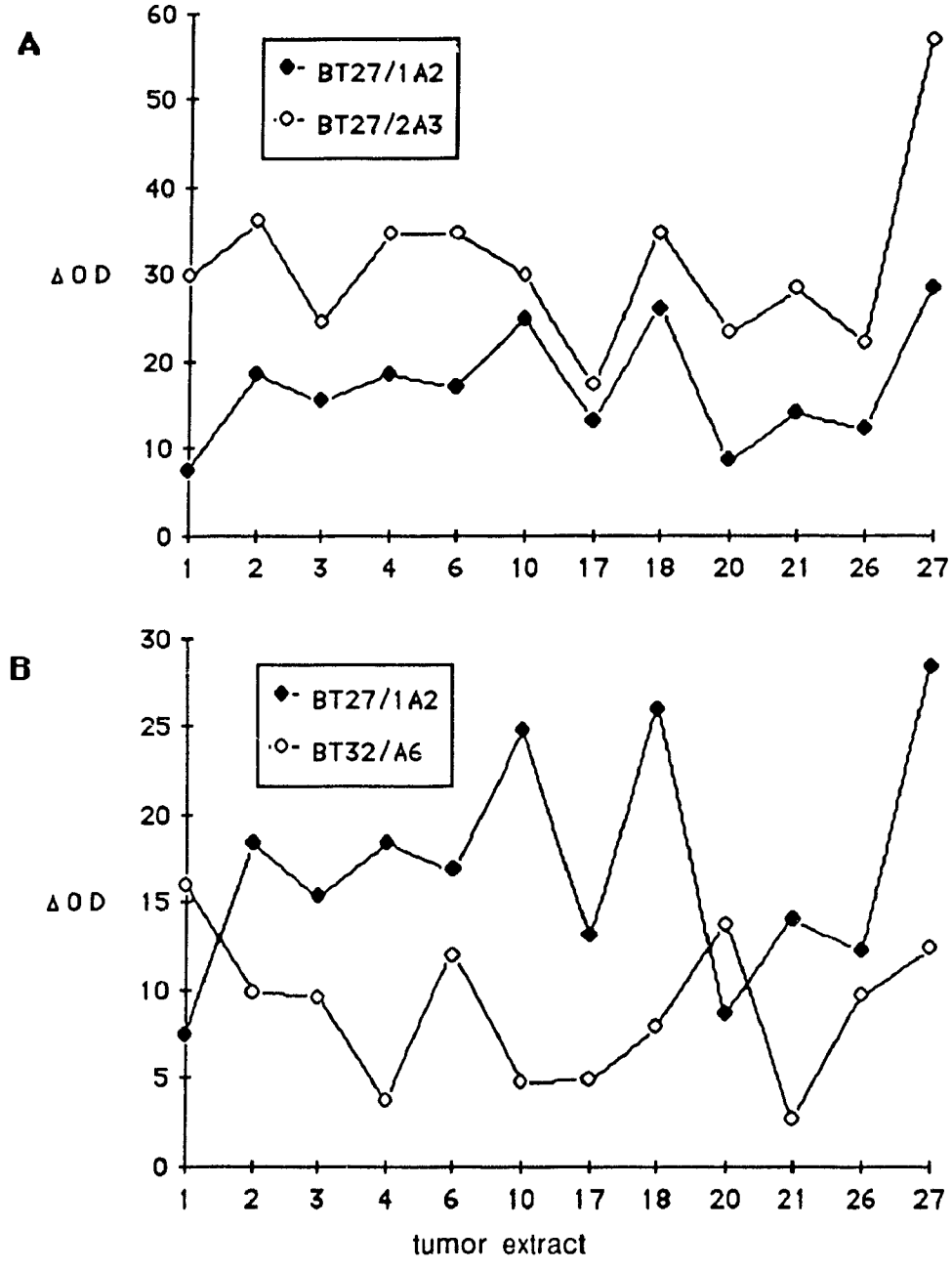
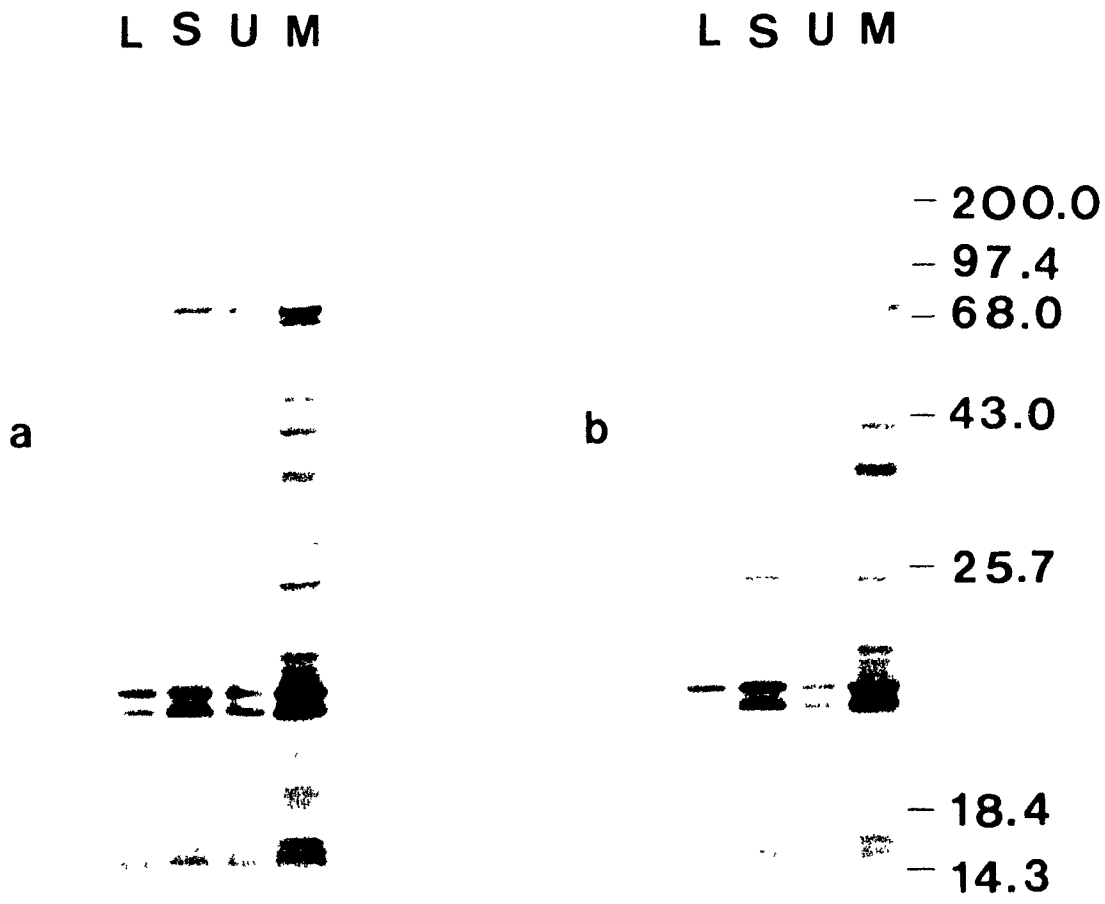


Figure 4.8: HmAb reactivity with extracts of allogeneic tumors

Abscissa is tumor extract BT number (see Table 3.1 for diagnosis), ordinate is O.D. (405 nm) above backround. See section 4.9 for details.

Figure 4.9: Western blots of tumor antigens

Western blots of cell membrane vessicles were prepared as outlined in section 2.2.9. Each lane represents one of the cell lines tested, L=LN-340 ($GFAP^-$ glioma), S=SK-MG-1 ($GFAP^-$ glioma), U=U-373 ($GFAP^+$ glioma), M=M-4 (melanoma). On the left (a) is BT27/2A3, and on the right (b) is control IgM, BT27/2D2. No appreciable difference in banding pattern is apparent. Molecular weight standards (kDa) are to the right of the diagram.


L S U M
a
L S U M
b
200.0
97.4
68.0
43.0
25.7
18.4
14.3

protein and glycoproteins were observed after prolonged development.

Dot blots of total lipid extracts from cultured neuroectodermal cell lines are represented in Figure 4.10. LN-340 and M-4 are glioma and melanoma cell lines which have been previously shown to be unreactive with any of the five HmAbs (see section 4.6). Compared to control HmAb BT27/2D2, there was some indication of potential reactivity for all five HmAbs when tested against glycolipids from the glioma cell line U-373. As for the glioma line SK-MG-1, all the HmAbs except for BT54/B8 seemed to exhibit some degree of reactivity to the lipid extract, although the quality of these dot blots was not entirely satisfactory.

Figure 4.11 illustrates immunochromatography on total lipid extract of SK-MG-1, using BT34/A5 and the parental line, TM-H2, as control. There is a single specific band with $R_f \approx 0.60$. As well, a non-specific band with $R_f \approx 0.80$ was evident in both the BT34/A5 and TM-H2 lanes. In separate experiments, similar results were obtained for BT27/1A2 and BT27/2A3 compared to BT27/2D2, i.e. a specific band at $R_f \approx 0.60$. Immunochromatography with HmAb BT54/B8 failed to reveal the presence of any specific banding pattern, which is puzzling, but consistent with the dot blot experiment.

Figure 4.10: Lipid dot blots of tumor antigens

Dot blots of total lipid extracts of the same cell lines used in the Western blots (Fig. 4.9) were prepared as described in section 2.2.10. Each dot blot was exposed to one of the five HmAbs (BT27/1A2, BT27/2A3, BT32/A6, BT34/A5, and BT54/B8), and control IgM (BT27/2D2). For all 5 HmAbs, the intensity of the DAMB reaction product is significantly above backround in the case of U-373, but unchanged from control for LN-340 and M-4. All HmAbs except BT54/B8 appear to react positively with total lipid extract of SK-MG-1. This pattern of reactivity is similar to that observed with flow cytometry (section 4.6), and suggests that the antibodies recognize a cell surface glycolipid.

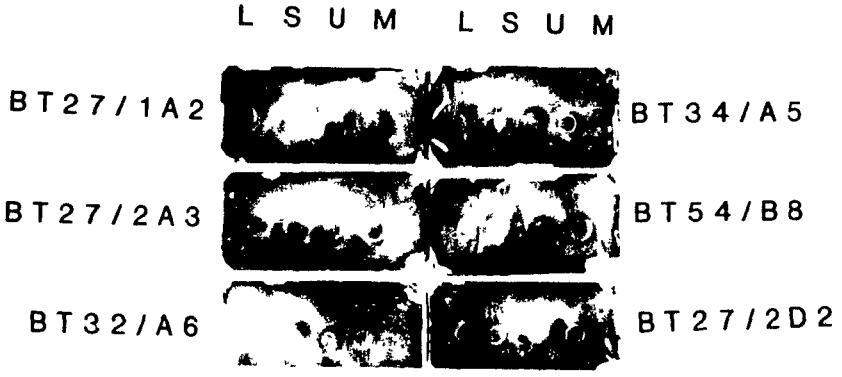

L S U M L S U M
BT27/1A2
BT34/A5
BT27/2A3
BT54/B8
BT32/A6
BT27/2D2

Figure 4.11: Immunochromatography of SK-MG-1 lipid extract

Total lipid extracts were prepared from SK-MG-1 cells, and chromatographed onto HPTLC plates. The plates were exposed to either BT34/A5, BT54/B8, or TM-H2 (control) supernatant. Following detection using an immunoperoxidase reaction, both specific and non-specific labelling was observed. There is a specific band with R_f = 0.61 in the BT34/A5 lane, which represents the glycolipid identified by this antibody. No such labelling was observed for BT54/B8.

