This thesis is dedicated to all the brave children from the Hospital for Sick Children, Great Ormond Street, London and The Queen's Medical Centre, Nottingham who played a part in this research.
IN VITRO STUDIES ON THE GROWTH AND CHEMOSENSITIVITY
OF SHORT-TERM CULTURES DERIVED FROM
CHILDHOOD BRAIN TUMOURS

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ABSTRACT

Clinical evidence indicates that many tumours of the central nervous system in children respond to radiotherapy. There is less evidence that these tumours are sensitive to chemotherapy. Unfortunately, there is a paucity of established cell lines from most types of paediatric brain tumour in which to investigate chemosensitivity in vitro. There have been no reports of cell lines derived from benign astrocytomas and only one derived from a malignant astrocytoma of childhood. Of the other tumour histologies, only two cell lines from ependymomas and a small number from medulloblastomas have been established.

The aim of this study was to carry out a systematic examination of the conditions necessary to grow childhood brain tumours in vitro for chemosensitivity studies. Tumour biopsy material was enzymatically disaggregated with collagenase and plated onto substrates known to enhance the attachment and growth of human tumour cells, including Cell-tak, fibronectin, laminin, Matrigel and vitrogen. Marked differences were noted between the substrates in their ability to promote in vitro growth of these tumour cell lines. Cell lines grew best on plastic, fibronectin and laminin and reached passage levels in excess of 20.

The phenotype of cells growing on a variety of substrates was examined. The intermediate filament composition of a number of cultures was determined using monoclonal antibodies against glial fibrillary acidic protein, neurofilament protein, vimentin, cytokeratin and desmin. Additionally, a polyclonal antibody against fibronectin and synaptophysin was used. Short-term cultures derived from tumours of glial origin expressed glial markers, for example astrocytomas expressed GFAP. Those cultures of neuronal origin (e.g., medulloblastomas) expressed neuronal markers such as neurofilament protein and synaptophysin. However, unexpected expression of certain markers was also observed, where a small number of astrocytoma-derived cultures expressed neurofilament protein (albeit to a small degree) and medulloblastoma-derived cultures occasionally expressed desmin.

The chemosensitivity of these cultures was determined using the MTT assay. Neuroblastomas were extremely sensitive to vincristine, while ependymomas were markedly resistant. Astrocytomas and medulloblastomas were of similar sensitivity. Cultures derived from neuroblastoma were sensitive to CCNU, whilst the other tumour groups had similar sensitivities. There was little difference between the chemosensitivities to procarbazine but some medulloblastomas were very resistant to this drug. These results show similarities of chemosensitivity with adult malignant gliomas. This indicates the need for the identification of new drugs using in vitro screening based on short-term cultures derived from paediatric brain tumours.
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ABBREVIATIONS

5-FU = 5-Fluorouracil
AA = Anaplastic astrocytoma
ACNU = 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea
ADR = Adriamycin
ALL = Acute lymphoblastic leukaemia
ARA-C = Cytosine arabinoside
BBB = Blood brain barrier
BCNU = Carmustin (Bischloronitrosourea)
BM = Basement membrane
C-M assay = Courtenay-Mills assay
CCNU = Lomustine (Chloroethylchloroexylnitrosourea)
CCSG = Children's Cancer Study Group
CDDP = Cisplatin
CENU = Chloroethylnitrosourea
CFA = Colony-forming assay
CFE = Colony-forming efficiency
CLL = Chronic lymphoblastic leukaemia
CM = Collection medium
CNS = Central nervous system
COL = Collagen
CSF = Cerebrospinal fluid
CUSA = Cavitation ultrasonic surgical aspirator
CYT = Cytokeratin
DBD = Dibromodulcitol
DES = Desmin
DiSC = Differential staining cytotoxicity assay
DMBA = Dimethylbenzanthracene
DMSO = Dimethylsulphoxide
DNA = Deoxyribonucleic acid
DOX = Doxorubicin
DTIC = Dacarbazine
ECM = Extracellular matrix
EGF = Epidermal growth factor
EGFR = Epidermal growth factor receptor
EGL = External granule layer
EHS = Engelbreth-Hohn-Swarm
ELISA = Enzyme-linked immunosorbent assay
EMF = Electromagnetic field
ENU = Ethyl-nitrosourea
FAPC = Familial adenomatous polyposis
FCS = Foetal calf serum
FGF = Fibroblast growth factor
FITC = Fluorescein isothiocyanate
FN = Fibronectin
GAG = Glycosaminoglycan
GB = Glioblastoma
GBM = Glioblastoma multiforme
GFAP = Glial fibrillary acidic acid
GM = Growth medium
GMEM = Glioma-mesenchymal ECM
GS = Glutamine synthetase
H-S assay = Hamburger-Salmon assay
HBSS = Hank's balanced salt solution
HDIAL = High-dose intraarterial chemotherapy
HGA = High grade astrocytoma
IF = Intermediate filament
LCS = Leukaemic cell survival
LGA = Low grade astrocytoma
LMN = Laminin
LOH = Loss of heterozygosity
mAMSA = 4-(9-acridinylamino)methanesulphon-m-anisidide
MB = Medulloblastoma
MCC = Merkel cell carcinoma
MCNU = 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea
MDR = Multi-drug resistance
MF = Mafosfamide
MGMT = O6-methylguanine-DNA methyltransferase
MMC = Mitomycin c
MNU = Methyl-nitrosourea
MoAb = Monoclonal antibody
MRI = Magnetic resonance imaging
mRNA = Messenger ribonucleic acid
MTT = (3-(4,5-dimethylthiazol-2-yl). 2,5-diphenyltetrazolium bromide
N-CAM = neural cell adhesion molecule
NB = Neuroblastoma
NF = Neurofilament
NF-H = High neurofilament protein
NF-I = Neurofibromatosis type I
NF-II = Neurofibromatosis type II
NF-L = Low neurofilament protein
NF-M = Middle neurofilament protein
NNE = Nonneuronal enolase
NSE = Neuron-specific enolase
P-gp = P-glycoprotein
PBS = Phosphate buffered saline
PCB = Procarbazine
PDGF = Platelet-derived growth factor
PDT = Population doubling time
PL = Passage level
PLL = Polylysine
PNET = Primitive neuroectodermal tumour
PNS = Peripheral nervous system
POG = Paediatric Oncology Group
PPC = Peak plasma concentrations
RB = Retinoblastoma
RBC = Red blood cell
RFI = Relapse free interval
RGD = Arg-Gly-Asp
RLFP = Restriction length fragment polymorphism
RNA = Ribonucleic acid
RTOG = Radiation Oncology Group
SF = Surviving fraction
SIOP = The International Society for Paediatric Oncology
SPNET = Supratentorial primitive neuroectodermal tumour
SRB = Sulphurhodamine
SYN = Synaptophysin
TGF-β1 = Human transforming growth factor
TN = Tenascin
VCR = Vincristine
VHL = von Hippel-Landau
VIM = Vimentin
VM = Ventricular matrix
VM-16 = Teniposide
VP16-213 = Etoposide
VT = Vitronectin
WHO = World Health Organization
Chapter 1

Clinical aspects of childhood brain tumours

Incidence

Cancer is the second most frequent cause of death in children under 15 years of age (Boring et al, 1991). After leukaemia, primary brain tumours are the second most common neoplasms of childhood and account for 20% of all cancers in children (Young & Miller, 1975; Boring et al, 1991) and are responsible for 20% of childhood cancer deaths (Gold, 1982).

Comparative mortality rates for brain tumours in patients of all ages indicate that the lowest rates occur in countries such as Mexico, Chile, Poland and Japan where the incidence is between 0.7 - 2.3 per 100,000, whilst higher rates are seen in Denmark (5.6 per 100,000) and Israel (6.5 per 100,000) (Gold, 1982). This apparent variation in incidence is probably due to the differences in medical care, diagnosis and cancer reporting between countries. However, the incidence of primary brain tumours amongst children aged 15 or younger is remarkably consistent between studies, at about 2.1 to 2.5 cases per 100,000 population (Bunin, 1987). Recently a trend of higher incidence in more affluent areas has been reported for Scotland, where the incidence is 4x higher in the most deprived compared to the affluent areas (McKinney et al, 1994).

Most, perhaps 75% of childhood brain tumours are glial in origin (Gold, 1982; Packer, 1995) and the most common type of brain tumour is astrocytoma, which accounts for 20-49% of all childhood brain neoplasms. The next most common tumours are medulloblastoma (MB) (ranging from 16% to 29%), ependymomas (6-17%) and glioblastoma (GB) (4-20%) (Farwell et al, 1978). In most countries, this distribution appears to be similar for histologically confirmed cases (Dohrmann & Farwell, 1976). However, in Far Eastern countries, teratomas and MBs appeared to be more common than astrocytomas (Oi et al, 1990). Brain metastases in children are comparatively uncommon (Lacayo & Farmer, 1991).

In adults, brain tumours arise most commonly in the cerebrum, whereas in children they are more frequently located in the cerebellum (Gold, 1982; Lacayo & Farmer, 1991). The incidence of tumours occurring in the cerebellum decreases approximately linearly with age, from almost 70% in patients under 10 years of age to about 50% in those patients aged 20-25 years and to nearly zero for those older than 55 years. However, for cerebral tumours there is an increase from about 30% of all tumours among children less than 10 years of age to about 45% to 50% in the 20 to 25 year age group and to essentially all brain tumours occurring in those 55 years and older (Gold, 1982).
Epidemiology

Genetic syndromes
Although the majority of childhood brain tumours appear to be sporadic, there are studies which indicate that a genetic predisposition can occur in a minority of cases. The occurrence of brain tumours in identical twins (Joughin, 1928) and in families have been reported (Wald et al., 1982; Farwell & Flannery, 1984; Honnorat et al., 1993). There are also a number of well characterised genetic disorders in which a greater than expected incidence of brain tumours in children is observed.

Neurofibromatosis: Types I and II
Type I neurofibromatosis (von Recklinghausen's disease, NF-I), is the most common autosomally inherited genetic disorder. Individuals with this condition have a higher risk of developing CNS tumours, including gliomas, acoustic neuromas and meningiomas as well as optic nerve gliomas and malignant schwannomas (Matsui et al., 1993). Children with NF-I also have a tendency to develop non-lymphocytic leukaemia (Clark & Hutter, 1982), neuroblastoma and Wilm's tumour (Riccardi et al., 1982). The NF-I gene is on the long arm of chromosome 17 (Seizinger et al., 1987) and has been shown to encode a protein related to guanosine triphosphatase (GTP-ase activating protein) (Xu et al., 1990).

In contrast, type II neurofibromatosis (NF-II) is associated with the development of bilateral acoustic neuromas, meningiomas and gliomas, but this condition is uncommon in children. The NF-II gene functions as a tumour suppressor gene of the retinoblastoma (RB) type (Seizinger et al., 1987).

Gardner syndrome and familial polyposis
These two diseases are manifested by adenomatous polyps and have been associated with the development of MB and GB. Familial adenomatous polyposis coli (FAPC), is characterised by the appearance of more than 100 polyps in the colon (Bussey, 1979), whereas the association of FAPC with sebaceous cysts, osteomas and supernumerary cysts are characteristic of Gardner's syndrome. Recently, it has been suggested that there may be a variant of Gardner's syndrome or familial polyposis, in which there is a strong association with the development of medulloblastoma (Cervoni, L et al., 1995).