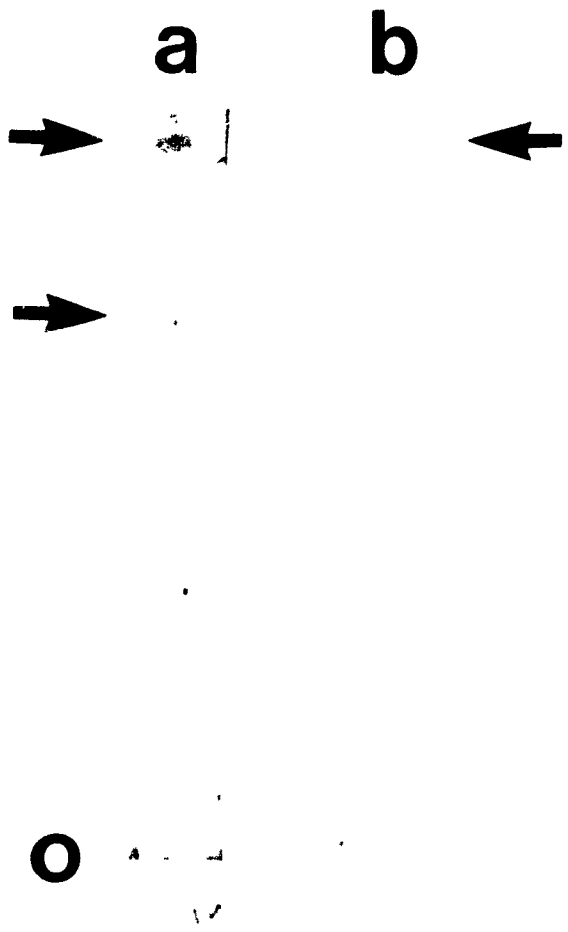
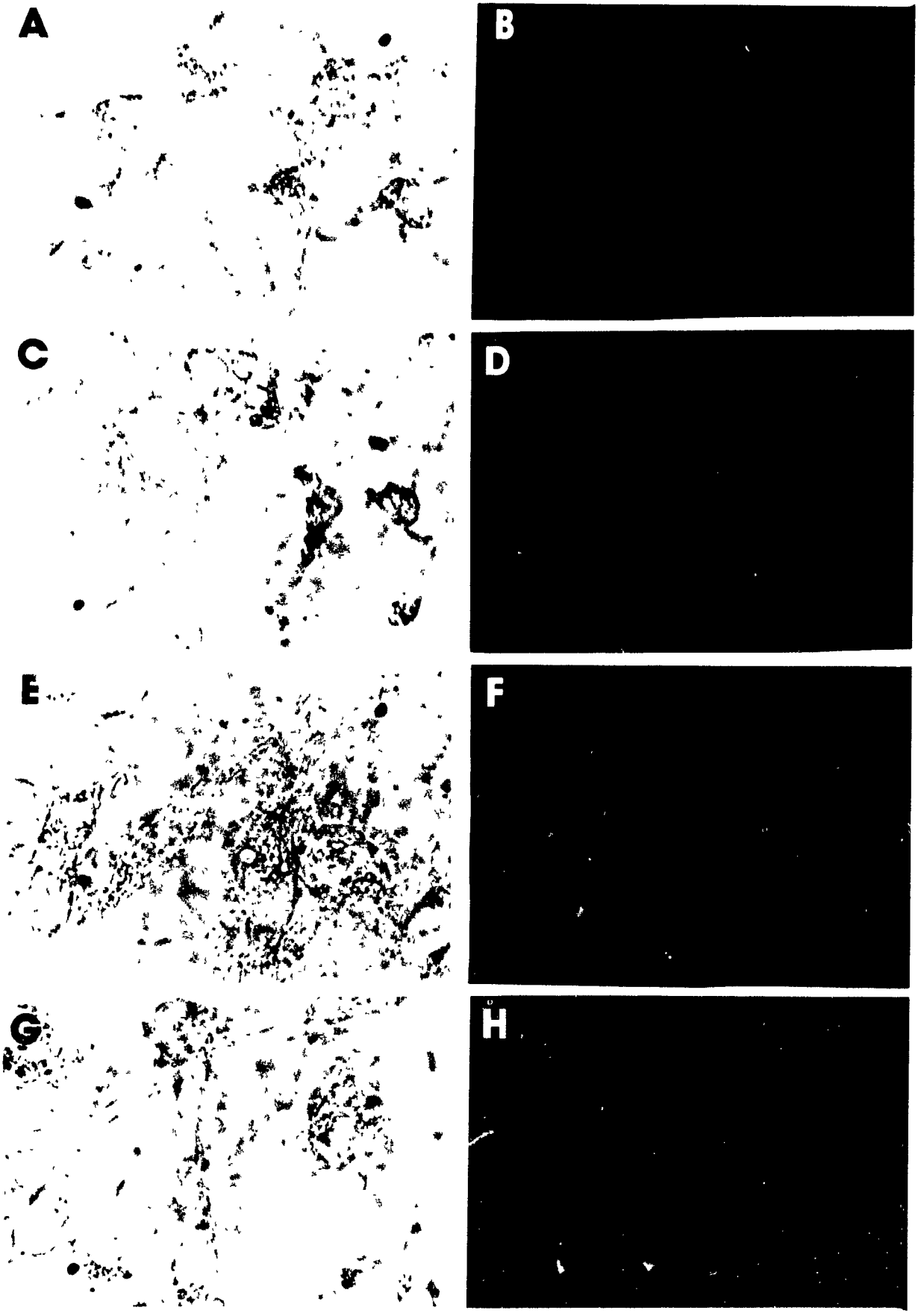

a
b
o

Figure 4.12: Reactivity with normal human astrocytes

Cultured human normal astrocytes derived from patients undergoing craniotomy for epilepsy were grown on glass coverslips and reacted with each of the three HmAbs. Panels (a) and (b) are control IgM antibody BT27/2D2, (c) and (d) are BT27/1A2, (e) and (f) are BT27/2A3, and (g) and (h) are BT32/A6. The left hand panels are phase contrast photographs of the astrocytes, with immunofluorescence represented on the right. None of the HmAbs appear to react with normal astrocytes. All exposure times for fluorescence photography were the same.


A
B
C
D
E
F
G
H

These results suggest that the HmAbs recognize a determinant of a glycolipid, or ganglioside, which was not detected in association with glycoproteins.

4.11 Reactivity with Normal Human Astrocytes

Initial attempts at FCM analysis using single cell preparations of non-neoplastic cerebral tissue obtained from patients undergoing craniotomy for epilepsy were unsuccessful, largely due to the poor quality of the preparations. Cultured human astrocytes were therefore assessed for labelling with the HmAbs. All reagents were checked for immune reactivity against an established positive human glioma cell line (U-373). None of the HmAbs BT27/1A2, BT27/2A3, and BT32/A6 appeared to label cultured astrocytes from two different individuals, as represented in Fig. 4.12. Antibodies BT34/A5 and BT54/B8 also failed to label normal human astrocytes on at least one occasion.

4.12 Discussion

Reports of HmAbs from patients with malignant gliomas have appeared in the literature, however, none of these antibodies have been particularly well characterized. Sikora, et al. (1982) were the first to report the production of HmAbs reactive to 0.25% glutaraldehyde-fixed glioma cells. These investigators obtained intratumoral lymphocytes from 12 patients undergoing craniotomy for

malignant glioma, and fused them to the EBNA$^+$, 8-azaguanine-resistant human lymphoblastoid line LICR-LON-HMy2, which is known to secrete γ and λ chains. A total of 71 hybridomas were obtained from 5 patients.

Hybridoma supernatants were found to contain κ, μ, α, δ, and ε chains in addition to the expected γ and λ chains. Proof of the hybrid nature of the cells was established by FCM determination of DNA content. In all cases, hybridoma DNA content was approximately twice that of the parental line, LICR-LON-Hmy2.

No subcloning experiments were reported in this particular study, and no attempt was made to prove the monoclonality of the hybridomas. In a subsequent paper (Phillips, et al. 1983), the same group of authors reported a single heavy and light chain in the supernatant of one of their anti-glioma HmAbs, LGL1-1D6, using SDS-polyacrylamide gel electrophoresis of ^{131}I-labelled immunoglobulin.

Antibody titration curves for concentrated LGL1-1D6 were reported by Sikora, et al (1983), using the same RIA used to screen the hybridomas. No statistical comparisons were made at each dilution level, however, the curve tended to converge with the control (LICR-LON-HMy2) at a dilution of 1/64. No initial plateau was seen. The authors concluded that the antibody was of a low affinity because

of the high concentrations which were necessary to demonstrate reactivity with targets.

Variation in antigenic expression with cell proliferation and cell density has been described in others systems using FCM (Leong, et al. 1985); however, the results appear to be opposite to those observed with SK-MG-1. Using a murine mAb (MM2-3C6) to a murine B16 melanoma-associated surface antigen, the authors reported a 1.68-fold increase in antigen density (estimated by FCM) during the logarithmic growth phase, followed by a drop to 37% of peak value after the cells had been confluent for 3 days. In contrast, the present study observed a significant increase in antigenic expression after the SK-MG-1 cells reached or had been left at confluence.

The fact that all five HmAbs in this study were of the IgM isotype may be significant. Previous work with TM-H2 (Sullivan, et al. 1982) has shown that this particular myeloma-like cell line will fuse equally well with B-lymphocytes of all isotypes, unlike lymphoblastoid cell lines, which fuse more readily with IgM secreting cells (see section 1.6.1). Secondly, at least three studies (Tokumaru and Catalano, 1975; Mahaley, et al. 1977; Bhondeley, et al. 1988) have shown that whereas overall Ig levels are normal in most patients with intracranial tumors, elevated pre-operative levels of IgM are frequently detected in patients with glioblastomas. Another

explanation for finding only IgM HmAbs may lie with the glioma immunosuppressive mechanisms described in section 1.3.5, which might prevent the elaboration of a secondary (IgG) immune response in these patients. Finally, the fact that all five HmAbs were IgM may simply be a reflection of the type of antigen which they recognize (i.e. a carbohydrate).

Cross-reactivity of anti-glioma HmAbs with other tumor cell lines was reported in the original publication by Sikora, et al. (1982). Out of 9 HmAbs studied with a panel of tumor cell lines, 4/9 reacted to a colon carcinoma (HT-29), 3/9 with the lung carcinoma MOR, and 1/9 with the lung carcinoma CALU-1. This should be compared with only 2 out of 19 non-gliomatous tumor cell lines exhibiting cross-reactivity with BT27/1A2, BT27/2A3, and BT32/A6, and only 1 out of 12 non-gliomatous tumor cell lines cross-reacting with BT34/A5 and BT54/B8.

In addition, there is no mention if any of the Sikora, et al. (1982) hybridomas were proven to be monoclonal in nature. The possibility of multiple Ig moieties with multiple reactivities must be raised, although there is ample evidence in the literature of murine monoclonal hybridoma antibodies with multiple tumor reactivities.

Other HmAbs cross-reactive with glioma-associated antigens have been produced and characterized. Houghton, et al. (1983) described a series of fusions between human

lymphocytes from patients with malignant melanomas and the human lymphoblastoid cell lines, LICR-LON-HMy2 and GM 4672, or the human myeloma line SKO-007. A number of stable clones were generated which produced HmAbs reacting with cell surface, cytoplasmic, or nuclear antigens on a variety of different normal and malignant cells.

One particular IgM anti-melanoma HmAb (Ma4) was found to cross-react weakly with cell surface antigens on two human glioma cell lines, SK-MG-1 and U-373. Ma4 was derived from a fusion between regional lymph node lymphocytes and LICR-LON-HMy2. It has been subcloned (1 cell/well) four times, and has maintained stable production of IgM (5 μg/ml) and IgG (2 μg/ml) over a 12 month period. The Ma4 cell line is tetraploid by FCM and contains only human chromosomes by karyotypic analysis. SDS-PAGE analysis demonstrated two distinct heavy chains, μ and γ, and light chains κ and λ. Preliminary characterization of the Ma4 antigen revealed it to be a glycolipid

A second IgM anti-melanoma HmAb (M304) was described as reacting to a cytoplasmic antigen found in 9/9 human glioma cell lines. In general, reactivity to cell surface antigens was noted infrequently (6/771 Ig-secreting cultures), whereas reactivity to intracellular antigens was more common (27/771 cultures). These results are in agreement with those of the present study (see section

3.5). The same group later went on to describe four more HmAbs which cross-reacted with glioma cell surface antigens (Cote, et al. 1986).

Perhaps the most extensively studied HmAb cross-reactive with a glioma cell surface antigen is the IgM(κ) molecule produced by the human B-lymphoblastoid cell line L72 (Irie, et al. 1982). This cell line was derived from a patient with a malignant melanoma by Epstein-Barr transformation of PBL. It secretes IgM at a level of 9 μg/ml in the spent medium of 1 x 10^6 cells within 4 to 5 days of subculture.

The L72 antibody binds weakly in an IA assay to four cultured human glioma lines: AS, LA-N-1, A172 MG, and SK-MG-3, but not to SK-MG-1 (Katano, et al. 1983). The antigen recognized by the L72 antibody was determined to be ganglioside GD2 (Cahan, et al. 1982). Given the lack of cross-reactivity with SK-MG-1, it seems unlikely that any of the three HmAbs discussed in the present study recognize GD2.

In contrast with these results is a study by Watanabe, et al. (1982) in which serum from a patient (AH) with a malignant melanoma was reported to cross-react with SK-MG-1 and U-373 cells. Partial purification of the gangliosides from autologous melanoma cells (SK-MEL-13) revealed that one of the minor components, which co-migrated with GD2 in thin layer chromatograms, was able to inhibit AH serum

reactivity. Using an IA assay and SK-MEL-13 ganglioside fractions (Pukel, et al. 1982), 1 µg of purified GD2 was determined to be sufficient for a 50% reduction in reactivity.

If AH serum contains an anti-GD2 antibody, then the serum must also be reacting with something other than GD2 on SK-MG-1 cells if we are to believe that the L72 antibody is also anti-GD2, and unreactive with SK-MG-1. In support of this viewpoint that AH serum contains other antibody activities, is a report by Cheresh (1985) in which a murine mAb, 126, of established anti-GD2 specificity was found not to react with U-373, whereas AH serum does.

An alternative explanation for the contradictory results concerning the presence of GD2 on SK-MG-1 cells is suggested by the work of Nores, et al (1987). These authors reported the production of a murine mAb, M2590, derived from mice immunized with syngeneic B16 melanoma cells. The M2590 antibody was subsequently shown to be directed to ganglioside GM3. Studies on the reactivity of M2590 with various cells having different GM3 densities at their cell surface indicated that: 1) M2590 reactivity depends greatly on the density of exposed GM3, 2) there is a threshold density that is recognized in an all-or-none fashion, 3) M2590 reacts not only with GM3, but also with GM3 lactone, only much more strongly, and that the latter may be the true immunogen since GM3 is widely distributed

in many types of animal cells and would not be thought of as normally eliciting an immune response.

The role of glycolipids and carbohydrate structures in cellular interaction, differentiation, and oncogenesis has received much attention in the past decade, and has been extensively reviewed elsewhere (Hakomori, 1981; Hakomori, 1984; Feizi, 1985; Reading and Hutchins, 1985; Feizi and Childs, 1988). A subclass of glycolipids known as gangliosides (Fig. 4.13) may play an important role in the regulation of cellular adhesion and proliferation.

The disialogangliosides GD2 and GD3 are well represented on surface of cultured melanomas and neuroblastomas. Using mAbs directed to the carbohydrate moieties of these two molecules, Cheresh, et al (1986) were able to inhibit cell attachment of cultured melanoma and neuroblastoma cells to various extracellular matrix proteins, e.g. collagen, fibronectin, and laminin.

Gangliosides have also been implicated as surface receptors for tetanus and cholera toxins (van Heyningen, 1974), interferon (Besançon and Ankel, 1974), Sendai virus (Markwell, et al. 1981), and influenza A virus (Suzuki, et al. 1985). In addition, the receptor for platelet-derived growth factor can be functionally modulated by changes in gangliosides (Bremer, et al. 1984). Glycosidic side chains reportedly have roles in regulation of insulin receptor affinity (Podskalny, et al. 1984) and in the function of

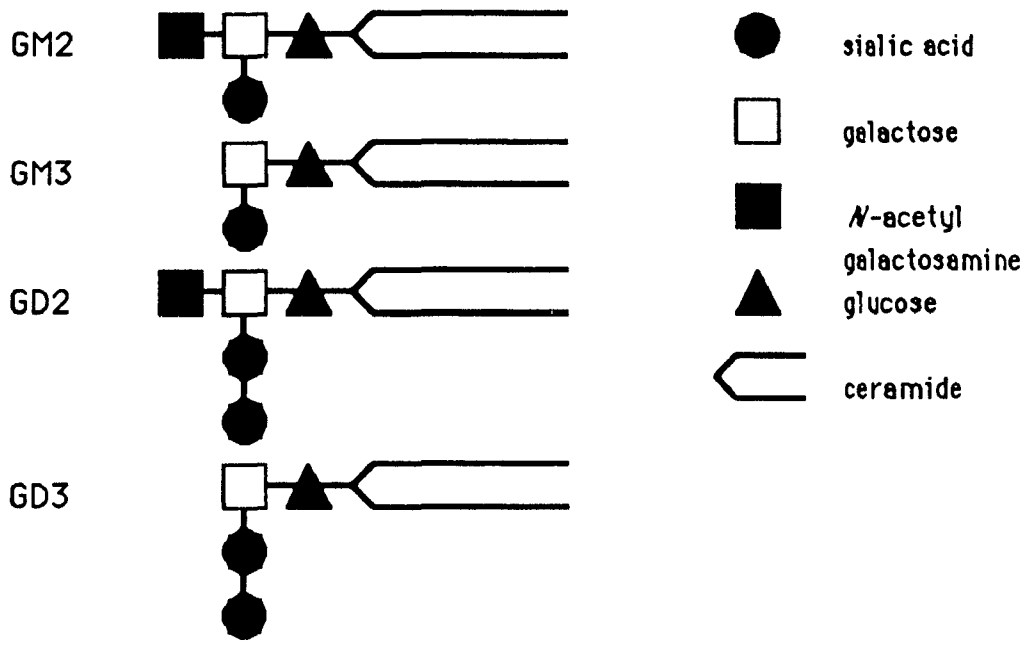


Figure 4.13: Structures of tumor-associated gangliosides

Schematic representation of ganglioside structures. Adapted from Reisfeld and Cheresh (1987).

low density lipoprotein receptors (Kingsley, et al. 1986). Changes in blood group antigens can also be a feature of malignant transformation. Loss of A-antigen determinants on epidermal growth factor receptors reportedly results in high affinity receptors, enhanced tyrosine kinase activity, and a shorter receptor half-life (Defize, et al. 1988).

When a cell undergoes malignant transformation, there may be subtle qualitative and quantitative changes in the chemistry of its surface gangliosides. Berra, et al. (1985) examined the ganglioside content of freshly resected human astrocytomas ranging in grade from I to IV (glioblastoma multiforme), and noted a statistically significant increase in the content of ganglioside GD3 when passing from the lower to the higher grades. In addition, the authors noted a decrease in polysialylated species with increasing levels of malignancy.

Finally, ganglioside expression may be altered *in vivo* and *in vitro*, but appears to be an intrinsic property of the culture system being used. In a study of 52 biopsied and 28 cultured human melanomas, Tsuchida, et al. (1987) reported that GM2, GM3, GD3, GD2, and GT1b were present in 74% and 49% of the samples, respectively. When biopsied melanoma cells were then passaged *in vitro*, the ganglioside composition changed. After transplantation into nude mice, however, the ganglioside composition was converted back to the same pattern as in the original surgical specimens.

This would suggest that loss of ganglioside expression *in vitro* results from a change of culture environment, rather then selection of cells with one particular phenotype.

The five HmAbs described in this section all appear to be directed against cell surface glycolipids, based on the results of the dot blot and immunochromatography experiments. Loss of reactivity following treatment with neuraminidase would strongly suggest that they recognize the carbohydrate component of gangliosides.

It is interesting to note that despite some minor differences in the pattern of reactivity against a panel of human tumor cell lines (section 4.6), all five HmAbs appear to be recognizing strikingly similar molecular substances, both in terms of biochemical composition and biological distribution. It should be emphasized that these HmAbs were derived from four separate individuals as clinically diverse as a 14 year old male with von Recklinghausen's disease harboring a low grade astrocytoma, and a 53 year old alcoholic male with a glioblastoma. As well, the HmAbs appear to recognize a substance(s) which also appears on explant cultures of meningiomas and medulloblastomas (see section 4.7).

Complete characterization of the glycolipid(s) identified by these five HmAbs may shed some light on the host anti-tumor immune response in patients with neurological tumors, as well as permit the generation of

new mAbs against the same, or related substances. It may also be possible to immunize glioma patients with the gangliosides themselves, as others (Livingston, et al. 1987) have done with melanoma patients using a mixture of GM2 and bacille de Calmette-Guérin.

5 BIOLOGICAL SIGNIFICANCE OF A GLIOMA CELL SUBPOPULATION IDENTIFIED BY HUMAN MONOCLONAL ANTIBODIES

5.1 Recognition of a Subpopulation of Glioma Cells

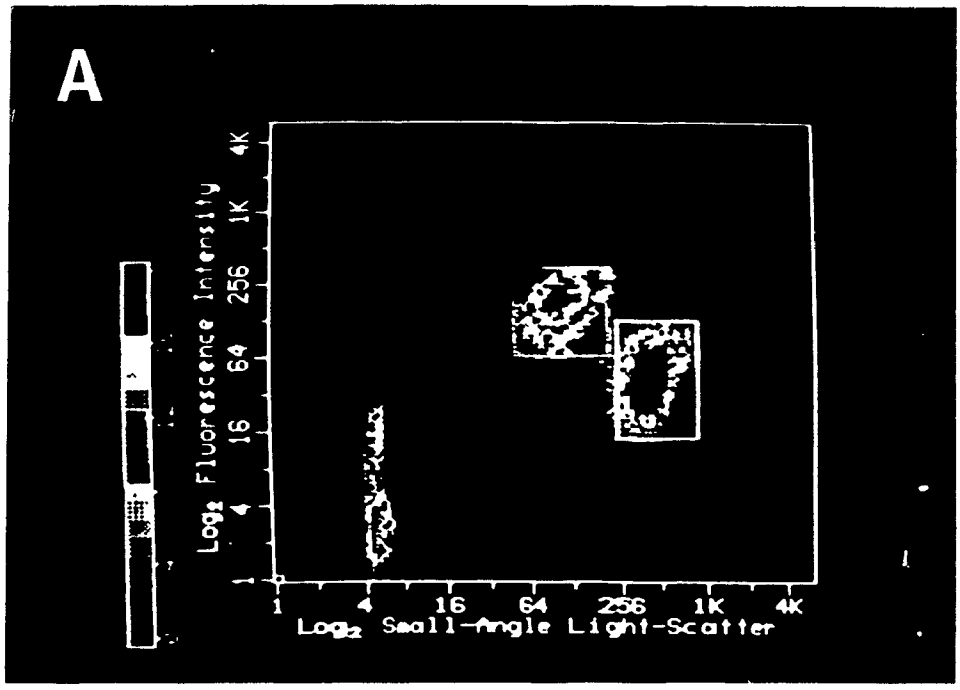
Initial studies of the human glioma line, SK-MG-1, using 2-dimensional FCM revealed two discrete subpopulations of cells (Fig. 5.1). These consisted of a smaller subpopulation, both proportionally and also in terms of small angle scatter, and a larger subpopulation, which contained the majority of the cells for this particular line. These will be referred to as S-cells and L-cells, respectively.

After reacting SK-MG-1 with either of the three HmAbs described in the previous chapters, there appeared to be selective labelling of only the S-cell subpopulation (Fig. 3.2). This selective labelling was consistently observed over many experiments, and was also observed in many other tumor cell lines which also contained two discrete subpopulations (e.g. HeLa, Fig. 5.2).

In another experiment to determine if a similar phenomenon existed in primary gliomas, a short term culture was prepared from a glioblastoma multiforme biopsy (BT-54) as outlined in section 2.2.5, then fluorescently labelled with HmAb BT32/A6, and analyzed in the FACS-III cell sorter

Figure 5.1: Flow cytometric analysis of SK-MG-1 cells

SK-MG-1 cells were analyzed by flow cytometry as outlined in section 2.1.9. Log_2 small angle scatter is represented on the abscissa, and log_2 fluorescence on the ordinate. The color scale to the left of each panel indicates relative cell number. Panel A represents control (BT27/2D2) labelled cells. For purposes of comparison, a red box has been placed around the 'small' cells, and a yellow box encloses the 'large' cells. In Panel B, the cells have been labelled with HmAb BT27/2A3. There is a significant shift in fluorescence affecting only the 'small' cell subpopulation.


A
Log2 Fluorescence Intensity
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4
16
64
256
1K
4K
Log2 Small-Angle Light-Scatter

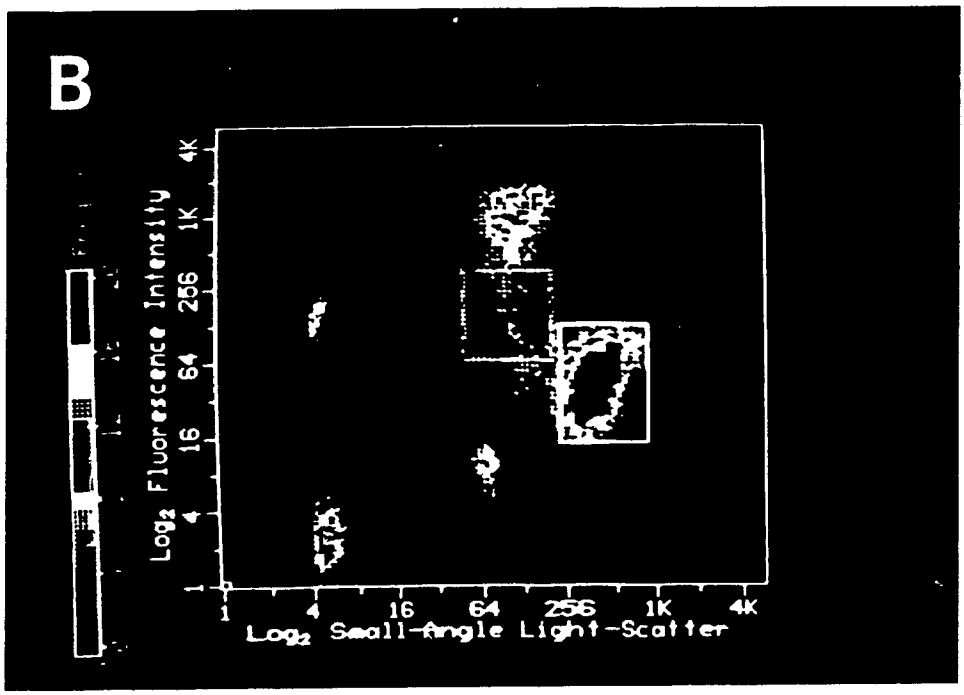
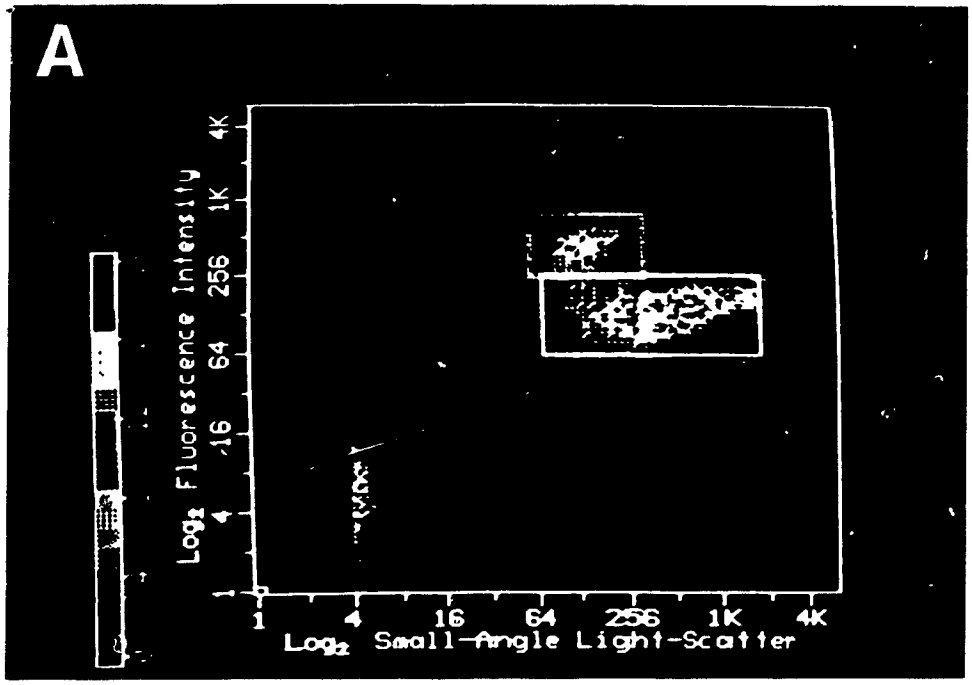

B
Log2 Fluorescence Intensity
1
4
16
64
256
1K
4K
Log2 Small-Angle Light-Scatter

Figure 5.2: Flow cytometric analysis of HeLa cells

HeLa cells were analyzed by flow cytometry as outlined in section 2.1.9. Log_2 small angle scatter is represented on the abscissa, and log_2 fluorescence on the ordinate. The color scale to the left of each panel indicates relative cell number. Panel A represents control (BT27/2D2) labelled cells. For purposes of comparison, a red box has been placed around the 'small' cells, and a yellow box encloses the 'large' cells. In Panel B, the cells have been labelled with HmAb BT27/2A3. There is a significant shift in fluorescence affecting only the 'small' cell subpopulation.