Turcot's syndrome
This uncommon disease with only 55 cases having been reported worldwide (Rutz et al., 1991) is characterised by an association between the development of polyposis coli and glioblastoma multiforme (GBM) (Cervoni et al., 1995). The prognosis for patients with this disease who develop GBM appears to be better than for those individuals who develop GBs sporadically (Rutz et al., 1991). It has been documented that tumour tissue from two patients
with Turcot's syndrome show somatic mutation in p53, but not germ-line mutations, suggesting that p53 may play a role in progression but not initiation of this disease (Kikuchi et al, 1993).

**Gorlin's syndrome**

This was first documented in 1960 and is also known as naevoid basal cell carcinoma syndrome and is an inherited autosomal dominant trait. Clinically it manifests itself as associations of multiple basal cell carcinomas of the skin, odontogenic keratocysts of the jaw and skeletal anomalies. A 20% risk of developing a MB has been associated with this syndrome (Gorlin, 1987) and these develop at an earlier age (mean age of approximately 2 years) than sporadic medulloblastomas (Evans et al, 1991b; Cowan et al, 1997). Evans et al (1991a) observed a MB and a high grade astrocytoma in two patients with Gorlin's syndrome and they suggested that children presenting with MB should be screened for this syndrome as well as their parents (Evans et al, 1991b). Allelic loss on chromosome 9 has been found in both sporadic and Gorlin-associated basal cell carcinomas (Farndon et al, 1992).

**Retinoblastoma**

The hypothesis that recessive genetic events are important in the development of many types of human cancer was first proposed for RB. This disease occurs in two forms, hereditary and sporadic, each of which occurs with about equal frequency. Sporadic cases usually present with development of a single unilateral tumour of the retina, whereas patients with the hereditary form usually develop multiple tumours in both eyes. Knudson (1971) proposed that the clinical observations could be accounted for by two genetic events within the target cells. In the hereditary form, the first event is inherited in the germ line and the second by random somatic mutation, but in the sporadic form both events occur in the somatic cells. Individuals with the hereditary form of RB have a higher than expected risk of developing a number of different tumours, including sarcomas and gliomas (Jensen & Miller, 1971; Draper et al, 1986).

**Tuberous sclerosis**

This disease affects about one in ten thousand live births and is characterised by multiple facial angio-fibromas, epilepsy and mental retardation (Gomez, 1991). It is inherited in an autosomal dominant manner but 75% of cases are new mutations from normal parents. The intracranial lesions which develop in this disease are either subependymal glial nodules or larger subependymal giant cell astrocytomas. At least two genes are involved, one on chromosome 9 and one on chromosome 11. These two gene abnormalities appear to produce indistinguishable phenotypes of the disease. The TSC2 gene product, tuberin, has been shown to have a region of homology to the GTP-ase activating protein GAP3, suggesting that this gene acts as a tumour suppressor gene (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993).
**Blue rubber-bleb naevus syndrome**

This syndrome predisposes individuals towards the development of medulloblastoma (Satya-Murti et al., 1986), and although this disease usually occurs sporadically, it may be inherited as an autosomal trait. Clinical manifestations include blueish, rubbery naevi which occur on the body surface. In addition to these skin lesions, vascular malformations of internal organs are common.

**von Hippel-Lindau disease**

This syndrome is associated with the development of a variety of tumours and is dominantly inherited. Tumours include haemangioblastomas of the CNS and retina, renal cell carcinoma and phaeochromocytoma (Neumann et al., 1992). Latif et al. (1993) have identified the von Hippel-Lindau (VHL) suppressor gene which is highly conserved and encodes two widely expressed transcripts of about 6 and 6.5 kilobases. The VHL protein may be localised on the cell membrane and may be involved in signal transduction or cell adhesion (Latif et al., 1993).

**Cytogenetics**

Cytogenetic studies on primary or short-term cultures have provided a valuable tool for detecting non-random genetic events which provide useful clues to the genetic lesions important in the pathogenesis of many kinds of brain tumours.

**Astrocytomas**

From the literature, it has been shown that 70-80% of published karyotypes from GM in adults possess abnormal stemlines (Bigner et al., 1990b). Most frequently seen are gains of chromosome 7, losses of chromosome 10, and deletions and translocations involving the short arm of chromosome 9. Less commonly, losses of chromosome 22 and the presence of double minutes have been reported (Bigner et al., 1988). In one series, one of two anaplastic astrocytomas in children showed a more complex karyotype, involving the gain of chromosomes 7 and 2 and the loss of chromosome 14 (Griffin et al., 1988).

Low grade astrocytomas in adults usually have a normal stemline, but many have small numbers of abnormal clones with chromosome abnormalities comparable with those seen in the higher-grade tumours (Rey et al., 1987; Jenkins et al., 1989). Other structural abnormalities associated with chromosome 1 have been reported in childhood astrocytomas as well as other types of brain tumour (Douglass et al., 1985; Griffin et al., 1988). Recently, in 33 low grade astrocytomas in children, the most frequent numerical abnormalities were trisomy 5, 11 and 7 (Neumann et al., 1993). Also no loss of chromosome 10 was seen nor double minutes in all the low grade astrocytomas which were analysed (Neumann et al., 1993).
Studies suggest that mutations in the p53 gene may be associated with the progression of tumours from low grade to GBs in adults (Chung et al, 1991), or recurrence of GB (Hayashi et al, 1991; Sidransky et al, 1992). However, in paediatric brain tumours, p53 mutations are much less common. They are extremely rare in ependymomas and in only 12% of oligodendrogliomas is the gene mutated (Ohgaki et al, 1991). Although, high grade astrocytomas in adults and children are indistinguishable histologically, their genetic basis appears to be different. Cogen (1991) reported 17p loss in 4/6 children with highly anaplastic astrocytomas but found infrequent mutations in the p53 gene, suggesting the presence of an additional tumour suppressor gene on 17p in paediatric brain tumours. Whilst LOH for chromosome 10 and gene amplification are common in GBM in adults (Rasheed et al, 1994), in childhood gliomas, there are fewer losses for 17q and 10, little or no gene amplification and infrequent p53 mutations. Louis et al (1993a) described loss of sequences on chromosome 17p and mutations in the p53 gene in a high proportion of brain stem GBs in children, indicating that these tumours may have a different pathogenesis from paediatric astrocytomas elsewhere in the brain.

**Medulloblastomas**

The presence of an isochromosome 17q is the most consistent abnormality in MBs (Griffin et al, 1988; James et al, 1990). Short-term cultures derived from MBs are commonly diploid with near normal karyotypes, although structural or numerical abnormalities of chromosome 17, loss of a sex chromosome, loss of all or the long arm of chromosome 6, additional copies of chromosome 7, and structural abnormalities involving chromosomes 5, 11, and 16 have been reported (Griffin et al, 1988; Biegel et al, 1989). A case of an isolated unbalanced translocation between chromosomes 1 and 6 has been described by Stratton et al (1991).

Loss of sequences on chromosome 17p has been found in nearly 50% of MBs and interestingly, this appears to be associated with a less favourable outcome (Cogen et al, 1990). Mutations of p53 are uncommon in MBs (Saylors et al, 1991). It has been suggested that, while losses in 17p undeniably occur in MBs, these do not involve the p53 gene, and that a second tumour suppressor gene exists on 17p distal to p53 which is important in the development of these tumours. Analysis has confirmed that non-random loss of alleles from the long arms of chromosomes 6 and 16 occur frequently in MBs, although which genes are involved is as yet still unknown (Thomas & Raffel, 1991).

**Ependymomas**

Karyotypes obtained from a small number of short-term cultures derived from these tumours, indicate that loss of chromosome 16 or deletions of 16q13, loss or structural changes of gonosomes and losses of chromosomes 10, 13, 14 and 22 occur frequently (Rey et al, 1987; Dal Cin & Sandberg, 1988; Griffin et al, 1988; Jenkins et al, 1989; Stratton et al, 1989a). Involvement of
chromosome 17 is frequent, but studies in four ependymomas showed that changes in chromosome 22 occur, as well as losses from chromosome 13 (James et al., 1990). Involvement of chromosome 1 in certain types of ependymomas has also been reported (Sawyer et al., 1994).

High grade ependymomas have been shown to have losses in chromosome 10 (Dal Cin & Sandberg, 1988) as have recurrent tumours (Bown et al., 1988). These findings indicate that chromosome 10 alterations may be associated with the progression towards anaplasia in these tumours. Cytogenetic evidence has also suggested that genes of chromosome 6 may be important in the early development of ependymomas (Bown et al., 1988).

Thiel et al. (1992) showed that in cultures derived from seven ependymomas two contained simple clonal abnormalities and four had complex abnormalities. They found monosomy 22 in four cases and del (22)(q13) in another. Similar observations have been documented (Griffin et al., 1992). Jenkins et al. (1989) found a breakpoint in an ependymoma and this abnormality has been observed by others (Sainati et al., 1992; Griffin et al., 1992).

Environmental and dietary exposure

Parental occupation
Parental occupation has been suggested as a risk factor in the development of brain tumours. A case control study by Wilkins & Sinks (1990) found an excess risk of developing brain tumours associated with paternal employment in agriculture, transportation, construction or where there was exposure to metals, paints, hydrocarbons and nitro-compounds. However, Giuffre et al. (1990) analysed 200 cases of histologically verified brain tumours in patients under 15 years of age, but were unable to confirm any association between the occupation of either parent and the subsequent development of brain tumours in their offspring. Although, Preston-Martin et al. (1982) previously reported a possible association with high dietary intake of nitrosoamines and their precursors and the development of a brain tumour, this was not confirmed by a recent report in a case-controlled study of maternal diet (Bunin et al., 1993).

Ionizing and non-ionizing radiation
There are many reports implicating irradiation as a risk factor in the development of cerebral neoplasms in children. Worldwide, 250,000 children received irradiation for Tinea capitis between 1910 and 1959 at a dose from 0.7 to 2 Gy (Werner et al., 1968). There appears to be a strong relationship between these small doses of radiation and the subsequent development of meningiomas (Modan et al., 1974; Rubinstein et al., 1984) and gliomas (Shore et al., 1976) and this has been confirmed in studies which showed that radiation doses in the order of 1-2 Gy can significantly increase the risk of neural tumours (Ron et al., 1988). A slight to moderately increased risk of developing a brain tumour in childhood has been associated in a number of
reports with *in utero* exposure to diagnostic irradiation (MacMahon, 1962) and Howe et al (1989), reported a six-fold to eight-fold higher risk of developing brain tumours in children who had undergone skull radiographs within the 5 years prior to diagnosis.

Exposure to low-level electric fields has been shown to result in changes in DNA synthesis, enzymatic activity and transcription in cell culture (Byus et al, 1987). It has been postulated that exposure to electromagnetic fields (EMFs) may be carcinogenic (Wertheimer & Leeper, 1979) although unsubstantiated.

**Other factors**

A higher risk of childhood brain tumour has been associated with a number of miscellaneous factors, such as exposure to farm animals and insecticides and a number of birth characteristics, such as being first born or having a high birth weight (Gold et al, 1979), but has not been confirmed in all studies (Howe et al, 1989). Head trauma has also been reported as increasing the risk of brain tumour development by a factor of two (Howe et al, 1989).

Barbiturates which are employed as medication for the treatment of epilepsy have also been associated with an increased risk of developing brain tumours (Gold et al, 1978). Goldhaber et al (1990) was unable to demonstrate an increased risk of developing brain tumour following *in utero* exposure, but was able to show a relationship with barbiturate use in childhood.

A population-based study using the Connecticut Tumour Registry data (between 1957-1961) showed an increased risk for CNS neoplasms, particularly medulloblastomas in children, whose mothers were vaccinated, during pregnancy with polio vaccine inadvertently contaminated with the SV40 virus (Farwell et al, 1978).

**Aetiology of brain tumours**

**Induction of experimental brain tumours with polycyclic hydrocarbons**

Seligman & Shear (1939) successfully produced brain tumours by using methylcholanthrene, a pure polycyclic hydrocarbon implanted directly into the brains of rats. Brain tumours developed in 20% of the rats within a year of treatment. The type of tumour produced was governed by the site of implantation. If the carcinogen was implanted in the frontal white matter, oligodendrogliomas were produced; in the ventricles ependymomas and in the subcortical regions of the parietal white matter, astrocytomas were produced while MBs developed after implantation in the cerebellum. Another polycyclic hydrocarbon, dimethylbenz-anthracene (DMBA) can be administered intravenously or orally, resulting in the development of tumours in the offspring of pregnant rats.
Induction of experimental tumours with alkylating nitroso compounds

Intravenous administration of methyl-nitrosourea (MNU) was found to produce tumours in the nervous system of adult rats (Druckery et al., 1965). Intravenous injection of MNU produces tumours in 90-100% of animals over a 32-36 week period. These are usually anaplastic tumours including astrocytomas, oligodendrogliomas and mixed gliomas. The tumours tend to be sited in the periventricular regions, hippocampus and subcortical white matter. Ethyl-nitrosourea (ENU) however, is a more potent carcinogen than MNU. For example, a single dose (5mg/kg) of ENU given to pregnant rats in the latter half of pregnancy results in nervous system tumours in the majority of their offspring. ENU-induced tumours are similar to those induced by MNU, including mixed astrocytomas and oligodendrogliomas in the CNS and anaplastic neurinomas in the peripheral nervous system (PNS) (Swenberg, 1976).

Exposure of the target macromolecules to the carcinogen must be short, since nitrosoureas have an in vivo half life of about 8 minutes. This has proved to be an advantage as the exact time from the point of exposure is known and a number of investigators have been able to perform studies on ENU-induced tumour development (Lantos & Pilkington, 1979). The one disadvantage of using systemic carcinogens is that location of tumour is unpredictable and many tumours are also produced in the PNS. Nevertheless, researchers have used the explants of ENU induced tumours in a wide variety of experimental studies (Schiffer et al., 1976; Spence & Geraci, 1981).

Virus induction of experimental brain tumours

Vasquez-Lopez (1936) reported that brain tumours could be induced in chickens by injecting a cell-free extract of the Rous sarcoma which is known to be induced by a retrovirus, the avian sarcoma virus. This and similar studies have resulted in the discovery of a number of DNA and RNA viruses capable of inducing brain tumours in experimental animals.

DNA viruses

In hamsters, mice and rats, brain tumours have been induced using human adenovirus 12, simian adenoviruses 7 and 20 and the avian adenovirus (CELO). The incidence of tumours ranges between 8 and 93%, and the latency period can be more than 9 months. Unfortunately, the tumours produced are similar morphologically to sarcomas and undifferentiated neuroectodermal tumours rather than malignant astrocytomas. Intracerebral inoculation of Syrian hamsters with polyoma virus induces sarcomas. Other papovaviruses such as murine papovavirus, bovine papilloma virus, simian vacuolating virus (SV40) and the human SV40-related papova viruses (PML strains 1-4, JC BK) appear to result in various tumour types being induced, usually mesenchymal tumours, with occasional reports of high grade astrocytomas, ependymomas (Corallini et al., 1977) and MBs (Padgett et al., 1977). In non-human primates, the JC virus has induced malignant astrocytomas and mixed glial-neuronal tumours. PNS embryonal neuroepithelial tumours have been produced in rodents after intraperitoneal
inoculation with the human adenovirus 12 (Ogawa, 1989). In a recent report, 10 of 20 human choroid plexus tumours and 10 of 11 ependymomas expressed a segment of the T antigen related to the monkey polyoma virus, SV40, suggesting that this virus or a closely related one may play a role in the development of these tumours (Bergsagel et al, 1992).

RNA viruses

Bigner et al (1975), using the avian sarcoma virus were able to produce a uniform and reproducible model where 100% of neonatal rats inoculated intracerebrally developed anaplastic astrocytomas, and because of the model's uniform range of mortality this has proved to be an important system for therapeutic investigations. Murine sarcoma virus induces sarcomas and glioblastomas in rats and the simian sarcoma viruses produce malignant astrocytomas in marmosets which are similar histologically to human malignant glioma.

Although it has been shown that oncogenic viruses are capable of producing tumours in experimental animals which are similar to human brain tumours and that in vitro such viruses can transform human glial cells, the role of viruses in inducing human brain tumours remains unclear (Ibelgaufts, 1982). Using gene transfer into normal neural tissue it has been possible to produce brain tumours. The transfer of SV40 large T gene into CNS grafts produced tumours which resemble human medulloblastomas (Aguzzi et al, 1991) or retinoblastomas (Theuring et al, 1990).

Classification of brain tumours

Neoplasms of the central nervous system may originate from four main types of neuroepithelium: neuronal, glial, pineal and retinal cells. Gliomas represent the single most important and most common group of tumours, and can be further subdivided into astrocytomas, oligodendrogiomas and ependymomas.

Astrocytomas

Astrocytomas are derived from astrocytic cells and can in children arise in either the cerebral hemispheres or more commonly, the cerebellum. In the latter site, they are almost always well circumscribed and tend to be cystic, with the neoplasm confined to a small intramural nodule. Pilocytic astrocytomas are composed of two cell types, stellate and elongated bipolar astrocytes. The relative proportion of these two types of cell varies between neoplasms. Vascular endothelial proliferation is common, as is a degree of nuclear pleomorphism. Low grade fibrillary astrocytomas also occur in the cerebral hemispheres and in the brain stem of children. The morphology of the tumour astrocytes often display ill-defined borders, although stellate forms do occur. The cells have multiple cytoplasmic processes which form a
fibrillary matrix. High grade astrocytomas comprise perhaps 10-15% of all supratentorial tumours in children. Histologically they closely resemble their adult counterparts.

Other astrocytic tumours of childhood, include, pleomorphic xanthoastrocytoma (PXA), subependymal giant cell astrocytoma and dysembryoplastic neuroepithelial tumour (DNT) (Kleihues et al, 1993). DNTs are commonly located in the temporal lobe and associated with intractable complex partial seizures in young patients. They resemble gangliogliomas with respect to their location, clinical behaviour and pathological appearance, but may be confused with pilocytic astrocytoma, in terms of morphology and behaviour (Leung et al, 1994).

**PNET and medulloblastoma**

Macroscopically MBs are soft, friable and fairly well demarcated from the remainder of the cerebellum. Generally, they infiltrate the floor or lateral wall of the 4th ventricle and extend into its cavity. Microscopically, the tumour is highly cellular, consisting of small cells with hyperchromatic nuclei and scanty cytoplasm. Mitoses are numerous and areas of tumour necrosis are quite common.

It has been suggested that medulloblastoma be considered a PNET although there is little evidence that these tumours are related to other small cell embryonic tumours of either the CNS or PNS. Similarly, it suggests that there is a hypothetical pluripotent progenitor cell involved in the development of these tumours. Elegant studies using two novel neuronal markers, class IIβ-tubulin (βIII) and calbindin D28K, which are expressed during normal cerebellar development have shed light on the pathogenesis of medulloblastoma (Katsetos & Burger, 1994). The normal cerebellar cortex is derived from two distinct sources; a neuroepithelial matrix of the subventricular surface of the roof membrane of the cerebellar plate known as the the ventricular matrix (VM) and a distinct matrix on the outside of the cerebellum known as the external granule layer (EGL). The EGL expresses βIII-tubulin but never calbindin, which is expressed only in the VM. This provides a ration basis for studies aimed at determining the cell of origin of medulloblastomas. Calbindin immunoreactivity is restricted to populations of cells in classic medulloblastoma and medulloblastoma with ganglion cells and βIII-tubulin staining is seen in desmoplastic variants of medulloblastoma which contain evidence of a differentiating neuronal phenotype. This strongly suggests that different populations of cells are targets for the development of these variant forms of medulloblastoma and that there does not seem to be a need to invoke the existence of a pluripotent stem cell as the cell of origin of medulloblastoma.

The presence of photoreceptor antigen expression on some MBs has been reported, suggesting a linkage with RBs, and tumours which express this antigen appear to have a better prognosis (Czerwionka et al, 1989). Patients with the desmoplastic variant of MB
appear to have conflicting survival results in different studies (Chatty & Earle, 1971; Caputy et al., 1987; Goldberg-Stern et al., 1991).

Ependymomas

Ependymomas are histologically characterised by the presence of perivascular pseudorosettes and, occasionally, by true ependymal rosettes with central lumina and blepharoplasts. Their microscopic appearance ranges from relatively bland to that of a highly cellular neoplasm with mitoses and variable amounts of cytological anaplasia and necrosis. Histological grading of ependymomas still continues to be of questionable clinical value (Ross & Rubinstein, 1989; Goldwein et al., 1990b). In some series it has been shown that patients with "high-grade" tumours have shorter survival times, but other reports showed no such correlation (Reyes-Mugica et al., 1994).

Criteria employed for defining the more malignant tumours include, cellular anaplasia with increased mitoses, increased cellularity and mitoses with necrosis, and a combination of increased mitoses and cellularity, cellular pleomorphism, loss of differentiation, necrosis and vascular endothelial proliferation (Zulch, 1979; Rorke et al., 1985; Nazar et al., 1990). Survival analysis of a large series of 298 ependymomas showed that the number of mitoses, endothelial hyperplasia, necrosis, intracranial site, age less than 4 years were highly significant prognostic factors (Schiffer et al., 1991). Goldwein and colleagues (1990a), have proposed that histology of the ependymoma at the time of relapse provides a more reliable indication of prognosis than the original tumour specimen. The site of the tumour also appears to have some importance with respect to survival. It has been observed that patients with myxopapillary ependymomas in the spine, have a much better prognosis than patients whose tumours were located in the cerebral hemispheres.