A
Log2 Fluorescence Intensity
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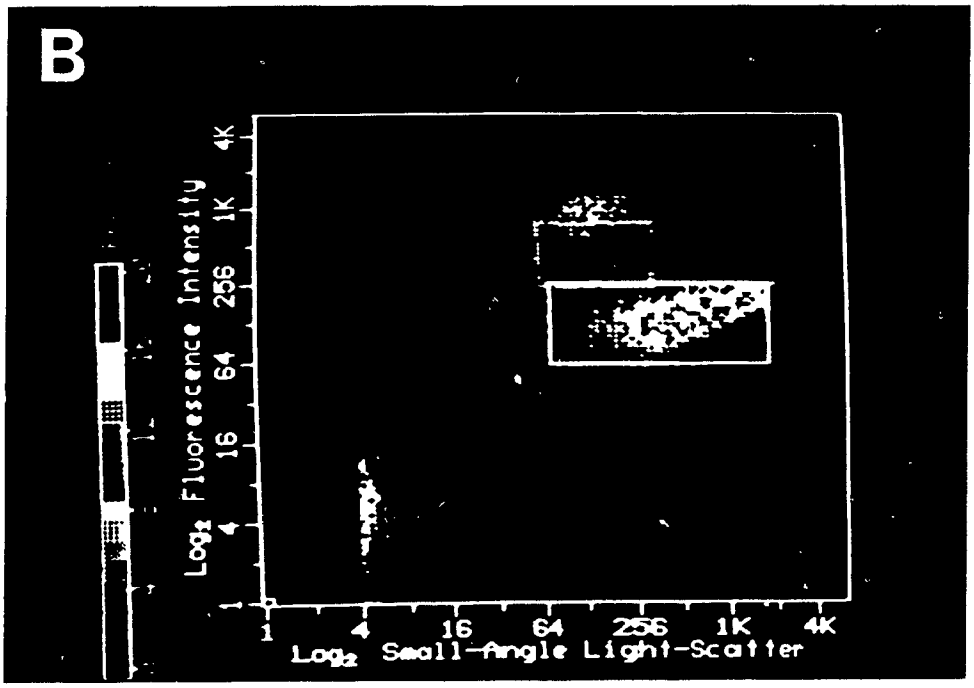
Log2 Small-Angle Light-Scatter


B
Log2 Fluorescence Intensity
1
4
16
64
256
1K
4K
Log2 Small-Angle Light-Scatter

using multiple parameters. With gating set on total cell scatter minus debris, it was first confirmed that labelling occurred in this particular tumor.

Next, the gating was switched to the high fluorescence range, and scatter data was collected for both labelled and control samples. The results are displayed as two overlapping scatter diagrams for each of the two samples, and indicate a significant 'shift' towards the apparent S-cell peak for the BT32/A6 specimen (Fig. 5.3). This implies that labelling in this sample was more significantly associated with the S-cell region.

5.2 Generation of Sublines from SK-MG-1

At first it was felt that the S- and L-cells might represent two functionally distinct lineages. In order to address this possibility, ten randomly generated sublines of SK-MG-1 (SK-MG-1.1 to SK-MG-1.10) were established by dissociation of an existing culture, followed by dilution and seeding into 96-well plates. Careful observation of the developing colony morphology ensured the selection of only monoclonal sublines. Ten were chosen at random, expanded to the T25 flask stage, labelled with BT27/2A3, and analyzed by FCM.

Out of ten randomly generated sublines, all sublines were observed to contain S- and L-cell subpopulations in varying proportions. In addition, there was a strong

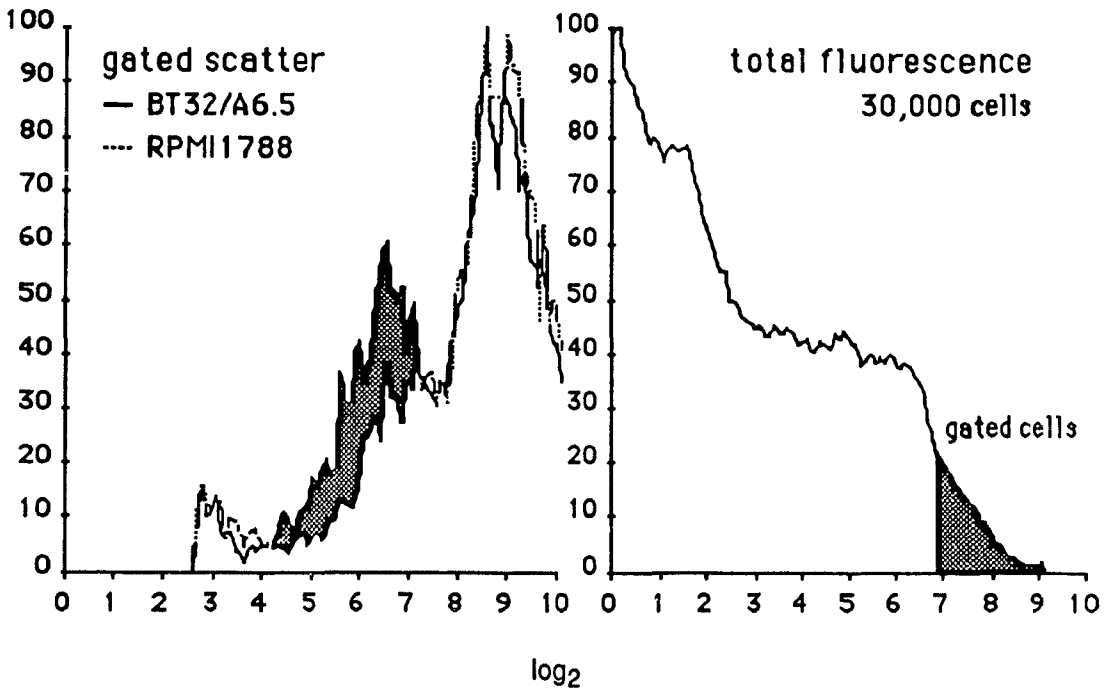


Figure 5.3: BT32/A6 labelling of an allogeneic glioma explant

Tumor tissue from a patient with a malignant glioma was grown in short term culture and labelled with BT32/A6 as outlined in section 2.2.5. The cells were analyzed by FCM with gating (shaded area) set on high fluorescence (right hand side of the diagram; $\log_2$ fluorescence on the abscissa, arbitrary units, and relative cell number on the ordinate, plotted to peak height). The corresponding scatter distributions for BT32/A6 and control IgM (RPMI 1788) labelled cells are shown on the left hand side of the diagram ($\log_2$ small angle scatter on the abscissa, arbitrary units, and relative cell number on the ordinate, plotted to peak height). The shading indicates that a predominance of cells with low small angle scatter and high fluorescence intensity were found in the BT32/A6 labelled sample.

correlation between the percentage of S-cells, and overall reactivity with antibody BT27/2A3 (r = 0.936; $p<0.01$) among the ten sublines. This relationship is illustrated in Figure 5.4.

The appearance of S- and L-cell subpopulations in 10 out of 10 randomly generated monoclonal sublines of SK-MG-1 favored a stem cell or a cell cycle explanation of the double subpopulation phenomenon. To investigate this, attention was focused on the phenotypic differences between the two cell types.

5.3 Effect of FCS Concentration on Tumor Subpopulations

As postulated by the tumor stem cell hypothesis, (outlined in section 1.1.4), a stem cell would be phenotypically more anaplastic than its differentiated progeny. This may be reflected in a number of ways, including preferential growth at low fetal calf serum concentrations.

To examine whether there were such phenotypic differences between the S- and L-cell subpopulations of SK-MG-1, an equal number of SK-MG-1 cells were seeded into tissue culture flasks containing different concentrations of FCS, and incubated under standard conditions for 4 days. The cells were then scraped off and analyzed by FCM. The

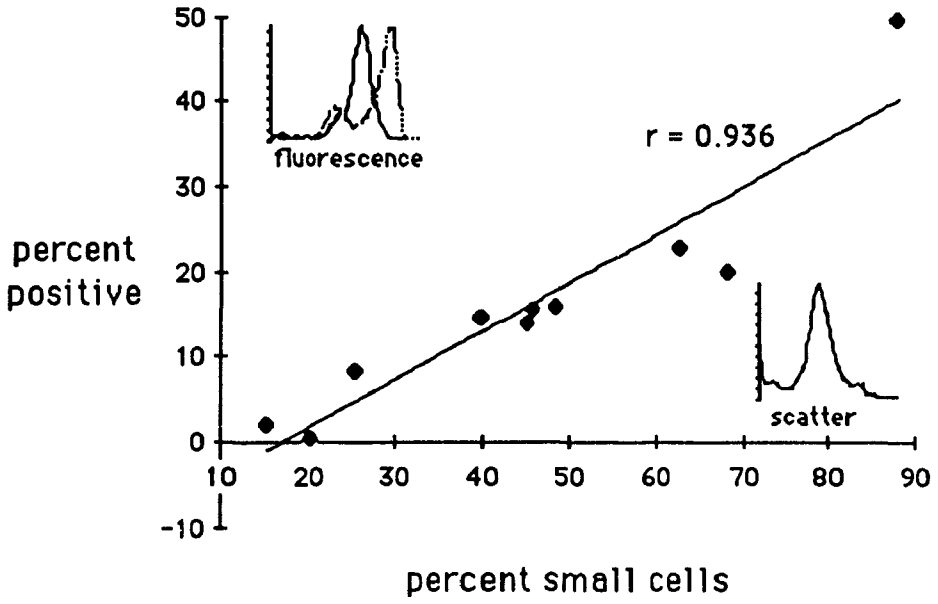


Figure 5.4: Sublines of SK-MG-1

Ten randomly selected monoclonal sublines of human glioma line SK-MG-1 were labelled with BT27/2A3 and analyzed by FCM. All 10 sublines contained small and large cell populations in varying proportions. The degree of positivity in each subline (ordinate) was directly proportional to the percentage of small cells (abscissa). Insets are examples of small angle scatter and fluorescence distributions for one particular subline, SK-MG-1.1, whose composition was predominantly of small cells. Solid line represents labelling with control antibody BT27/2D2, and dotted line represents labelling with BT27/2A3.

proportion of S- vs. L-cells was estimated and plotted as a function of FCS concentration.

Results are shown in Figure 5.5, which also shows the total number of viable cells per flask, and the estimated number of small cells. These results suggest that the S- and L-cell subpopulations possess a different growth response to FCS concentration. At low concentrations, the L-cell subpopulation is decreased compared to high FCS concentrations; whereas the growth of S-cells appears unaffected by FCS concentration.

5.4 Generation of Subpopulation-Derived Sublines from SK-MG-1

Earlier studies of monoclonally-derived sublines of SK-MG-1 involved the generation of randomly selected, unsorted clones. In these experiments, monoclonally-derived sublines were established from sorted S- and L-cells of SK-MG-1 (Fig. 5.6). The flow cytometric properties of the resulting monoclonal S- and L-cell derived sublines (S101-10, and L101-10, respectively) were then compared to those of the parental line, SK-MG-1. Three important conclusions were drawn:

1) The colony forming efficiency *in vitro* for sorted S- and L-cells was equivalent (approximately 1%).

2) All S- and L-cell derived sublines were found to contain S- and L-cells.

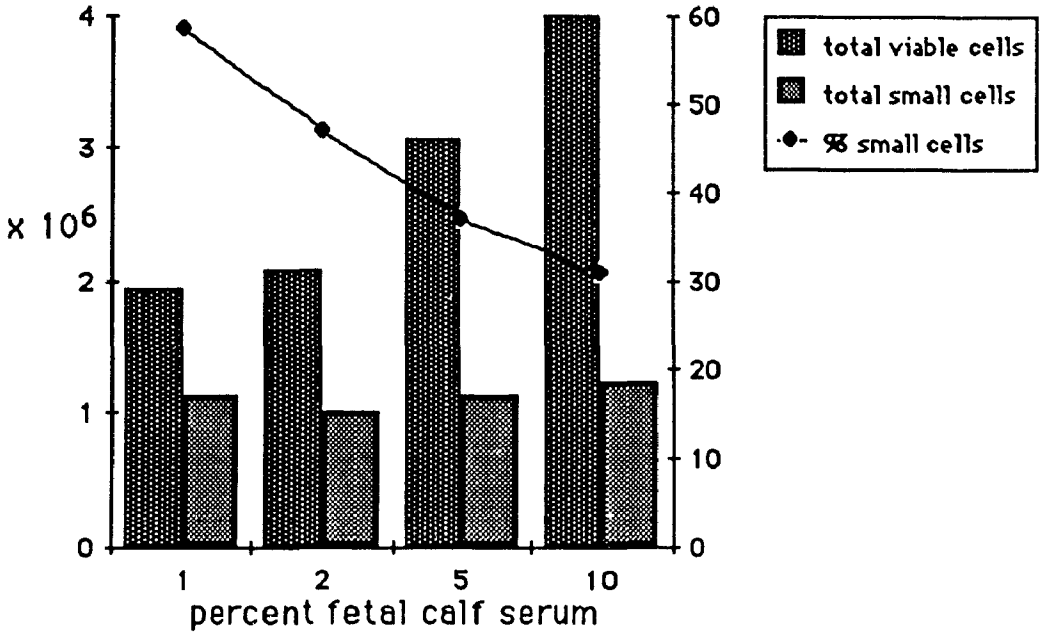


Figure 5.5: Effects of serum concentration on small cell population size

Human glioma line SK-MG-1 was seeded into T80 flasks at 10^6 cells/flask, and grown under standard conditions for 4 days at different FCS concentrations (abscissa). The total number of viable cells (left ordinate) was then counted by the dye exclusion method, and the percentage of small cells (right ordinate) estimated using FCM. The total number of small cells per flask (left ordinate) was then estimated and compared. Results indicate that the total number of small cells per flask remained constant for different concentrations of FCS, whereas the number of large cells present per flask was decreased when FCS concentrations was low.