The majority of ependymomas express GFAP, and additionally some may express epithelial antigens, providing further evidence for a possible association with choroid plexus tumours (Mannoji & Becker, 1988), but the clinical importance of these observations have not yet been established. Some workers suggest that the pattern of expression of these two groups of antigen appears to be associated with histology. GFAP positive and epithelial antigen negative tumours are likely to be "better differentiated" while the more malignant and less differentiated tumours are more likely to express the epithelial markers (Kaneko et al., 1990). However, other authors have demonstrated that epithelial membrane antigen was expressed in the better differentiated tumours (Uematsu et al., 1989).
Clinical features and treatment of brain tumours in childhood

Signs and symptoms
Overall, chronic or frequent headaches are the most common symptoms in children with a brain tumour prior to hospital presentation. The incidence of this symptom appears to be compartment-specific, occurring in 58% of children with supratentorial tumours and in 70% of children with infratentorial tumours (The Childhood Brain Tumor Consortium, 1991). In children with supratentorial tumours, headache is associated with diplopia, coma, stiff neck, pupillary abnormalities, and abnormalities of personality, academic performance, or speech. In these patients, seizures are also common (Gilles et al, 1992). Virtually all children with cerebellar astrocytoma and about 90% of children with MB develop hydrocephalus (Albright et al, 1989).

Treatment
Astrocytomas
Low grade astrocytomas
Pilocytic cerebellar astrocytomas are usually curable surgically, provided the surgeon is able to resect the entire tumour and that invasion into other structures, such as the brainstem or peduncle has not occurred. If there is residual tumour and the risk of morbidity low, then a further operation may be indicated. Residual tumour may also be treated by limited-field external beam irradiation, although there is little evidence that this will significantly prolong survival (Austin & Alvord, 1988; Garcia et al, 1989).

The prognosis for completely excised tumours is excellent, as 90-94% of patients with juvenile pilocytic astrocytomas remain disease-free 20 years post-operatively (Austin & Alvord, 1988; Forsyth et al, 1993). However, whilst long-term survival of children with the pilocytic astrocytomas is good, the survival of children with the diffuse astrocytoma is very much poorer (Hayostek et al, 1993).

Chemotherapy has not been widely used in these tumours although, carboplatin and iproplatinum in patients with recurrent low grade astrocytomas (Friedman et al, 1992b) appear to significantly prolong stable disease. Gajjar et al (1993) showed that an alkylating agent or platinum-based chemotherapy can successfully delay the growth of low grade astrocytomas and suggested that it be included in the primary treatment of these tumours.

High grade astrocytomas
Generally, gross total surgical resection or extensive removal is the aim of surgery, although with tumours that infiltrate into adjacent tissues, this may not be possible. Radical resection as opposed to biopsy improves survival (Artico et al, 1993). In a recent study conducted by the
Children's Cancer Group (CCG-945), the 3-year progression-free survivals were 50% and 28% for radical and less radical resections respectively (Wisoff et al, 1993).

The importance of conventional radiotherapy in the treatment of high grade astrocytomas in adults has been confirmed in randomised clinical trials (Walker et al, 1980; Green et al, 1983), however no large scale trials have been carried out in children. Typically, a conventional radiation regimen comprises of a total dose of 50 to 60 Gy, fractionated at doses of 1.8 to 2.8 Gy per day, over a five day week. A dose-response relationship in children with anaplastic astrocytoma (Marchese & Chang, 1990) has been demonstrated. In patients who received at least 54 Gy, the 5-year survival is 60% as opposed to only 14% in patients who received 35-50 Gy.

A number of single and multi-institution series have shown the effectiveness of nitrosourea-based chemotherapy in extending survival in adults with high grade astrocytomas (Walker et al, 1978; Sheline, 1990; Vega et al, 1992). In children with newly diagnosed high grade astrocytomas adjuvant chemotherapy treatment with CCNU, VCR and prednisone, significantly prolonged overall and disease-free survival with 46% of patients surviving at 5-years versus 18% of patients treated with radiation only (Sposto et al, 1989).

Complex multidrug chemotherapy protocols (e.g., 8 drugs in 1 day, consisting of CCNU, VCR, cisplatin, hydroxyurea, procarbazine, (PCB), prednisone, cytosine arabinoside and dacarbazine (DTIC)) have not been shown to be more efficacious than the standard chemotherapy regimes, such as CCNU, VCR and prednisone (Finlay et al, 1991; 1995). Similarly, high dose chemotherapy with agents such as busulphan and thiopeta (Kalifa et al, 1992), or high dose thiopeta with cyclophosphamide (Heideman et al, 1993) both followed by autologous marrow transplantation have failed to improve survival in children with malignant astrocytomas.

More recent studies have indicated that combination chemotherapy using alternating cycles of cyclophosphamide and VCR, followed by cisplatin and etoposide are effective in the adjuvant treatment of these tumours. The 2-year progression-free rate was 54% and the overall survival rate of 65% for these children with malignant astrocytoma (Duffner et al, 1993). This result compared more favourably with other studies in older children treated with post-operative radiation alone, where the progression-free survival was 20% and overall only 40% (Shrieve et al, 1992). It has been reported that children less than 3 years of age had higher response rates to chemotherapy than older children, indicating that chemotherapy can be used to treat children without the need to irradiate (Geyer, 1995).
Brain stem gliomas (Astrocytomas)

Brain stem gliomas usually occur in patients under the age of 18 years. Pathologically, they may be pilocytic or diffuse. The peak incidence of these tumours is in the latter half of the first decade of life and there is no sex predilection. These tumours can be subdivided into three groups, including diffuse intrinsic, tectal and cervicomedullary. Although overall prognosis is poor, a number of factors have been found to be of prognostic importance (Stroink et al., 1986). It has been reported that surgery is not viable in the diffuse intrinsic tumour, and that biopsy may even increase morbidity (Albright et al., 1993). In children with brain stem gliomas, either conventional or higher dose hyperfractionated radiation therapy is given.

Ependymomas

Treatment involves surgery followed by radiation therapy to the primary tumour in low grade tumours or to the whole craniospinal axis in high grade tumours (Dohrmann et al., 1976; Pierre-Kahn et al., 1983; Goldwein et al., 1990b; Kovalic et al., 1993). Long survival after surgery alone is rare. Kovalic et al (1993) showed that cranial irradiation and pathology were significant prognostic factors, whilst another report stressed that the extent of surgical resection was a strong prognostic factor (Healey et al., 1991). The supratentorial ependymomas usually have a poorer prognosis than the infratentorial tumours. For posterior fossa tumours there appears to be an association between histological features and the probability of spinal seeding, although this does not seem to correlate with overall prognosis (Rawlings et al., 1988). Knowledge of the microanatomical relationship between the tumour and the surrounding structures has shown to improve the postoperative outcome of children with posterior fossa ependymomas (Ikezaki et al., 1993). The probability of recurrence is correlated with degree of removal of the tumour. Most tumour recurrences are local, and occur either 1-2 years after incomplete removal or 2-3 years after complete excision of the tumour (Tomita et al., 1988a; Healey et al., 1991; Hoppe-Hirsch et al., 1993).

Radiation therapy with doses of between 50 and 54 Gy to the posterior fossa is the usual treatment (Pierre-Kahn et al., 1983; Goldwein et al., 1990b; Vanuystel et al., 1992; Rousseau et al., 1994). However, the value of craniospinal irradiation remains controversial (Salazar et al., 1983; Goldwein et al., 1990b). The 5-year survival rate for children with ependymoma is in the region of 40-70%, comparable with those with MB. Treatment of recurrent ependymomas is difficult. Chemotherapy with cisplatin and etoposide (VP-16) has been shown to be of some benefit (Goldwein et al., 1990b), as with combinations of cisplatin and MCNU (Tamura et al., 1990). Adjuvant CCNU and VCR has not increased survival of patients with recurrent ependymoma significantly (Krischer et al., 1991).
**Medulloblastomas**

MBs represent 20 to 30% of all childhood brain tumours. Over the last 60 years, survival rates have changed dramatically. Early poor results have been significantly improved by advances in surgical technique, instrumentation, anaesthesia, and the addition of radiotherapy to the treatment to the tumours (Duffner & Cohen, 1984). A number of series have reported 5-year survival rates ranging from 40% to 60% (Bloom et al, 1969; Bloom & Bessell, 1990). A review of survival rates of children with MB in Britain has showed less success where the 5-year survival rate for a large group of patients was only 18% between 1962 and 1970, 27% between 1971-1974, and 38% between 1975-1978 (Bloom et al, 1990).

Prognosis is better in patients who were older at time of diagnosis, who had total removal of tumour, and had no evidence of extension into the brain-stem and no dissemination through the neuroaxis. However, patients with significant post-operative tumour burden, under 5 years of age, dissemination through the neuroaxis, and possibly evidence of cellular differentiation, have a poor prognosis (Allen & Epstein, 1982; Evans et al, 1990).

Radiation therapy has been responsible for the major improvements in the survival of children with MBs (Bloom et al, 1969). MB is radiosensitive and radiotherapy remains the most significant determinant of outcome in patients with this disease (Leibel & Sheline, 1987). The recommended doses for tumour control are 50 Gy to the posterior fossa and a minimum of 30 Gy to the remaining neuraxis (Berry et al, 1981; Garton et al, 1990).

A number of large studies which evaluated the effectiveness of chemotherapy have been published over the last decade. Analysis of a large series of children with brain tumours from the Royal Marsden Hospital treated between 1950-1981, indicated that of those patients who received radiotherapy alone, the survival rate was 42% at 5 years, 33% at 10 years and 29% at 15 years (Bloom et al, 1990). The use of adjuvant chemotherapy did not improve survival in these patients (Bloom et al, 1990).

The Children's Cancer Study Group (CCSG) and the Radiation Oncology Group (RTOG), have evaluated results of post-operative radiation and vincristine, CCNU, and prednisone compared with surgery and radiation therapy alone (Evans et al, 1990). The 5-year survival rate was approximately 65% for both groups. However, those patients with more extensive tumours and who had received chemotherapy had better progression-free survival, but this was not statistically significant.

Larger scale randomised studies indicate a small but insignificant difference in disease-free survival in favour of chemotherapy. However, chemotherapy did seem to be of benefit in a particular subgroup of patients including those with total rather than subtotal resection, those whose age was greater than 2 years, and those with evidence of
dissemination in the brain-stem and neuroaxis (Duffner & Cohen, 1992). The addition of cisplatin to this regimen may also extend event-free survival (Packer, 1994).

Kovnar et al (1990) have evaluated a pre-radiation chemotherapy regimen comprising cisplatin and VP-16 in 11 children with newly diagnosed MBs, pineoblastomas, and cerebral neuroblastomas. There were responses in all eight children with medulloblastomas treated and none showed signs of progression 18 to 48 months after diagnosis. Another study on 30 patients who underwent pre-irradiation chemotherapy with VCR, cisplatin and cyclophosphamide followed by craniospinal irradiation demonstrated complete or partial response in 13/30 (43%) patients, but the 2-year progression-free survival for these patients was still only 40% (Mosijczuk et al, 1993).

Preoperative chemotherapy in brain tumours using drug combinations including dibromodulcitol (DBD) proved to be effective for the treatment of MB (Schuler et al, 1992). A subsequent study showed that preoperative therapy enabled a more radical resection to be carried in the majority of MBs and in some ependymomas (Schuler et al, 1993). A recent Phase II study using ifosfamide has shown some activity in childhood brain tumours, including MBs, although it does produce high neurotoxicity limiting its use in recurrent progressive tumours (Chastagner et al, 1993).

Cerebral PNET

Hart and Earle (1973) introduced the term primitive neuroectodermal tumour (PNET) to describe poorly differentiated embryonal tumours having the histological features of a MB, but which occur in areas of the brain other than the cerebellum. The prognosis of these tumours is poor. Hart and Earle (1973) reported a case study of six patients, where only one patient lived 5 years after onset of symptoms, and the mean survival of the remainder was only 10 months. Parker et al (1975) observed that from a total of six patients, four died per-operatively, two died 18 and 21 months post-operatively, and only one patient was alive after 3 years. Another study showed that less than 40% of patients were alive after 6 months, and less than 10% at 1 year even following subtotal resection, radiation and chemotherapy using VCR, prednisone and CCNU (Kosnik et al, 1978).

Even though cerebral PNETs are histologically similar to cerebellar medulloblastomas, there is a clear difference clinically with respect to response to treatment and the subsequent survival between the two groups of patients. Patients with MBs show better response to treatment than patients with cerebral PNET (Tomita & McLone, 1986; Tomita et al, 1988b). However, one study reported long-term survival with radical resection in children (Halperin et al, 1993). A phase III trial conducted by Children's Cancer Group in North America (CCG-921 study) comparing posterior fossa PNET (mainly MB) with
supratentorial PNETs (of various diagnoses) were randomised to one of two chemotherapy regimens (all patients had surgical resection and post-operative craniospinal irradiation with a boost to the primary tumour site). Chemotherapy was either with CCNU, VCR and prednisolone or the "8 days in one drug" (including CCNU, VCR, CIS, hydroxyurea, PCB, cytosine arabinoside, cyclophosphamide and methylprednisolone). Initial results confirm previous reports in that supratentorial PNETs had a worse progression-free and overall survival than patients with posterior fossa NET (i.e., MB) (49% vs 59%, p=0.03 respectively, Zeltzer et al, 1993)

Seeding into the CSF has been observed by a number of authors. In one study, 9/13 patients had CSF seeding (Ganti et al, 1983), and other authors demonstrated that in all autopsied patients diffuse CSF dissemination of neoplastic cells was present (Parker et al, 1975; Kosnik et al, 1978). This suggests that tumour cells had already disseminated at the time of detection, but in another study, only 1/8 patients was seen to have CSF seeding on postoperative staging (Tomita et al, 1988b). This illustrates the need for whole neuroaxis irradiation together with frequent CSF cytology studies and assessment by myelography in the follow-up period.

Side effects of treatment
Problems with sequelae due to radiation treatment in the very young is a major concern. Severe effects on growth and neurological development have been reported (Duffner et al, 1993). In the few studies that have been carried out, initial results have indicated that chemotherapy can be used to delay (and in some cases omit) the need for radiation therapy in children for both benign and malignant tumours (Duffner et al, 1993).
Established cell lines from human brain tumours

Established cell lines (passage level >70) have been produced from a number of different types of human brain tumours including malignant astrocytomas and oligodendrogliomas (Ponten & Macintyre, 1968; Giard et al, 1973; Westermark et al, 1973; Bigner et al, 1981a; Collins, 1983; Rutka et al, 1987c). Most of these have been established from adult malignant astrocytomas and include some of these most widely used for in vitro studies (e.g., U138MG, U251MG, U373MG among others). However, there has been a marked lack of established cell lines from malignant astrocytomas from children and benign astrocytomas from both children and adults. In fact, there is only one established cell line derived from an 8-year old boy with a frontal glioblastoma, SF-188 (Rutka et al, 1987c) but none from the lower tumour grades.

Established cell lines from medulloblastoma are uncommon (Batzdorf & Gold, 1974; Friedman et al, 1985; Jacobsen et al, 1985; Rutka et al, 1987a; Friedman et al, 1988a; Tamura et al, 1989a; Bigner et al, 1990a; Darling, 1990; Oakes et al, 1990; Pietsch et al, 1994). Initial studies in the growth of medulloblastoma in vitro have concentrated on the description of short-term cultures from these tumours as either monolayers, where satellite colony formation was observed (Batzdorf & Gold, 1974), or in organ culture where astrocytic differentiation was produced (Herman & Rubinstein, 1984). The description of well characterised lines derived from medulloblastomas have been published and are summarised in Table 1.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Growth in vitro</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D283 Med</td>
<td>Biopsy-abdominal metastasis</td>
<td>Suspension</td>
<td>Friedman et al, 1985</td>
</tr>
<tr>
<td>D341 Med</td>
<td>Biopsy-cerebellum</td>
<td>Suspension</td>
<td>Friedman et al, 1988a</td>
</tr>
<tr>
<td>D384 Med</td>
<td>Biopsy-cerebellum</td>
<td>Suspension</td>
<td>Bigner et al, 1990a</td>
</tr>
<tr>
<td>D425 Med</td>
<td>Biopsy-cerebellum</td>
<td>Suspension</td>
<td>Bigner et al, 1990a</td>
</tr>
<tr>
<td>DAOY</td>
<td>Biopsy-cerebellum</td>
<td>Adherent</td>
<td>Jacobsen et al, 1985</td>
</tr>
<tr>
<td>ONS-76</td>
<td>Biopsy-cerebellar tumour</td>
<td>Adherent</td>
<td>Tamura et al, 1989a</td>
</tr>
<tr>
<td>ONS-81</td>
<td>Biopsy-cerebellar tumour</td>
<td>Adherent</td>
<td>Tamura et al, 1989a</td>
</tr>
<tr>
<td>MHH-MED-1, 2, 3 and 4</td>
<td>Biopsy-cerebellar tumour</td>
<td>Suspension</td>
<td>Pietsch et al, 1994</td>
</tr>
</tbody>
</table>
The most important difference between the five cell lines established at Duke University and those established by others is that the cell lines grow in suspension as loose aggregates of floating cells or larger aggregates resembling multicellular tumour spheroids (Friedman et al, 1985, 1988a, Bigner et al, 1990a, He et al, 1991). In comparison, Daoy, ONS-76 and ONS-81 grow as adherent monolayers (Jacobsen et al, 1985; Tamura et al, 1989a). D283 Med was established from a mixture of digested tissue fragments and ascites cells. D341 Med was produced from a mechanically disaggregated single cell suspension. Adherent cells only persisted to passage level 3 but the non-adherent cells continued to grow. Both D384 Med and D425 Med were established from material removed using a Cavitron Ultrasonic Surgical Aspirator (CUSA) and put into culture without further digestion. Daoy, ONS-76 and ONS-81 were from mechanically disaggregated tumour biopsies without enzymatic digestion.

Recently, Fults et al (1992b) established a cell line, PFSK from a PNET in the cerebral hemisphere of a 22 month old boy. To date this is the only example of an established PNET cell line derived from the cerebral hemisphere. Initially, cells proliferated as a monolayer but after 10 days in culture, foci of cells were growing as multilayered aggregates on the monolayer surface. These foci were then picked and transferred to microtitre wells which produced sublines; PFSK-1 and PSFK-2C. The PFSK-2C cells were passaged independently giving rise to the PFSK-2 subline which grows in clumps with poor surface adherence. Recently, Pietsch et al (1994) has established a further five cell lines from human PNETs, three were generated from cerebellar MB (MHH-MED-2, MHH-MED-3 and MHH-MED-4) and one from a PNET located in the spinal cord (MHH-MED-5) and another established from cells obtained from CSF of a patient with a recurrent MB (MHH-MED-1). All biopsies were minced and placed into culture and adherent cells removed by two consecutive steps of plastic adherence for 90 mins. The four MB cell lines grew in suspension as floating aggregates or as slightly adherent cells (Pietsch et al, 1994). One of the earliest described medulloblastoma cell line, TE671 (Mc Allister et al, 1977), has subsequently been shown to be identical to the RD cell line, originally derived from a rhabdomyosarcoma (Stratton et al, 1989b).

Only two established cell lines have been described from ependymomas. One is NU2-26 (Nakagawa et al, 1983) derived from a cerebellar ependymoma and the other, KM-SII (Mihara, 1986) was established from a recurrent ependymoma developed in the 4th ventricle of a 4 year old boy. Both cell lines were produced by mechanical disaggregation and treatment with trypsin and grew as adherent cultures.

Morphology
The morphological characteristics of cells derived from human brain tumours have been reviewed over the years (Lumsden, 1971; Unterharnscheidt, 1972). Low grade astrocytomas have been shown to produce slowly proliferating cells with marked morphological evidence of astrocytic differentiation. These cells have long processes which form a loose network. On the
other hand, malignant gliomas tend to produce morphologically heterogeneous primary cultures. More homogeneous morphological patterns of appearance develop with subsequent passaging (Bigner et al, 1981b, Yang et al, 1990). Some workers have grouped established cell lines into four classes on the basis of their appearance after multiple passages. These groups are designated epithelial (polygonal shape with clear, sharp boundaries; "pavement-like"), glial (cell body and processes), fascicular and fibroblastic (spindle-shaped, bipolar or stellate and multipolar) (Bigner et al, 1981b).

Cultures derived from malignant astrocytomas show classical cytological characteristics of anaplasia, including mitoses, giant cells, nuclear overlapping, heterogeneity in nuclear size and shape as well as evidence of cell piling (Giard et al, 1973; Maunoury, 1977; Bigner et al, 1981b; Rutka et al, 1987c). In vitro, ependymoma cell cultures have an epithelial-like morphology and formation of rosette-like patterns when density is high (Nakagawa et al, 1983; Mihara, 1986). In these cultures there is little cytological evidence of malignancy.

Medulloblastomas, on the other hand, grow relatively well in short-term culture and cells may persist in vitro for many months (Wilson et al, 1966). Unfortunately, with time, these cultures tend to degenerate or adventitious cells dominate (Lumsden, 1971). There have been no comprehensive studies on the identification of these cells grown from surgical biopsy specimens. However, Waghe et al (1973) demonstrated the presence of proliferating cells using tritiated thymidine. In primary cultures, morphological cell types include small spindle shaped or triangular cells with round, oval nuclei and scant cytoplasm. A study by Mackillop et al (1985) observed that 3 MB cultures grew in monolayer with the presence of floating aggregates. The cells from these cultures were elongated but lacked the long processes seen in the astrocytomas. The "Duke" series of medulloblastoma cell lines all grew in suspension and formed aggregates spontaneously. Three sublines of PFSK-1 were shown to have "multinucleated cells with broad flat morphology", whereas PFSK-2 grew in clumps and adhered less well to plastic (Fults et al, 1992b). Three cell lines (MHH-MED-1, 2 and 3) grew as cell aggregates in suspension, the cell line MHH-MED-4 was slightly adherent and MHH-PNET-5 mainly comprised of adherent cells with spindle-shaped or stellate morphology (Pietsch et al, 1994).

Cell line kinetics

Bigner et al (1981a) demonstrated that established glioma cell lines reached higher saturation densities than normal human glial cultures. The doubling times of short-term cultures derived from malignant astrocytoma are comparable to those of normal adult brain (Pertuiset et al, 1985) and it is apparent that there is a stabilisation in doubling times over the first five passages in vitro (Pertuiset et al, 1985). Reports in the literature indicate that established cell lines derived from human gliomas show wide variation in their doubling times, between 24-264 hours (Pertuiset et al, 1985; Cook et al, 1987; Zupi et al, 1988).
The heterogeneous morphological appearance of cultures at quite high passage levels, suggests that various cell types are still present in these cultures, indicating that a mechanism is in operation that regulates the growth of these cells within a culture. Other studies have shown that small subpopulations of endothelial cells were incorporated stably into short-term cultures of human glioma for many passage levels (Guner et al, 1977). However, some mixed cultures have been shown to be stable in culture, therefore indicating that doubling times are similar for different cell types.