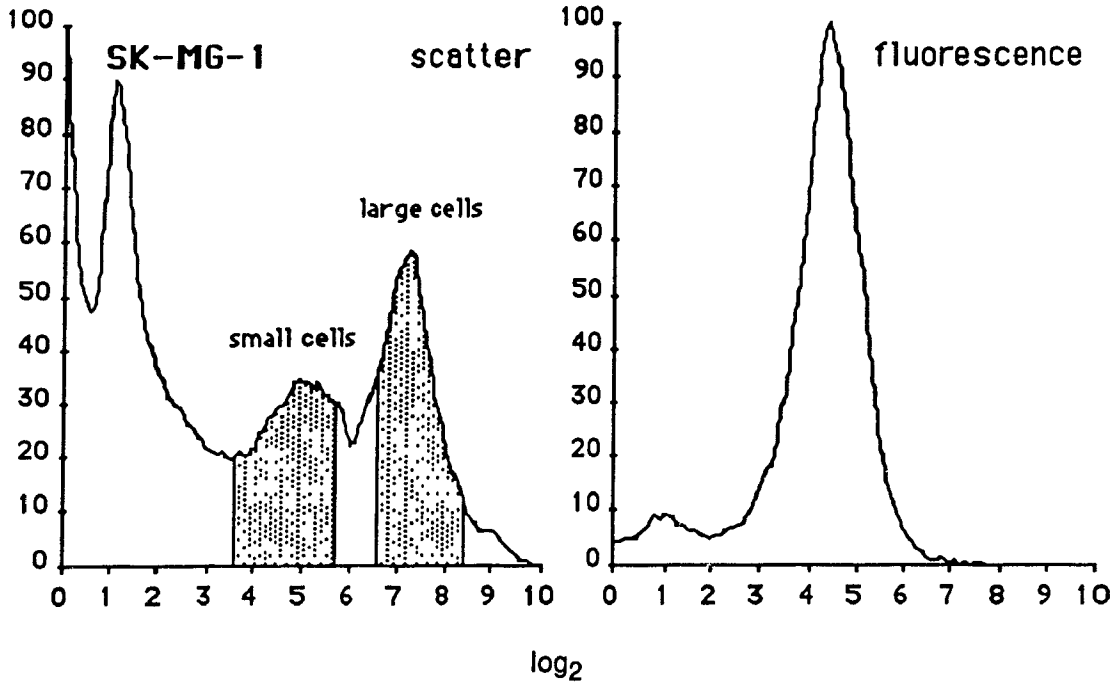


Figure 5.6: SK-MG-1 cell sorting

Shaded areas indicate the small angle scatter gating used to sort small and large cells (left hand side of diagram; $\log_2$ small angle scatter on abscissa, arbitrary units; relative cell number on ordinate). The right hand side of the diagram indicates the total fluorescence distribution for SK-MG-1 ($\log_2$ fluorescence on abscissa, arbitrary units; relative cell number on ordinate).

3) The small angle scatter (optical size) of the S-cells in the L-cell derived sublines was significantly lower ($p<0.05$) than the small angle scatter of the S-cells in the parental line, SK-MG-1. In contrast, the FCM profile of the S-cell derived sublines appeared identical to that of SK-MG-1.

Table 5.1 compares the modal small angle scatter channel number for both S- and L-cells in SK-MG-1 with 8 S-cell derived and 5 L-cell derived sublines. Figure 5.7 is a composite of three small angle scatter distributions: SK-MG-1, and one typical S- and L-cell derived subline. The L-cell peaks for SK-MG-1, S106, and L102 appear to map together, as do the S-cell peaks for SK-MG-1 and S106. The S-cell peak for L102, however, is seven channels lower (44.5% lower after conversion to a linear scale) than the corresponding S-cell peak of SK-MG-1.

Figure 5.8 gives examples of typical FCM sortings of S- and L-cells, centrifuged onto glass slides and stained with Giemsa. Occasional mitotic figures were observed in FCM sortings of L-cells, but very rarely among the S-cells.

These results suggest that there exists a high degree of phenotypic similarity between the S-cell derived sublines and the parental line, SK-MG-1, compared with the L-cell derived sublines. Additional similarities and differences between SK-MG-1 and its S- and L-cell derived

Table 5.1: Small angle scatter data[a] for SK-MG-1 and its small and large cell derived clones

	small cells	*large cells*
SK-MG-1 (parental)	76.75 ± .49	95.50 ± 1.85
	(*n* = 4)	(*n* = 4)
small cell clones	77.75 ± .63	95.94 ± 1.00
	(*n* = 8)	(*n* = 8)
large cell clones	72.80 ± 1.20[b]	98.00 ± .95
	(*n* = 5)	(*n* = 5)

[a]results expressed as mean ± standard error for modal channel number, 128-channel $\log_2$ scale; *n* refers to the number of FACS distributions pooled to obtain these values

[b]$p<0.05$, compared with SK-MG-1 using a two-tailed Student's *t*-test

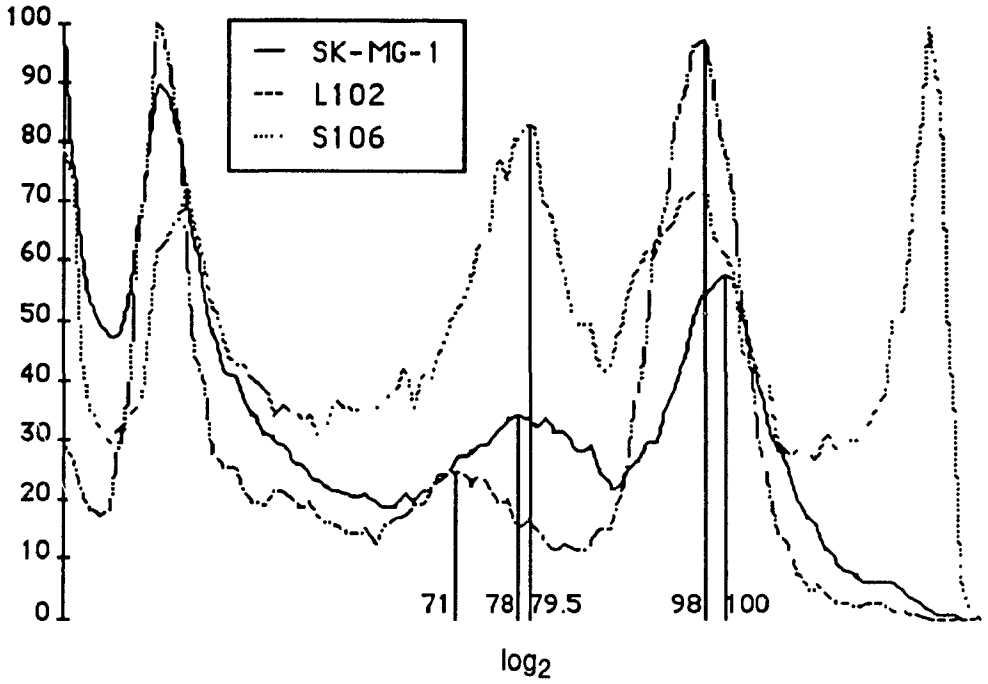
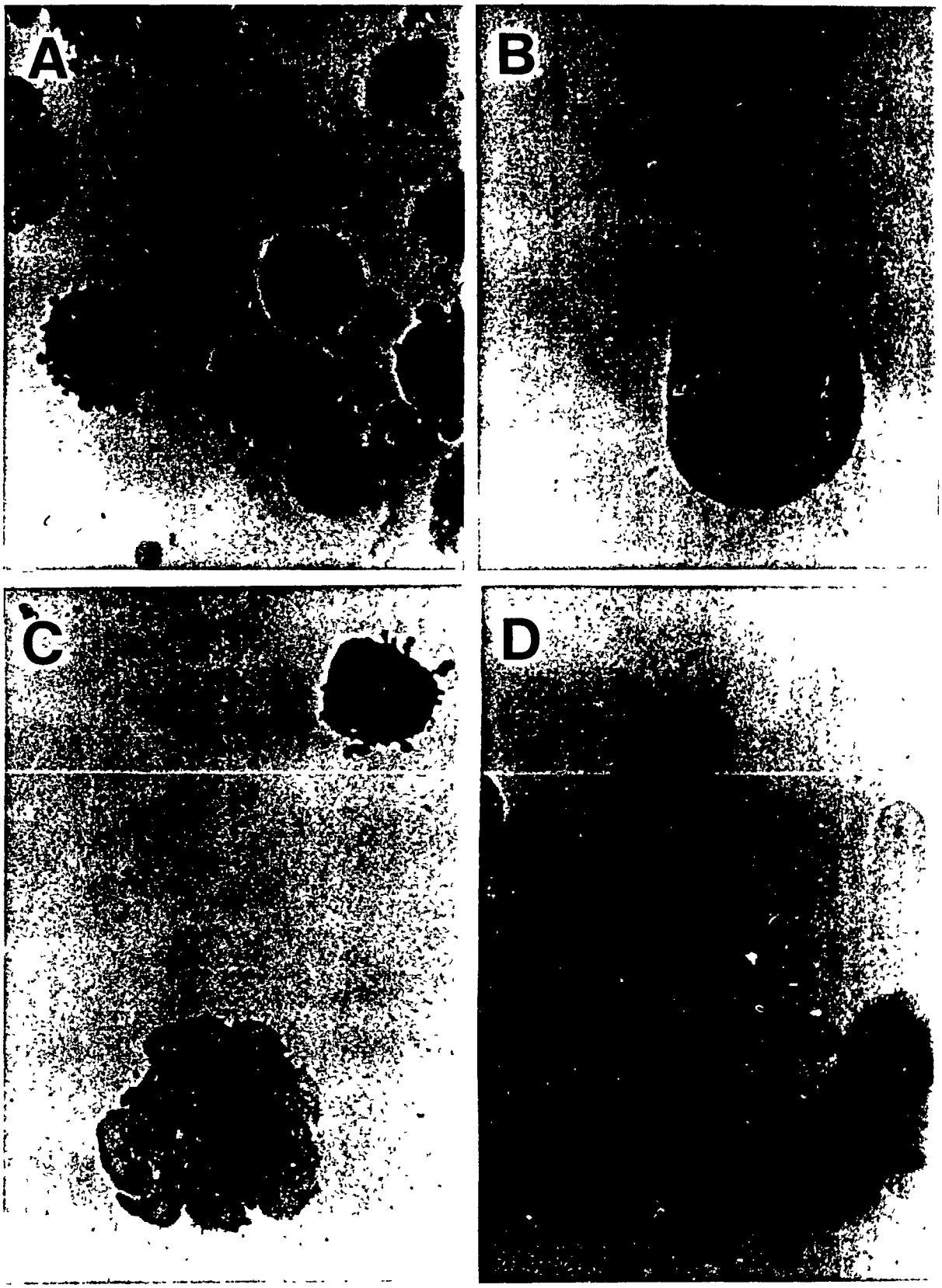


Figure 5.7: Small angle scatter distributions for SK-MG-1, S106, and L102

Log_2 small angle scatter distributions for SK-MG-1 (parental cell line, represented by solid line), L106 (L-cell derived clone, heavy broken line), and S106 (S-cell derived clone, light broken line) are superimposed for comparison. All three cell lines contain a L-cell population in the 98-100 channel range. The S-cell population of S106 overlaps the S-cell population of the parental line, SK-MG-1, however, the S-cell population of L102 is significantly lower than either. Peaks of scatter to the left of S-cell and L-cell subpopulations are dead cells and debris; the peaks of scatter to the extreme right are clumps, cell aggregates.

Figure 5.8: Sorted 'small' and 'large' cells of SK-MG-1

SK-MG-1 cells were sorted according to small angle scatter and fluorescence as outlined in section 2.3.3, centrifuged onto glass slides, and stained with Wright's stain. Panel A represents sorted 'small' cells, corresponding to the red box in Figure 5.1, Panel A; Panel B represents 'large' cells, which were derived from the yellow box in Figure 5.1, Panel A; Panel C is unsorted 'small' and 'large' cells; and Panel D represents unlabelled debris from the red box in Figure 5.1, Panel B. Differences in 'large' cell staining intensity between panels B and C are due to variability in staining technique with the Wright's stain, and not due to inherent differences in the cells.


A
B
C
D

sublines were revealed when total DNA content and cell cycle state were examined.

5.5 Cell Cycle Analysis of SK-MG-1 and Its Sublines

DNA histograms for SK-MG-1 and its S- and L-cell derived sublines were generated by labelling ethanol-fixed cells from confluent cultures with propidium iodide, and sorting based on small angle scatter using the FACS-III flow cytometer. Analysis of sorted S- and L-cells of SK-MG-1 demonstrated clearly that the S-cell subpopulation contained cells which were almost exclusively in the G_1 or G_0 ('resting') portion of the cycle, (Fig. 5.9). The L-cell subpopulation, however, contained cells primarily in the G_1 and S phases, suggesting that they were actively dividing.