Population doubling times for established medulloblastoma cell lines fall into two groups. Daoy, D283 Med, D341 Med, ONS-76 and ONS-81 have doubling times between 19-53 hours, while cell lines D384 Med, D425 Med and D485 Med have doubling times in the region of 58-83 hours (Tamura et al, 1989a; Bigner et al, 1990a; Oakes et al, 1990). Of the most recently established MHH-MED series, doubling times ranged between 61-72 hours. The population doubling times for PFSK cell line is 30 hours for sublines 1 and 2C and 48 hours for subline 2, while the ependymoma cell lines, NU2-26 and KM-SII have rapid doubling times in the region of 24 hours.

Karyotypic analysis
Established glioma cell lines generally contain stemlines or modal chromosome numbers in the near-triploid region (Mark et al, 1974; Bigner et al, 1983). This seems to be relatively stable at high passages although some changes do occur, including gains or losses of copies of entire normal or abnormal chromosomes as well as the appearance of new chromosomal markers (Rey et al, 1983; Rey et al, 1989). Medulloblastoma cell lines from the "Duke" series are near-diploid and often have structural abnormality of chromosome 17, including an isochromosome 17 (Bigner et al, 1990b).

Amplification of the c-myc gene in cell lines was reported (Bigner et al, 1990a) but no amplification of N-myc, EGFR or gli was detected. An 150-fold increase in the amplification of the N-myc gene was observed in a new medulloblastoma cell line, MTS (Wasson et al, 1990). It appears that medulloblastoma cell lines have a high frequency of c-myc amplification and this may be a requisite for such tumours to establish in vitro (Wasson et al, 1990). In contrast, Daoy is tetraploid and has been shown to have amplification of the erbB1 gene (Wasson et al, 1990). The PFSK-1 cell lines are hypotetraploid with many chromosomal aberrations, whereas the PFSK-2 has a pseudodiploid karyotype with translocations t(1:11), t(3:10) and t(17:22). Trisomy of chromosome 8 of the PFSK-2C cells was also noted (Fults et al, 1992b). Cytogenetic analysis of the cell lines in the MHH series showed near diploid stem line karyotypes with clonal structural abnormalities. A partial trisomy of 1q was found in two lines (MHH-MED-1 and MHH-MED-4) and chromosomal regions 16q24, 11q24 and 10q24 exhibited structural rearrangements, including translocations in more than one line (Pietsch et al, 1994).
Interestingly the cell lines MHH-MED-2, 3 and 4 had no detectable losses on chromosome arm 17p. The MHH-MED-2 cell line showed double minutes and amplification of c-myc as well as over-expression of c-myc mRNA and protein (Pietsch et al, 1994). One ependymoma cell line, KM-SII is hyperdiploid with a modal number of 56 and the other, NU2-26 is near diploid with a chromosome modal number of 46. In a cytogenetic analysis of cultures derived from four ependymomas with different degrees of malignancy (I-IV), numerical changes were seen in chromosomes 17 and 22 and structural changes were seen involving chromosomes 10, 12, and X (Rogatto et al, 1993).

**Extracellular matrix**

Advances in research methods in culturing cells in vitro have promoted culturing of "delicate" cells with the aid of substrates such as feeder layers (Ponten & Macintyre, 1968) or coating of tissue culture-ware with semi-defined attachment components of the extracellular matrix (Bottenstein & Sato, 1980; Lundgren et al, 1985; Terranova et al, 1986).

**Feeder layers**

Feeder layers are cells which have been killed either by X-ray treatment or using mitomycin C. This inhibits cell division but maintains the metabolic integrity of the cells. The cells from tumour biopsies or cell lines can then be grown on these feeder layers. They are used for a number of purposes, including a source of attachment factors, as a source of nutrients or growth factors. However, there is one disadvantage with such a system in that it is difficult to determine which of the above cellular factors was responsible for enhancing attachment or proliferation of a particular cell line.

**Properties of ECM components**

It has been shown that cells when maintained on an extracellular matrix (ECM) derived from endothelial cells resemble their in vivo counterparts more closely than when maintained on plastic or glass (Vlodavsky et al, 1980).

The ECM is the naturally occurring substrate upon which cells migrate, proliferate and differentiate in vivo (Gospodarowicz et al, 1978; Reid & Jefferson, 1984). It acts as a biological adhesive that maintains the normal cytoarchitecture of different tissues and defines the important spatial relationships among dissimilar cell types (Reid & Jefferson, 1984). Cells adhere to each other through surface components such as those involved in the formation of junctions. These cell-matrix interactions are necessary for embryonic development.

The structural composition of the ECM has been widely reviewed (Timpl & Dziadek, 1986; Reichardt & Tomaselli, 1991). The major structural components of the ECM have been
shown to be synthesised and deposited by cells during the very early stages of embryonic development (Kleinman et al, 1981). The component parts include collagen of various types, the non-collagenous glycoproteins (laminin and fibronectin), the glycosaminoglycans (GAGs) such as hyaluronic acid and heparin sulphate and the proteoglycans. Collagen types I-III are the interstitial collagens found in skin, bone, blood vessels and connective tissue septae. Collagen type IV is found only in basement membranes (BM) and usually forms the scaffolding to which linking proteins such as laminin and fibronectin bind cells (Reid & Jefferson, 1984; Giordana et al, 1985; Laurie et al, 1986). The non-collagenous glycoproteins, such as laminin, fibronectin, entactin (Nidogen) and vitronectin are relatively large compounds and can bind either directly to cells to mediate their effects, or link cells to other ECM macromolecules.

Using immunocytochemical approaches on normal and pathological human CNS specimens, ECM proteins are predominantly deposited at the junction between glial and mesenchymal elements (Bellon et al, 1985; Giordana et al, 1985; Rutka et al, 1987d).

The ability of normal and neoplastic cells of neuroectodermal origin to adhere to components of the normal leptomeningeal ECM, primarily fibronectin, laminin and collagen I, III and IV (Rutka et al, 1987a) has been widely reported (Hall et al, 1987; Dedhar & Gray, 1990) and these observations are important with regard to studies on neuronal migration (Tuttle et al, 1989). Calof & Lander (1991) employed a model system consisting of neuronal cells of the mouse embryonic olfactory epithelium (OE) in which to study regulation of the migratory behaviour of these cells by ECM components. Following plating onto glass coverslips with laminin or fibronectin, only neurons plated on laminin migrated away from an epithelial explant but both laminin and fibronectin supported cellular attachment. Another experiment included glass coverslips with stripes of single ECM molecules, including laminin, fibronectin, collagen I and IV and a laminin-like protein, merosin. Explants attached to all substrata but extensive cell migration occurred only on stripes of laminin or merosin. Neurite outgrowth was also greater on laminin and merosin compared with other substrates. Fishman & Hatten (1993) showed that granule neurons migrated rapidly on laminin fibres, moved quite slowly on collagen fibres but not at all on collagen fibres. This is in contrast to other studies with neural crest migration where both laminin and fibronectin provided a receptor system for cell migration (Perris et al, 1989).

**Collagen types**

This family of highly characteristic ECM glycoproteins comprise more than 25% of mammalian total protein. At least 11 genetically distinct collagen types are known to be distributed in connective tissues (Mayne & Brewton, 1993). All collagen (COL) molecules have a characteristic stiff triple-stranded helical structure, that aggregate in fibrils (minute fibres) or sheets in extracellular spaces. The fibrils, which assemble in various highly ordered arrays, provide
structural support and are known to bind cells. COL types I, II and III are the fibrillar COLs, whereas COL IV is found exclusively in the basal laminae where, instead of forming fibrils, it assembles into a sheetlike meshwork that forms the major component of all basal laminae. COL IV is composed of at least five different polypeptide chains (Zhou et al, 1992). The adhesive capacity of COL has been shown to affect the adhesion growth, morphology and differentiation of a wide range of cell types in vitro (Montesano et al, 1983; von der Mark & Kuhl, 1985).

Type I COL has been commonly used as a cell-culture substratum. Myoblasts, spinal ganglia, hepatocytes, embryonic lung, heart explants, fibroblasts, endothelial cells, islet cells and limb rudiments have been successfully cultured on films or gels of type I COL (Leifer et al, 1984; Cannella & Ross, 1987; Yong et al, 1988). Used as a thin coating on culture-ware, studies on the adhesion of keratinocytes (Adams & Watt, 1991) and the migration of endothelial cells (Leavesley et al, 1993) have been carried out.

Type IV COL which forms large networks within the BM, is thought to provide mechanical stability to matrices. Several studies indicate that COL type IV promotes adhesion and growth of normal and transformed cell lines of keratinocytes, hepatocytes, endothelial cells, myocytes, tumour cells and macrophages (Murray et al, 1980; Aumailley & Timpl 1986; Mc Kay et al, 1992). It was more effective than LMN in enhancing adhesion of aortic endothelial cells (AEC) (Herbst et al, 1988). The molecular basis of these interactions and possible requirements for mediator proteins have as yet not been determined. Recently, COL IV-treated culture-ware was shown to affect growth cone elongation of sensory neurons (Lamoureaux et al, 1992) as well as migration of smooth muscle cells (Di Milla et al, 1993). Scarpa et al (1987) examined ECM synthesis by small round cell tumours of childhood in vitro. It was observed that all 8 neural tumours synthesised FN, LMN and COL IV (only two lacked COL IV synthesis). However, no stromal COL (I/III) synthesis was seen in these tumours.

Similar studies with COL VI indicate that it is also a strong substrate for promoting cell adhesion. This was first observed for baby hamster kidney cells adhering to a partially purified preparation of COL VI (Carter, 1982). COL VI also induced adhesion of a variety of normal and transformed cells (Aumailley et al, 1989). Although adhesion to COL IV was shown to require an intact helical structure (Aumailley & Timpl, 1986), COL VI in its triple helical and unfolded form could promote cell adhesion in a similar way (Aumailley et al, 1989).

Non-collagenous glycoproteins

**Fibronectin**

Fibronectin (FN) is a very large ($M_r$=500,000) dimeric glycoprotein composed of two similar subunits (each almost 2500 amino acid residues long) linked by a pair of disulphide bonds (Skorstengaard et al, 1986). It constitutes between 1-3% of cellular protein and may account for
around half of all membrane glycoproteins. It is an extremely effective broad range natural adhesive factor (Ruoslahti, 1988; Yamada, 1989; Aota et al, 1991) and promotes the attachment and spreading of many cell types, including BHK and CHO cells (Barnes & Sato, 1980), neurons (Mc Guire & Seeds, 1990), fibroblasts (Tremble et al, 1993) and smooth muscle cells (Di Milla et al, 1993). A component of both plasma and cell secreted matrix (Vaheri & Mosher, 1978; Hynes & Yamada, 1982), FN functions as a cellular adhesion factor by binding to COL, heparin and other cell glycosaminoglycans (Aota et al, 1991). The assembly of FN into the ECM has been reviewed (Mosher, 1993). FN has been added to serum-free culture systems to improve cell adhesion and growth and more recently shown to be useful in the growth of normal lung and cystic fibrosis epithelial cells in low serum culture (Bames & Sato, 1980; Schwiebert et al, 1992). It may be used either as a thin coating for tissue culture plastic or as a soluble media additive.

In short or long-term culture it has been demonstrated that FN expression is lost on transformed glial cells to some degree (Bigner et al, 1981a; Sherbet et al, 1982). It has been suggested that the absence of surface FN may account for the inability of human glioma cells to interact and attach to the basement membrane of capillaries, hence explaining the lack of systemic metastases in these tumours (Sherbet et al, 1987). This subject will be discussed in more detail in the immunocytochemistry chapter.

Laminin
Laminin (LMN) is a large (Mr=900,000) glycoprotein and is the major non-collagenous glycoprotein component of BMs and is composed of three polypeptide chains with a multi-domain structure (Beck et al, 1990). It belongs to a family of proteins with several genetically distinct subunit chains that are present in numerous isoforms (Tryggvason, 1993). The structure and properties of LMN have been widely described (Martin & Timpl, 1987; Engel, 1992; Tryggvason, 1993). It interacts with other BM components, including type IV COL, proteoglycan, entactin, heparin sulphate and related polysaccharides (Timpl et al, 1983; Engel, 1992). LMN has been shown to have a number of biological activities in vitro, including the ability to influence cell adhesion, growth, morphology, differentiation, matrix assembly and cell migration (Kleinman et al, 1985; Beck et al 1990) of a variety of cells. It promotes the attachment of various epithelial cells to plastic or to type IV COL-coated surfaces (Terranova et al, 1980; Vlodavsky & Gospodarowicz, 1981). LMN may regulate the growth of a variety of cell types, including macrophages (Mc Kay et al, 1992), keratinocytes (Adams & Watt, 1991), melanoma cells (Yamamura et al, 1993), ras-transformed cells (Chambers et al, 1993) and mouse embryo stem cells (Cooper et al, 1991). LMN is also thought to be important in development and has been identified in the 4-cell stage embryo (Cooper & Mac Queen, 1983).

LMN has been reported to be involved in the growth of neurons (Kleinman et al, 1988), in the growth cone elongation of sensory neurons (Lamoureux et al, 1992) as well as influencing neuron migration, morphology and adhesion (Calof & Lander, 1991). Kleinman et al (1988)
reported that radioactively labelled LMN binds specifically to the surface of NG108-15 neuroblastoma/glioma cells via three membrane proteins of 110 kDa, 180 kDa and 67 kDa. The 110 kDa protein occurs in a variety of epithelial cells and in brain, whereas the 180 kDa protein is neuron specific. Antibodies against the 110 kDa and 180 kDa proteins inhibit LMN-induced neurite outgrowth, whilst antibodies directed against the 67 kDa protein do not.

Unlike FN, LMN is able to bind either to the cell surface or to COL, although it appears to be more effective as an attachment protein for epithelial cells when bound to type IV COL. Certain fibroblasts do not recognise LMN and will not survive in culture if excess laminin is present (Terranova et al, 1983).

As well as adhesive properties other changes observed include changes in cell shape and have been related to changes in cell growth (Folkman & Moscona, 1978; Gospodarowicz et al, 1978). Sugrue and Hay (1981) observed that LMN altered cellular morphology and that epithelial cells without a BM formed blebs on their surface indicating that the basal plasmalemma was unstable. Observations from other studies include elongation of Schwann cells (Palm & Furcht, 1983) and spreading of HT-1080 cells (Gold & Pearlstein, 1980). LMN therefore possibly stimulates a morphological feature of these cells, which is more characteristic of the in vivo morphology for these particular cell populations.

Tenascin
Tenascin (TN) also known as cytotactin is another ECM glycoprotein but its tissue distribution is more limited than that of the other ECM glycoproteins (Chiquet-Ehrismann et al, 1986; Erickson & Bourdon, 1989). TN is transiently present in the dense mesenchyme surrounding several developing organs, i.e., mammary gland during embryogenesis (Chiquet-Ehrismann et al, 1986), kidney (Aufderheide et al, 1987) and in both CNS and PNS (Grumet et al, 1985; Bronner-Fraser, 1988) where TN expression is thought to be related to cell proliferation and migration as well as remodelling of the ECM (Bronner-Fraser, 1988; Erickson & Bourdon, 1989).

Tenascin appears to be part of a family consisting of three related proteins, tenascin-C (TN-C), tenascin-R (TN-R) and tenascin-X (TN-X), properties of which have been recently discussed (Erickson, 1993). TN-C is the first member of the TN family to have been characterised and is expressed in developing brain, cartilage and mesenchyme and is also re-expressed in tumours, wound healing and inflammation (Erickson, 1993). TN-R was originally described under the names restrictin in chicken (Rathjen et al, 1991) and as J1-160/180 in rat (Pesheva et al, 1989). TN-X was originally reported as a partial sequence encoded by gene X (Morel et al, 1989) and recently almost all of the sequence of this large protein has been reported (Bristow et al, 1993). TN-X mRNA is mainly expressed in foetal muscle (smooth muscle of gut, skeletal muscle and heart) and testis with a lower level in foetal adrenal gland, kidney and lung (Bristow et al, 1993).
Studies have shown that more than half of the TN molecule is homologous to the amino acid sequence of FN (Jones et al, 1988; Erickson & Bourdon, 1989). It seems that these two glycoproteins have opposing functions, FN has an inhibitory effect on chondrogenic and osteogenic differentiation in vitro and in vivo, whilst TN promotes such differentiation (Mackie et al, 1987). The attachment and spreading of cells on FN are inhibited by soluble TN (Bourdon & Ruoslahti, 1989; Lotz et al, 1989). TN was shown to inhibit neurite outgrowth from dorsal root ganglia on FN and LMN substrates (Crossin et al, 1990) whilst it was able to enhance neurite growth when neurons were cultured on PLL (Lochter et al, 1991). Recently, when cells were offered a mixed substrata with other ECM proteins, two TN-R isoforms and TN-C derived from mouse brain selectively inhibited FN-dependent cell adhesion and neurite outgrowth and affected cell morphology of different mesenchymal and neural cells (Pesheva et al, 1994).

**Vitronectin**

Vitronectin (VT) is a glycoprotein present in human plasma and serum and is capable of mediating attachment, migration, proliferation and spreading of a number of cell types, including fibroblastic and epithelial cells (Barnes & Silnutzer, 1983; Hayman et al, 1985) as well as promoting retinal neurite outgrowth (Neugebauer et al, 1991) and for studying cell migration (Leavesley et al, 1992). It is commonly referred to as the "serum spreading factor". During cell spreading, the attached cell flattens or spreads, usually assuming the shape characteristic of its differentiated state. This is induced by the formation of focal adhesions or sites of strongest adhesion at the cell surface that make contact with the substratum (Harris, 1973) due to cell attachment.

**Entactin/nidogen**

Entactin and nidogen are identical sulphated glycoproteins originally identified in cultured cells, as in mouse endodermal cell line and found in the surface of epithelial cells of tubules and glomeruli adjacent to BMs (Carlin et al, 1981) but subsequently also isolated from mouse EHS tumour (Timpl et al, 1983). Entactin has a Mr of 150,000 and was isolated from embryonic neurons from chick dorsal root ganglia (Carbonetto et al, 1983). Nidogen has been shown to have the potential to bind to other ECM components and is thought to be a major mediator in BM assembly (Aumailley et al, 1993). The LMN-nidogen complex binds the core protein of heparin-sulphate proteoglycan (Battaglia et al, 1992). This binding occurs via different globular domains of nidogen (Reindardt et al, 1993) and is considered to be an essential step in connecting networks of LMN and COL IV with BMs (Aumailley et al, 1993).

**Glycosaminoglycans and proteoglycans**

The glycosaminoglycans (GAGs) include four main groups divided according to their sugar residues, the type of linkage between these residues and the number and location of sulphate groups and include: (i) hyaluronic acid, (ii) chondroitin sulphate and dermatan sulphate, (iii)
heparin sulphate and heparin and (iv) keratin sulphate. These molecules are long unbranched polysaccharide chains composed of repeating disaccharide units. One of the two sugar residues in the repeating disaccharide is always an amino sugar. They play an important role in cellular attachment, provide cellular support and regulate cell biosynthesis and additionally have a role in cell proliferation and differentiation (Elias et al, 1988).

Proteoglycans are very large ECM macromolecules that contain 90-95% carbohydrate by weight in the form of many long unbranched GAG side chains attached to a core protein molecule (for review of structure see Kjellén & Lindahl, 1991). These molecules have been shown to be important in cell growth and adhesion, receptor binding and transformation (Margolis et al, 1975; Iozzo, 1984; Turley, 1984). Purified proteoglycans have been used to study their interaction with soluble components of cell culture media (Iida et al, 1992) and growth factors, such as FGF, EGF and TGF-β (Klagsbrun & Baird, 1991; Ruoslahti & Yamaguchi, 1991).

Artificial substrata

There have been a number of attempts to develop artificial substrata from mixtures of ECM components, some of which are discussed below.

Matrigel

Matrigel is a biopolymer comprising of 60% LMN, 30% type IV COL, 5% entactin (nidogen) and 3% heparin sulphate proteoglycan. It promotes the growth and differentiation of many cultured cells in vitro including melanoma cells, Schwann's cells and liver cells (Kleinman et al, 1986). Carey et al (1986) reported that rat Schwann cells when co-cultured with neurons on Matrigel matrix in a serum-free medium produced myelin segments. In the absence of the matrix the cells proliferated but did not differentiate. These cells when grown on the matrix also exhibited longitudinal spreading along the nerve fibres and this spreading only occurred in areas where the cells were in direct contact with the matrix. Grant et al (1989) found that endothelial cells formed capillary-like structures when plated on Matrigel. Glioma cells (U251MG cell line) have been shown to form network structures when cultured on Matrigel (Mori et al, 1991). Also Matrigel-treated culture-ware has shown to maintain the normal morphology of Sertoli cells (Dym et al, 1991). It also promoted the differentiation of mammary epithelial cells (Bergstraesser & Weitzman, 1993) and neurite regeneration (Pleasure et al, 1992).

Additionally, Matrigel has been used to enhance cell growth and differentiation of anchorage dependent cells for both normal and malignant tissues in vivo (Fridman et al, 1991; Pretlow et al, 1991; Noel et al, 1992). The in vivo effects of mixing Matrigel and cells before transplantation include enhanced heterotransplantation of human solid tumours to nude mice and acceleration of growth and enhanced differentiation (Pretlow et al, 1991). Matrigel enhances
establishment and maintenance of early B-lineage acute lymphoblastic cell growth in a subcutaneous xenograft model (Sterling-Levis et al, 1993).

Cell-tak

Cell-tak is a formulation of polyphenolic proteins extracted from a marine mussel, *Mytilus edulis* (Waite & Tanzer, 1980). These proteins are the key components of the glue secreted by the mussel to anchor itself to solid substrates in its marine environment.