No statistically significant differences were found between the DNA histograms of sorted S-cell derived clones and the parental line, SK-MG-1 (Table 5.2). The S-cell derived clones therefore, again appear to resemble SK-MG-1 phenotypically. By comparison, the average DNA content (as estimated from the G_0/G_1 peak) of both S- and L-cells in the L-cell derived clones was statistically greater ($p<0.05$) than the parental line.

These results suggested that the L-cells may represent divergent hyperploid sublines of SK-MG-1. To verify this

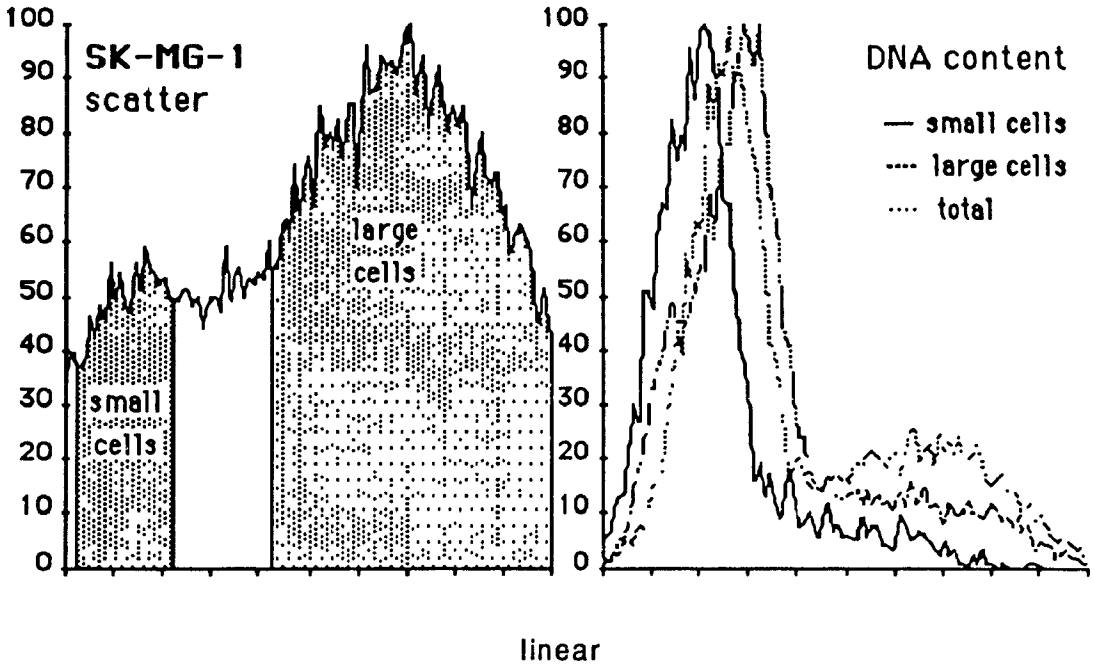


Figure 5.9: Cell cycle analysis of SK-MG-1

Ethanol-fixed SK-MG-1 cells were stained with propidium iodide and sorted according to small angle scatter (indicated by shaded areas). The S-cell population had the lowest G_0/G_1 peak, and almost no G_2 + M peak (represented on the right hand side of the diagram by the solid line), whereas the L-cell population (represented by the heavy broken line) had a shifted G_0/G_1 + S peak, and a more significant G_2 + M peak. These results collectively indicate that, comparatively, the S-cells are in a 'resting' state, and that the L-cells appear relatively more 'in cycle'. The abscissa of the left hand side of the diagram is linear scatter in arbitrary unit; the abscissa of the right hand side of the diagram is linear fluorescence of propidium iodide in arbitrary units. Abscissa scales are 128 channels and are delineated every 10 channels. The ordinate is relative cell number.

Table 5.2: DNA content[a] of SK-MG-1 and its small and large cell derived clones

	small cells	*large cells*
SK-MG-1 (parental)	50.33 ± .67	57.33 ± .67
	(*n* = 3)	(*n* = 3)
small cell clones	49.20 ± .73	55.50 ± .72
	(*n* = 5)	(*n* = 6)
large cell clones	57.10 ± .78[b]	63.33 ± .94[b]
	(*n* = 10)	(*n* = 9)

[a]ethanol-fixed cells were stained with propidium iodide as detailed in section 2.3.4; results expressed as mean ± standard error for modal channel number, 128-channel linear scale; *n* refers to the number of FACS distributions pooled to obtain these values

[b]$p<0.005$, compared with SK-MG-1 using a two-tailed Student's *t*-test

hypothesis, the karyotypes of SK-MG-1 and its S- and L-cell derived clones were studied.

5.6 Karyotypic Analysis of SK-MG-1 and Its Sublines

Metaphase chromosome spreads were prepared for SK-MG-1, and three different S- and L-cell derived sublines (Table 5.3). Statistical analysis revealed that:

1) There was no significant mean chromosomal difference between SK-MG-1 and its S-cell derived sublines, however,

2) The variance among S-cell derived sublines was significantly lower than that of the parental line.

3) L-cell derived sublines had, on average, a higher ploidy number than SK-MG-1 (compatible with the results of DNA labelling with propidium iodide).

4) One particular L-cell derived subline (L108), had a variance about the mean that was significantly greater ($p<0.05$) than for SK-MG-1.

These results suggest that the S-cell subpopulation of SK-MG-1 possess properties resembling those of a hypothetical tumor stem cell. For example, the S-cells are phenotypically smaller and more anaplastic than the L-cells. When S-cells are individually sorted, they establish sublines which are similar in terms of cell size, DNA content, and chromosome number to the parental line.

Table 5.3: Metaphase chromosomes of SK-MG-1 and its small and large cell derived clones

	mean ± SD[a]	*mode*	*range*[b]	*t*[c]	df[d]	*F*[e]
SK-MG-1 (parental)	57.4 ± 4.2	56	46-68			
S103	54.5 ± .93	51	50-64	-0.66	19	21.00[g]
S104	55.5 ± 1.0	55	49-66	-0.44	19	18.10[g]
S110	53.7 ± 1.3	50.5	46-68	-0.82	19	10.95[g]
L103	73.2 ± 5.3	60	46-146	2.32[f]	38	1.58
L107	66.2 ± 3.2	60.5	51-100	1.65	38	1.74
L108	96.2 ± 10.7	74.5	46->200	3.37[f]	19	6.32[g]

[a]results expressed as mean ± standard error for 20 metaphase spreads

[b]range represents the lowest to highest chromosome number counted

[c]calculated *t*-statistic

[d]degrees of freedom

[e]calculated *F*-statistic

[f]$p<0.005$, compared with SK-MG-1, using a two-tailed Student's *t* test

[g]$p<0.01$, compared with SK-MG-1, using a two-tailed *F* test with df = (19,19)

The L-cell subpopulation of SK-MG-1 is also capable of establishing colonies *in vitro* after sorting, a property which would not be expected if L-cells were differentiated 'end cells'. The fact that these colonies also contain a S- and L-cell subpopulation is somewhat perplexing. An explanation may lie with the fact that the L-cell derived sublines appear to be from a different stemline than that which predominates in SK-MG-1. The L-cell subpopulation may, in fact, include a mixture of differentiated 'end' cells derived from the S-cell subpopulation, and divergent minority sublines of SK-MG-1 with stem cell-like properties. Evidence in support of this is given by the finding of a significantly lower chromosomal variance about the mean among the S-cell derived sublines than among the SK-MG-1 parental line.

5.7 Discussion

The concept of a bifunctional chemotherapeutic molecule with selective cytotoxicity was first popularized in the early part of this century by Paul Ehrlich (1913). Although a detailed knowledge of antibody structure was lacking at the time, it seemed possible to envision 'immunotoxins' capable of seeking out and destroying cancer cells, while sparing normal tissues. This automatically raised the question of whether the histological differences between normal and malignant tissues could be accounted for

in molecular differences. Would immunologists be able to find markers of malignancy that were sufficiently sensitive and specific to be exploited clinically?

Monoclonal antibody technology has been a boon to cancer immunology in that it is capable of supplying the investigator with limitless quantities of monospecific antibody. Cancer cells, unfortunately, do not appear to have limitless quantities of monospecific antibody receptors (i.e. tumor-specific antigens).

The importance of this observation is illustrated by a study by Stavrou, et al. (1983). The authors reported the production of three murine mAbs, 13GC1, 14BC1, and 14FC3, which were derived from the fusion of X63-Ag8.653 myeloma cells with splenocytes from female BALB/c mice immunized with 79FR-G-41 glioma cells. The mAbs reacted selectively with the 79FR-G-41 glioma cells, as well as with other rat glioma cell lines, but not with fibroblasts, kidney, and brain cells from newborn F344 rats. At the tissue level, however, there was a significant heterogeneity of reactivity between individual tumor cells as assesed by immunofluorescence and immunoperoxidase staining. In a given rat glioma cell line, only a fraction (20-95%) of the total cell population was determined to be reactive to either of the three mAbs.

The explanation for these observations rests with the concept of tumor heterogeneity. Tumor heterogeneity may

arise through 'normal' mechanisms, or result from increased genetic instability intrinsic to the cancer cell (Nowell, 1976). It has been suggested that in the strictest sense, the term 'tumor heterogeneity' should be reserved for situations in which there are differences in cell lineage (Heppner, 1984). Cell cycle effects and other epigenetic phenomena add another level of complexity to the picture of tumor cell variability.

Clearly, it is not sufficient to establish that a particular mAb reacted with "9/12 gliomas, 3/11 melanomas, and 0/3 neuroblastomas". Information must also be sought as to *which cells* reacted, and *what* functional *role* (if any) do they play in the tumor "society"?

From a therapeutic viewpoint, it would be of great benefit to develop mAbs capable of recognizing the differentiation state of individual tumor cells. Such an approach would help to point the way to the actual tumor stem cells. One such system has been proposed by Cairncross, et al. (1982) using the mAbs AO10 and AJ8. These mAbs were derived from the immunization of BALB/c mice with the same GFAP$^-$ human glioma line used in these studies, SK-MG-1, and the GFAP$^+$ astrocytoma line SK-AO2. The AO10 and AJ8 antibodies appear to recognize different differentiation antigens on cultured glioma cells: 4/7 AO10$^+$ astrocytomas were GFAP$^+$, whereas 9/9 AJ8$^+$

astrocytomas were GFAP$^-$. The antigen phenotype of some immature astrocytes might therefore be AJ8$^+$ AO10$^-$ GFAP$^-$.

The FCM characteristics of immature astrocytes have been suggested in a round about way by Nishiguchi, et al. (1985). The authors fixed cultured human glioma and fetal brain (CHII) in 4% paraformaldehyde, and stained for the presence of GFAP. After confirming the existence of GFAP$^+$ cells in the CHII culture, only those cells with greater than background green fluorescence were sorted (analogous to the experiment described in section 5.1). Comparison of cell size (estimated by forward blue light scatter) with the unsorted CHII scatter distribution revealed that GFAP$^+$ cells were generally larger than GFAP$^-$ cells. A corollary of these findings would be that less differentiated astrocytes are smaller than well differentiated ones.

The results presented in section 5.2 would suggest that the human glioma cell line SK-MG-1 is composed of two interdependent, FCM-defineable subpopulations, one larger than the other. The HmAbs BT27/2A3, BT27/1A2, and BT32/A6 appear to recognize only the smaller of the two subpopulations. Since SK-MG-1 is a GFAP$^-$ line, no statement can be made about co-expression of this differentiation marker with the receptor recognized by the HmAbs. There is, however, evidence suggesting that the S-cell subpopulation consists of immature cells

representative of the main stemline of SK-MG-1, and capable of stem cell-like behaviour:

Cell size is often regarded as a characteristic feature of differentiation or anaplasia. In many normal tissues, terminally differentiated 'end' cells tend to be larger than their smaller undifferentiated precursors. Tumors, in a like fashion, can be viewed as a 'caricature' of normal tissue differentiation (Mackillop, et al. 1983).

In addition to size, preferential growth at low serum concentrations is an established property of transformed cells. Although the total number of S-cells did not change at low serum concentrations, the relative proportion of L-cells *decreased*, suggesting that the latter subpopulation behaves more like well-differentiated cells than do S-cells.

Clonogenicity, unfortunately, has not been shown to be a reliable indicator of differentiation state in malignant gliomas. At least one study has shown that the colony forming efficiency (CFE) of individual glioma populations labelled with a vital DNA stain (Hoescht 33342), and sorted in the flow cytometer according to DNA content, does not vary significantly from population to population within a given tumor (Hoshino, et al. 1982). These results may explain the lack of discrimination in CFE between sorted S-cell and L-cell subpopulations observed in section 5.4.

Tumor stem cells are often thought to reside primarily in a 'resting state' *in vivo* (Mackillop, et al. 1983). In section 5.5 it was shown that the S-cell subpopulation of SK-MG-1 appears to be primarily in the G_0/G_1 phase of the cell cycle in confluent cultures.

Finally, there is some evidence that the karyotypic pattern of individual glioma subpopulations may reflect their degree of differentiation. In an extensive study of freshly resected glioma biopsy specimens, Shapiro and Shapiro (1985) concluded that most high grade gliomas evolve from a relatively homogeneous population of near-diploid cells (35-57 chromosomes per cell) to the hyperdiploid state most frequently encountered at surgery. The hyperdiploid cells were noted to be unstable *in vitro*, tending to grow rapidly with short doubling times, but often demonstrating sensitivity to nitrosoureas. In contrast, the near-diploid cell populations were stable in culture with long doubling times, resistant to nitrosoureas, and behaved functionally as the tumor stem cells by repopulating the tumor mass *in vivo*.

Karyotypic studies of the S-cell derived clones, together with analysis of DNA content in sorted S-cells have shown that the S-cell subpopulation of SK-MG-1 contains near-diploid cells capable of giving rise to sublines which are genotypically and phenotypically similar to the parental culture (section 5.6).

In contrast, the L-cells are 1) larger than the S-cells, 2) are not optimally sustained at low serum concentrations, 3) divide actively even when they have reached confluence, 4) possess a hyperdiploid karyotype, and 5) give rise to unstable sublines with widely varying karyotypes.

The relationship between S-cells and L-cells appears to be complex. Cloning experiments have shown that each is capable of giving rise to the other (section 5.4); however, the S-cells of the L-cell derived clones are not the same phenotypically as the S-cells of SK-MG-1 or the S-cell derived clones. Karyotypically, the L-cell derived clones' distribution is hyperdiploid, with a high degree of variance. They have also not been observed to overgrow the culture after many passages *in vitro*. A possible explanation may lie with the phenomenon of clonal interaction and growth suppression. The latter concept arose from experiments which demonstrated that clonal tumor lines which behave in an unstable manner when grown in isolation, will stabilize when grown together (Poste, et al. 1981; Miner, et al. 1982).

The development of HmAbs capable of recognizing a glioma stem cell population would be an extremely important step towards the study and treatment of these aggressive and lethal tumors. As with all other forms of cancer, cytoreductive surgery followed by radiotherapy and,

sometimes, chemotherapy form the basis of current treatment. Unfortunately, the principal targets of such an approach are the more differentiated 'end' cells which make up the bulk of the tumor. Failure to deplete a tumor of its stem cell compartment inevitably results in tumor recurrence.

6 CONCLUDING REMARKS AND PERSPECTIVES

6.1 Diagnostic Imaging with Monoclonal Antibodies

Reports of radioimmunolocalization of malignant intracranial neoplasms using mAbs have been contributed by Phillips, et al. (1983), Goldman, et al. (1985), Bullard, et al. (1986), and Neuwelt, et al. (1987) (see Table 6.1). The first of these investigators isolated intratumoral lymphocytes from patients with malignant gliomas, and fused them to the human myeloma line LICR-LON-HMy2 (Sikora, et al. 1982), yielding human-human hybridomas. One such hybridoma, designated as LGL1-1D6, was derived from a patient with a cystic grade III astrocytoma, and was chosen for its reactivity with the human glioma cell line GCCM.

One mg of hybridoma antibody was purified by ammonium sulfate precipitation and ion exchange chromatography, and labelled with ^{131}I. Labelled antibody was then injected i.v. back into the original patient, after a tumor recurrence had been documented by CT scan. Rectilinear gamma scintigraphy, performed on day 4 after mAb administration, revealed a diffuse uptake of mAb in the area of the tumor. Repeated sampling of tumor cyst fluid indicated that labelled mAb persisted in the fluid for over 6 days.

Table 6.1: Antibodies suitable for radioimmunolocalization of human neural tumors

mAb[a]	*antigen*	*surface*	*target neoplasm*	*reference*
UJ13a	Pan-neuroectodermal normal and neoplastic cells[b], ?glycolipid	reactive	all neuroectodermal tumors[c]	Allan, et al. (1983)
UJ181.4	Embryonic and neoplastic neuronal cells	reactive	Neuroblastomas, medulloblastomas	Garson (1982)
M340	Neuronal cells	reactive	Primary pineal tumors	H.B. Coakham and J.T. Kemshead (unpublished data)
FD32.3	GMEM[d]	reactive	Glial neoplasms	Garson (1982)
81C6	GMEM[d]	reactive	Glial neoplasms	Bourdon, et al. (1983)

[a]Monoclonal antibody
[b]Except melanocytes
[c]Except melanomas
[d]Glioma-mesenchymal extracellular matrix

Adapted from Davies, et al. (1986)

Three weeks after the initial localization study, ^{131}I-labelled control myeloma protein (obtained from the culture supernatant of the parent myeloma) was administered. The latter was not detected in tumor cyst fluid in nearly the same quantities or for the same duration of time as the labelled mAb; however, neither labelled mAb nor control myeloma protein were recovered from CSF. (Unfortunately, the authors did not publish the control ^{131}I-labelled myeloma protein scan, so there is no way of verifying whether specific labelling of tumor parenchyma actually occurred).

The importance of using both specific and nonspecific mAbs for imaging of intracranial tumors was stressed by Bullard, et al. (1986) in their study of D-54 MG human glioma xenografts implanted into athymic rats. The authors administered ^{131}I or ^{125}I-labelled specific or nonspecific mAbs (81C6 and 45.6, respectively), and compared external gamma camera images (obtainable only with the ^{131}I-labelled antibody) to tissue levels for both mAbs. Although antibody 45.6 reached significantly higher levels in tumor than in normal brain ($p<0.05$), the levels were significantly lower in tumor than were the levels reached with specific antibody 81C6 ($p<0.005$).

Using nonspecific antibody 45.6. intracranial tumors weighing >300 mg were detectable by means of external gamma

scintigraphy. The specific antibody, 81C6, could detect tumors weighing as little as 20 mg. The authors speculated that there was 'false' localization of nonspecific mAb in the tumor due to breakdown of the blood-brain barrier, and nonselective entry of antibody into the tumor parenchyma. Tumor size, therefore, must be considered as an important factor in radioimmunolocalization studies, since even nonspecific antibodies will image intracranial tumors if the latter are large enough.

This observation was underscored by Mann, et al. (1984) who simultaneously injected two different ^{131}I or ^{125}I-labelled mAbs into nude mice bearing two different kinds of subcutaneously implanted tumors, to which either one or the other mAb was specifically directed. Although *in vivo* binding of mAb to tumor tissue correlated with *in vitro* labelling, computer analysis of percent radioactivity in the tumors showed that tumor images were related directly to tumor size and relatively uninfluenced by mAb specificity.

In a study aimed at elucidating the kinetics of radiolabelled xenogeneic mAbs in humans, Epenetos, et al. (1986) selected pairs of specific and nonspecific tumor-associated mAbs which were labelled with ^{125}I or ^{131}I, and given i.v. to 19 patients 1-26 days prior to surgical excision of primary and metastatic breast, ovarian, and gastrointestinal tumors. Quantitation of antibody uptake

was performed on resected normal and neoplastic tissues. Although good tumor:non-tumor ratios were obtained with the specifc mAbs, the absolute amount of radiolabel detected in tumors was unacceptably low, only in the order of 0.015% of total injected amount per gm of tumor, and maximal one day post administration. These results must be kept in mind when considering therapeutic applications of xenogeneic mAbs in humans.

Finally, Neuwelt et al. (1987) failed to observe distinct and persistant localization of intravenously administered ^{131}I-labelled melanoma-specific murine mAb in three patients with intracranial metastatic melanoma, even following osmotic modification of the blood-brain barrier (BBB). There seemed to be increased uptake of antibody in the tumor region after barrier modification in one patient, but antibody clearance from that region occurred at the same rate as from surrounding and apparently tumor-free brain. In another patient with carcinomatous meningitis, it was shown that the antibody bound to only a fraction of the antigen binding sites on tumor cells in the CSF after osmotic BBB modification.

6.2 Immunotherapy with Monoclonal Antibodies

Therapeutic applications of mAbs to the treatment of malignant gliomas include the administration of antibodies coupled to toxin molecules, chemotherapeutic agents, or

simply in the unconjugated form. Most toxins are naturally occuring substances which are capable of interfering with protein synthesis, even at very low concentrations. At present, the plant toxins ricin, abrin, and gelonin, and the bacterial toxins of diphtheria (*A*-chain toxin; DTA) and *Pseudomonas aeruginosa* toxin have been successfully coupled to mAb molecules (Bourdon, et al. 1984). Gilliland, et al. (1980) have reported successfully coupling DTA to a mAb directed against a colorectal carcinoma tumor-associated antigen. The conjugates were cytotoxic for the colorectal carcinoma cells *in vitro*, but not for a variety of cells which lacked the antigen.

Antibodies have also been successfully coupled to the drugs methotrexate, P-phenylenediamine mustard, chlorambucil, melphalan, triaziquinone, α-amanitin, vindesine, neocarzinostatin, adriamycin, and daunomycin (Bullard and Bigner, 1985). The rationale for this approach is to use the antibodies as tissue-specific agents, with avoidance of undue systemic toxicity.

An alternative approach would be to conjugate mAbs to ^{10}B in the form of anionic boranes, then 'activate' the conjugates with neutron irradiation. The resulting neutron capture reaction would cause the release of a 0.48 MeV photon 96% of the time, sufficient to cause local cell death (Tolpin, et al. 1975).

Elaborate schemes for coupling mAbs to tumoricidal molecules may eventually prove unnecessary, since there is now evidence that unconjugated mAbs may be entirely sufficient for effective treatment of tumor cells *in vivo*. Sears, et al. (1984) have reported their experience with 20 patients with metastatic gastrointestinal malignancies treated with the murine anti-colorectal cancer mAb, 1083-17-1A. All patients, with only two exceptions, received a single i.v. injection of unconjugated mAb in a dose range of 15-1,000 mg per patient. No untoward reactions to the injection were observed; and the murine mAbs circulated in the patient's blood for 2-50 days, depending on the dose given.

Eleven patients had documented hepatic or renal failure at the time they were given immunotherapy, and 9 of the 11 had previously undergone an abdominal surgical procedure. Nine additional patients with tumor metastases, but no evidence of hepatic or renal dysfunction, were also given immunotherapy and were alive 10-35 months after treatment. Most important, however, was that 3 of the 9 were alive with no evidence of disease at 10, 13, and 22 months after immunotherapy. Since this was only a phase I trial, no control group of conventionally treated subjects was studied for comparison.

In another study, Irie and Morton (1986) reported the effects of direct intralesional injections of a HmAb, L72,

into cutaneous metastases of malignant melanomas in 8 patients. A total of 21 nodules were treated with injections of 0.16-7.4 mg of purified antibody per nodule, on a daily or weekly basis, and clinical responses were determined 3-4 weeks after the last injection. Of the 21 nodules so treated, 16 responded either completely or partially, 3 stabilized, and 2 did not exhibit any response. The latter were derived from tumors which did not express ganglioside GD2, the target molecule for mAb L72 (Cahan, et al. 1982). As in the previous trial, none of the patients exhibited any serious side-effects from immunotherapy.

The therapeutic mechanism of action of the unconjugated mAbs may be different in both of these studies. Sears, et al. (1984) noted the complete disappearance of tumor metastases in 3 patients by 10 weeks at the earliest, long after the mAbs had been cleared from the circulation. In a follow-up study (Koprowski, et al. 1984) on the same cohort of patients, the presence of anti-idiotype antibodies was detectable in the sera of 3 patients who improved clinically and had long remissions from their disease following immunotherapy.

The anti-idiotype antibodies could serve as 'internal' images of the tumor, inducing an anti-anti-idiotype immune response which would in turn cross-react with the tumor itself (Raychaudhuri, et al. 1986), according to the immune

network theory proposed by Jerne (1974). The authors speculated that: "The presence of an internal image of an antigen on a human immunoglobulin molecule, in contrast to the presence of the antigen on a tumor cell, may change the conditions under which the immune system reacts to the tumor antigen".

The mechanism of tumor cell killing in the study by Irie and Morton (1986) may have involved complement-mediated lysis of melanoma cells, as suggested by the rapid development of a clinical response in some patients only 3 weeks following immunotherapy, and the requirement for complement together with antibody for an anti-tumor effect *in vitro*. The presence of anti-GD2 antibodies in the sera of 2 patients from this trial was detected within 1-3 weeks after the initial injection of mAb; therefore it appears that both direct and indirect immune mechanisms may have been involved in some patients.

Methods of improving the delivery of mAbs to normal rat and dog brain were reported by Bullard, et al. (1984), and Neuwelt, et al. (1985), respectively. Both groups proposed the use of intra-arterial infusions of hyperosmotic agents to temporarily open the blood-brain or blood-CSF barriers, followed by intra-arterial infusion of mAbs. In the study by Neuwelt, et al. (1985), selective perfusion of the vertebral arteries with 25% mannitol resulted in optimum delivery of ^{125}I-labelled mAb into the

CSF, whereas osmotic opening of the internal carotid artery territory maximized the delivery of antibody to the brain parenchyma itself.

Given that most malignant gliomas already demonstrate a variable degree of BBB disruption, and that the therapeutic mechanism of action of unconjugated mAbs *in vivo* may be a long-term effect on immune regulation rather than a direct effect, the ultimate role of intra-arterial hyperosmotic infusions in tumor immunotherapy with mAbs remains to be determined.

6.3 Advantages of Human Monoclonal Antibodies

Two important advantages of allogeneic over xenogeneic mAbs are greater biological compatibility and reduced clearence by the host reticuloendothelial system. In the study by Sears, et al. (1984), 8 of 9 patients who received the murine mAb, 1083-17-1A, in doses of <200 mg developed anti-mouse immunoglobulins, whereas 8 of 9 patients who received doses of 366-1,000 mg did not develop any such antibodies. Others (Schroff, et al. 1985), have noted the development of anti-mouse immunoglobulin responses in patients being treated with murine mAbs as early as 2 weeks after the onset of immunotherapy. The antiglobulins were primarily of the IgG class, and cross-reacted with most mouse IgG preparations. Despite the presence of anti-mouse immunoglobulins in patient's sera, antibody localization on

tumor cells by immunofluorescence staining was not observed to be affected in these patients.

Improved clearance kinetics of xenogeneic mAbs can be achieved by using Fab fragments instead of the whole immuglobulin molecule, since it is the Fc portion which largely determines uptake by the reticuloendothelial system. For purposes of immunotherapy, however, it may be desirable to have a bifunctional reagent capable of binding complement.

An allogeneic approach to tumor immunotherapy offers two more theoretical advantages: recognition of more subtle and specific tumor-associated antigens, and greater compatability with host cellular effector mechanisms. Although the latter remain to be proven in the context of human versus murine mAbs, it is hoped that the antibodies described in this dissertation will eventually play a role in the diagnosis and clinical management of malignant gliomas.

6.4 Future Prospects for Glioma Immunotherapy

The past three decades have seen little improvement in the quality of life or overall prognosis for patients with high grade gliomas, even with the advent of BCNU chemotherapy in recent years. The addition of immunotherapy to a multimodality treatment approach remains an elusive, yet ever-promising possibility.

Many immunotherapeutic approaches are possible, although few have been tested in a properly controlled fashion, and none have emerged as reproducibly effective. Current interests have focused on active immunization with lethally irradiated glioma cells, and non-specific immunopotentiators (Mahaley, et al. 1983a), and IL-2/LAK therapy (Jacobs, et al. 1986a). Therapy with anti-glioma monoclonal antibodies has not yet reached phase I trials in humans.

There are two important areas in glioma immunobiology that must be addressed in the next decade: the mechanism of glioma-induced immunosuppression, and the nature of glioma-specific antigens.

Progress is being made in the area of immunosuppression in studies on tumor infiltrating lymphocytes (Miescher, et al. 1988), and on the glioblastoma-derived T-lymphocyte suppressive factor which resembles transforming growth factor-β_2 (de Martin, et al. 1987).

Murine mAb technology has been extremely helpful in furthering our understanding of glioma-associated antigens and their relationships to other antigenic systems (e.g. hemopoietc), but has so far failed to yield any tumor specific markers. By constructing human-human B cell hybridomas from patients with malignant gliomas, the present study attempted to generate a series of HmAbs

against glioma-related antigens which were being recognized in some important way by the host immune system. The fact that 5 different IgM HmAbs from 4 different patients all appear to recognize similar glycolipid molecules may be highly significant and deserves further study.

In a more general sense, the 5 HmAbs presented in this dissertation are of potential clinical value in humans, and should be put to practical use where possible. Future efforts should be directed towards: 1) radioimaging studies using separate labels for control and specific antibodies, with *in vivo* verification of dosimetry at the time of surgery, 2) immunohistological studies aimed at verifying the distribution of reactivities across a range of normal and malignant tissues, as well as in human fetal tissue at various stages of development, 3) further characterization of the glycolipid antigens recognized by the five HmAbs, 4) preparation of immunotoxins and other immunoconjugates with *in vitro* toxicity testing against various normal and malignant tissues, 5) generation of murine idiotype and anti-idiotype mAbs for use in nude mice and possibly humans, if the HmAbs should prove too difficult to subclone or maintain in longterm production, 6) isolation of IgM to IgG class switch variants, 7) large scale production, isolation, and purification of the HmAbs with phase I testing in humans, and 8) immunization of glioma patients

with the gangliosides identified by the HmAbs in an attempt to boost host anti-tumor immune mechanisms.

CLAIMS TO ORIGINALITY

The following results presented in this thesis are original:

1) Stable human-human B cell hybridomas secreting human monoclonal antibodies (HmAbs) reactive with extracts of autologous and allogeneic malignant gliomas were produced by the fusion of peripheral blood lymphocytes from patients with neurological tumors and the human myeloma-like cell line, TM-H2-SP2.

2) Five hybridomas designated as BT27/1A2, BT27/2A3, BT32/A6, BT34/A5, and BT54/B8 were selected for detailed study. All five produced human IgM in a range of 2.4-44 μg/ml. HmAbs BT32/A6 and BT54/B8 appeared to bind to allogeneic glioma extract with low affinity at 4 °C.

3) All five HmAbs had a similar, but not identical, pattern of reactivity against a panel of human tumor cell lines when analyzed using 2-dimensional flow cytometry. Most labelled only gliomas when tested against a variety of neuroectodermal tumor cell lines, and none appeared to label any hematological cell

lines. HmAbs BT27/1A2 and BT27/2A3 appeared to have a similar pattern of reactivity, based also on quantitative comparisons of reactivity in ELISA and immuno-fluorescence staining patterns. HmAb BT32/A6 appeared to recognize a slightly different or related antigenic determinant from the former two, while HmAbs BT34/A5 and BT54/B8 presented a similar pattern of reactivity which differed slightly from the other three.

4) Flow cytometric analysis of tumor explant cultures passaged for short term *in vitro* was performed. Two astrocytomas, one meningioma, and one medulloblastoma cultures were successfully labelled with HmAb BT27/2A3. None of the five HmAbs, however, appeared to label cultured normal human astrocytes.

5) Preliminary antigen characterization suggested that at least four (BT27/1A2, BT27/2A3, BT32/A6, and BT34/A5) of the five HmAbs recognize a cell surface glycolipid, which in the case of BT27/2A3, was shown to be preferentially expressed *in vitro* at high cell densities.

6) Multiparameter flow cytometric studies revealled that the five HmAbs recognize a subpopulation of tumor

cells, both in established tumor cell lines and in tumor explant cultures passaged for short term *in vitro*.

7) The biological significance of the tumor subpopulation identified by the HmAbs was studied in cell sorting experiments. These suggested that the subpopulation labelled by the five HmAbs may share certain properties consistent with tumor stem cells, i.e. ablity to reconstitute the heterogeneity of the parental line (in terms of subpopulation size, DNA content, and chromosome number) when independently sorted and re-plated.

APPENDIX: Biotinylation of Human Monoclonal Antibodies

Initial attempts at labelling tissue sections and tumor biopsy specimen cell suspensions with human monoclonal antibodies (HmAbs) from hybridoma supernatants were unsuccessful, largely due to the high background levels of human immunoglobulin in the specimens. To overcome these difficulties, the following method of direct labelling was devised:

Saturated ammonium sulfate in phosphate buffered saline (PBS), pH 7.2 (70 g/100 ml) was added dropwise in equal volume to the hybridoma supernatants. The samples were allowed to stand in an ice bath for 30 min, except in the case of BT27/1A2, which was only allowed to stand until it became markedly turbid (about 10 min). The samples were then spun at 10,000 rpm for 10 min in a Sorvall High Speed Centrifuge, at 4 °C. The supernatant was decanted, and the pellets resuspended in 50% saturated ammonium sulfate in PBS, pH 7.2. The supernatants were again spun at 10,000 rpm for 10 min after which the supernatant was discarded. The pellets were resuspended in 2 ml of PBS, pH 7.2, for each 100 ml of the original supernatant. The samples were then dialyzed against 0.1 *M* $NaHCO_3$, pH 8.5, with three changes over 24 hours, at 4 °C. After dialysis the concentration of γ-globulin was measured

spectrophotometrically using the optical density (O.D.) of 1:20 dilutions of the samples at a wavelength of 280 nm.

The concentration of γ-globulin was adjusted to 300 µg/ml in 0.1 *M* $NaHCO_3$, pH 8.5. The biotin ester, biotinamidocaproate (caproylaminobiotin) *N*-hydroxysuccinimide ester (CAB-NHS), was dissolved in dimethylformamide to a concentration of 50 mg/ml. The biotin ester preparation was then incubated with the γ-globulin extract solution at a w/w ratio of 1:10 (biotin ester to protein) for one hour at room temperature, after which the reaction was stopped with excess 1 *M* NH_4Cl. The samples were then dialyzed in a solution of 10 m*M* Tris buffer, 50 m*M* NaCl, pH 7.4, to remove the free biotin.

The biological activity of the biotinylated antibody preparations was monitored using a modification of the ELISA procedure described in section 2.1.6. Briefly, 3 *M* KCl extract from an allogeneic glioblastoma multiforme (BT-34) was coated onto 96-well round bottom microtiter plates (Dynatech, Alexandria, VA) in the usual manner, then reacted with the biotinylated HmAbs diluted in 4% bovine serum albumin (BSA) at a final concentration of 0.5 µg/ml. Biotinylated nonspecific control antibody, BT27/2D2, was added as a control, as well as 4% BSA alone, to test for endogenous peroxidase activity in the diluent. Following the usual incubation procedure, the plates were incubated at room temperature with a 1:1250 dilution of peroxidase

linked biotin-streptavidin reagent (Amersham RPN.1043). The plates were then developed with 3,3',5,5'-tetramethylbenzidine (TMB), 0.1 mg/ml, in 0.1 *M* citrated PBS, pH 5.0, with 0.02% H_2O_2, and read using a Dyntech microELISA plate reader set at a wavelength of 450 nm.

Precipitation of the γ-globulin fraction of the hybridoma supernatants using the procedure described succeeded in preserving the activity of the HmAbs. It was found that BT27/1A2 was sensitive to the period of exposure to the saturated ammonium sulfate; however, by minimizing the exposure time, preservation of activity was achieved.

A dilution curve showing the relative activities at varying concentrations of each of the three biotinylated HmAbs is shown in Figure A.1. The ordinate of the curve displays specific activity, which is represented by the difference in O.D. units between total binding of the biotinylated HmAbs and the nonspecific binding of control HmAb, BT27/2D2, at the same concentration.

In a modification of the ELISA for glutaraldehyde fixed cells described in section 2.1.7, viable glioma cells from the cell line SK-MG-1 were attached via poly-L-lysine to polyvinyl chloride round bottom microELISA plates, and developed with the biotinylated HmAbs as outlined above. The results are presented in Figure A.2, and indicate specific labelling of live glioma cells.

The preservation of activity following direct labelling with biotin constitutes the first step towards the use of these HmAbs in immunohistochemical studies.

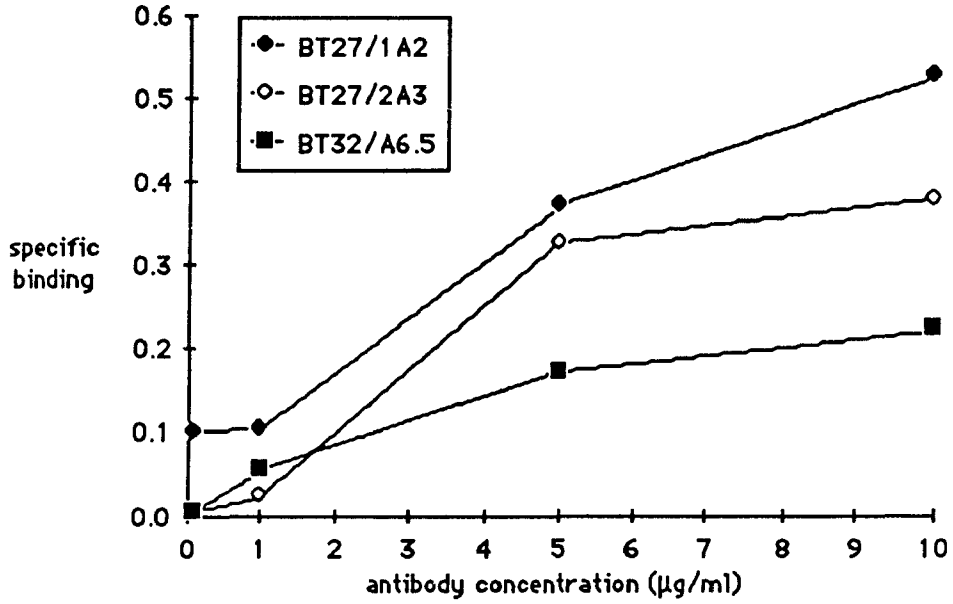


Figure A.1: Titration curve for biotinylated human monoclonal antibodies

Human monoclonal antibodies were precipitated from culture supernatants using saturated ammonium sulfate, and reacted with 3 *M* KCl extract of allogeneic tumor in a quantitative ELISA. Specific binding is the difference in optical density at 450 nm between the biotinylated antibodies and biotinylated control IgM antibody, BT27/2D2.

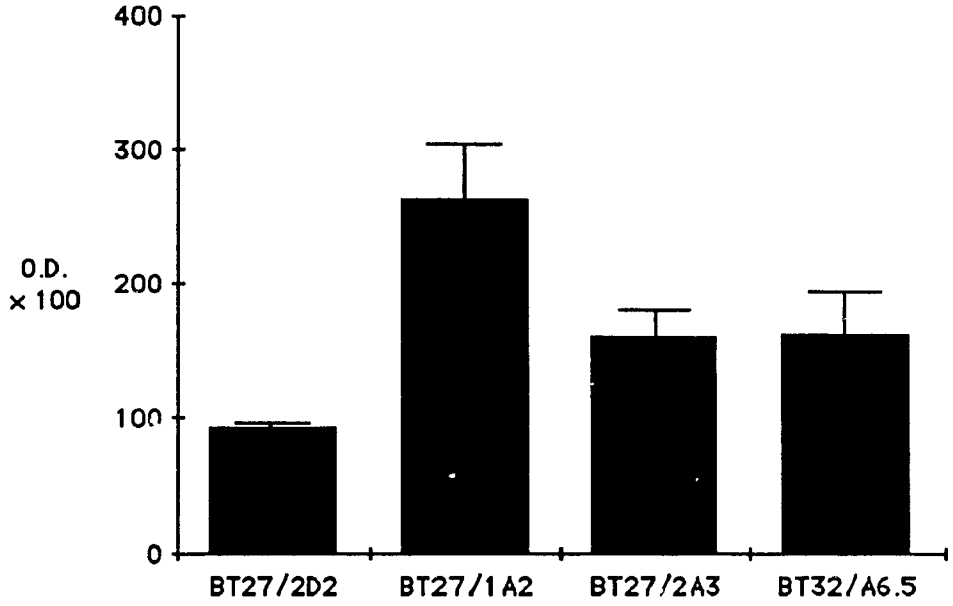


Figure A.2: Binding of biotinylated HmAbs to SK-MG-1 in ELISA

Live SK-MG-1 cells were coated onto microELISA plates and labelled with each of the three biotinylated HmAbs, as well as HmAb BT27/2D2 (IgM control). Results are expressed as OD_{450} x 100 ± standard error of the mean. Fifteen replicates per sample.

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