Cell-tak has been used to attach tissue, cells, isolated organelles or proteins to a surface. It has been shown to enhance the attachment of cells in primary cell cultures and cell lines, including embryonic hypothalamic cells, neuronal cell lines and kidney epithelial cell lines (Notter, 1988; Roth et al, 1988) as well as normal skin fibroblasts, rat osteosarcoma cells and liver cells (Barsony & McKoy, 1992).

Polylysine

Cells have an overall negative charge (Sherbet et al, 1972) and so positive-charged polymers can reduce the repulsive forces of conventional negative-charged tissue culture substrata, such as glass or plastic. A thin coating of positive-charged polymers like polyornithine, polyarginine, polyhistidine, DEAE-dextran, protamine and polylysine have been shown to improve cell attachment and proliferation. The most commonly used polymer is polylysine with both the D and L isomers being effective. However, the D isomer and other amino acid polymers are used more often (Cannella & Ross, 1987; Needham et al, 1988) in order to avoid interference by L-amino acids produced by degradation of the polymer during culture. Needham et al (1988) showed that PLL stimulated endothelial cells from pig aorta to release prostacyclin and cytoplasmic purines via a dose (charge)-dependent and molecular weight (size) dependent manner. Poly-D-lysine has been used to culture a rat phaeochromocytoma cell line (Tomaselli et al, 1987) and recently has been shown to maintain the growth of phaeochromocytoma cells in vitro in low-serum conditions (Doherty et al, 1993). It has also been used to study the mechanism of neurite outgrowth (Doherty et al, 1993).

ECM in human brain tumours

Until recently, it appeared that the adult brain had no organised ECM (Sanes, 1989). LMN has been shown to be produced by early astrocytes in primary culture (Liesi et al, 1983) and is synthesised by astrocytes after injury to adult CNS (Liesi et al, 1984). A few ECM components have been identified, including tenascin which is detectable in adult brain (Hoffman et al, 1988). Recently, Iwata & Carlson (1993) have identified a new, large chondroitin sulphate proteoglycan (T1 antigen) that acts like a general ECM protein in adult brain. It is present in both grey and white matter in the brain and surrounds the cell body of large neurons such as Purkinje cells in the cerebellum but no staining was observed inside the cells (Iwata & Carlson,
1993). However, adult brain is known to include the BM glycoproteins FN, LMN and VT as well as COL I, III and IV (Rutka et al., 1988a; Venstrom & Reichardt, 1993) but these molecules are mainly confined to major blood vessels and meningeal surfaces. COL VIII has been shown to be expressed in embryonal brain and GBs but is absent in adult brain (Paulus et al., 1991).

Bourdon and Ruoslahti (1989) demonstrated that glioblastomas express a glioma-mesenchymal ECM (GMEM) protein identical to TN. They previously reported that the human glioma cell line, U251MG could secrete large amounts of TN in vitro (Bourdon et al., 1983). Rettig et al (1992) found TN-C expression is high in all areas of bovine brain and mainly expressed in the molecular layer of the cerebellum in rat and mouse but is completely absent from the cerebellum of human brain. TN-R is thought to be restricted to CNS and is most prominent during development. It is found in the retina, cerebellum and spinal cord of embryonic chicken (Norenberg et al., 1992) and in cerebellum, hippocampus and olfactory bulb of postnatal rat, peaking at 7-14 days (Fuss et al., 1993) and such expression was also seen in adult rat cerebellum. TN-X is nearly absent from 22 week human foetal and also from 2-day postnatal mouse brain (Saga et al., 1992) but TN-C and TN-R are very prominent. TN-C and TN-R are expressed only in the CNS by oligodendrocytes and some neuronal subpopulations (Pesheva et al., 1989; Fuss et al., 1993). TN is expressed in embryonal brain and in GBs but not in adult brain (Erickson & Bourdon, 1989). Recently, both anaplastic astrocytomas and glioblastomas have been shown to express TN and this expression was mainly localised in the BM of the proliferating tumour vessels (Higuchi et al., 1993). In contrast to the in vivo situation, cultured gliomas and established glioma cell lines usually produce large amounts of ECM proteins such as COLs, LMN and FN (Bjerkvig et al., 1989). Grierson et al (1990) found that there are two morphologically distinct subtypes of hypothalamic astrocytes that produce TN in vitro. The 'rocky' astrocytes that comprise an uneven monolayer in culture were found to have high levels of TN expression, whereas the 'flat' astrocytes that constitute a smooth monolayer expressed low levels of TN.

VT is expressed in human brain tumours and was found to be localised to the parenchyma in glioblastomas but not in low grade tumours or normal glia (Gladson & Cheresh, 1991). Neurocan is one of the most abundant chondroitin sulphate proteoglycans of developing rat brain (Rauch et al., 1991) and its primary structure has recently been determined (Rauch et al., 1992). Also molecular cloning of SCI which encodes a putative calcium binding glycoprotein was found in large amounts in developing and adult rat brain but not in kidney or liver (Johnston et al., 1990). SCI showed some structural similarity to the ECM glycoprotein SPARC/osteonectin which has been suggested to be a modulator of ECM, affecting the shape, together with other proteins such as TN and thrombospondin (Sage & Bornstein, 1991). SCI shows high levels of expression in adult brain and may be important in the developing nervous system as well as in the functioning of adult brain (Mendis et al., 1994). Many in vivo studies have also shown that in rat and human embryonic and neonatal brain cortex and cerebellum,
differentiation is associated with a decrease in hyaluronic acid levels (Jenkins & Bachelard, 1988; Laurent & Fraser, 1992).

ECM or individual components derived from the ECM (derived from bovine corneal endothelium) have been shown to enhance the growth in vitro of a variety of human brain tumours. Westphal et al. (1987) demonstrated that 80% of primary glioma cultures showed a marked improvement in initial plating efficiency, colony formation and speed of attachment when plated on ECM derived from bovine corneal endothelium. Individual components of the ECM, including FN and LMN promoted cellular adhesion, growth migration and differentiation of glioma cells, e.g., U251MG and C6 (Liesi, 1984; Rutka et al., 1987b).

Cells from established human glioma cells are able to migrate on FN and LMN coated surfaces. Glioma cells exhibit highly migratory responses to FN, at a relatively low concentration (0.5 g/ml), but migration to LMN required a higher concentration and not all cell lines showed this response (Ohnishi et al., 1991). Studies by Rutka (1991) examined the effects of purified FN, LMN, COL types I and IV as individual substrata for proliferation of U343MG cells and found that both COL I and IV inhibited glioma cell proliferation and induced marked morphological and biochemical differentiation in these cells, while LMN and FN had no effect on growth. Giese et al. (1993) showed that human glioma cell lines (SX 763 and SX 767) attached to COL, FN, VT and LMN to varying degrees and in a dose dependent manner. It was also noted that human LMN induced the most rapid attachment in vitro of these glioma cell lines.

Bogenmann et al. (1983) demonstrated medulloblastoma cells derived directly from human biopsies attached to and grew well on smooth muscle cell ECM and Rutka et al. (1987a) showed that medulloblastoma cells adhered to and grew on monolayers of leptomeningeal cells which produced an ECM composed of LMN, FN and COL types I, II and IV. In contrast, Wikstrand et al. (1991) documented that attachment of five suspension medulloblastoma lines, of neuronal lineage ("Duke" series) to normal leptomeningeal ECM (FN, LMN and COL IV) substrates, assessed in vitro, was a rare occurrence compared to that of established glioma or neuroblastoma cell lines or Daoy. Subsequent studies showed under in vitro conditions, attachment of the 5/6 MED cell lines to this ECM was not associated with integrin receptor mediated binding (Wikstrand et al., 1993).

Integrins

Integrins are a superfamily of αβ heterodimeric proteins (cell adhesion receptors) which have the ability to bring about cell-cell and cell-matrix interactions and such interactions can result in changes in cell shape or migratory behaviour (Cheresh, 1993). Integrins form associations with cytoskeletal elements (Sastry & Horwitz, 1993) and so function as transmembrane links
between extra- and intra-cellular compartments. To date, there are at least 14 α-subunits which can link (noncovalently) with at least 8 β-subunits to form a functional receptor. Some heterodimers recognise only one ligand (i.e., α5β1 binds FN) whereas others can bind several ECM components (e.g., α3β1 binds LMN, COL IV and FN). Ligand specificity may also differ depending upon the cell type in which a given integrin is expressed (Kirchhofer et al., 1990). The β family is widely expressed in embryonic and adult tissues and includes the majority of known receptors for ECM (Hynes, 1992).

Integrins play a role as mediators of the effects of ECM on cell morphology, motility, proliferation, differentiation and gene expression (Hynes, 1992; Ruoslahti, 1992; Adams & Watts, 1993). Some integrins (e.g., FN receptor) recognise the triplet peptide sequence RGD (Arg-Glycine-Asp) which appears to play a key role in cell adhesion (for review, Ruoslahti & Pierschbacher, 1987). Other domains of FN involved with the adhesion process also interact with COL, heparin and cell surface GAGs (Aota et al., 1991). Several binding sites have been identified for LMN and include an IKVAV (Ile-Lys-Ala-Val) and RGD site in the A-chain and a YIGSR (Tyr-Ile-Gly-Ser-Arg) site in the B1-chain (Massia et al., 1993). Like many adhesion molecules, VT binds to cells through an interaction of the RGD sequence in its cell binding domain (Pierschbacher & Ruoslahti, 1984) with the VT-specific cell surface integrin, v3 and v5 (Fitzgerald et al., 1985) and a recent review discusses the properties of some newly discovered integrins for VT (Felding-Habermann & Cheresh, 1993). A possible receptor for hyaluronan in adult human brain, H-CAM has been reported and has been shown to be present in a subset of glial cells (Vogel et al., 1992). Even though several adhesion molecules and receptors have been characterised for a number of ECM components (as described above) the molecular basis which brings about the many interactions between normal and neoplastic neuroectodermal and cerebral ECM is as yet still unclear (Couldwell et al., 1992). The LMN receptor α6β1 was originally isolated from a human glioblastoma cell line RuGli (Gehlsen et al., 1988). Other studies using GBs in situ and the established cell line, U251MG showed expression of αv and β3 but not β5 (Gladson & Cheresh, 1991). Also in rat and human glioma cell lines, the integrins α1β1, α3β1, α4β1 and αvβ5 were identified using immunoprecipitation (Bednarczyk & McIntyre, 1992; Malek-Hedayat & Rome, 1992).

Recent studies have shown that increased expression of different integrins in vivo and in vitro was found in neoplastic astrocytes compared with their normal counterparts (Paulus et al., 1993). Astrocytoma cell lines, U138MG and U373MG revealed a strong attachment to COL types I and VI and undulin, which was inhibited by antibodies to β1 but not by those to α2, α3, α6 and αv (Paulus et al., 1993). The results therefore showed that attachment to ECM components by astrocytomas was solely mediated by β1 integrins (Paulus et al., 1993). In a subsequent in vitro invasion study, human glioblastoma cells derived from either primary cultures, those at passage level 5 and established cell lines were able to migrate through Matrigel (Paulus & Tonn, 1994) and such observations have been seen by other authors using
rat C6 astrocytoma cells (Bernstein et al, 1991). Invasion of U138MG cells was reduced with antibodies to α7, αv, β1 and β3 integrin chains and markedly increased by anti-α5, whereas invasion of U373MG cells was reduced by antibodies to α3, αv, β1 and β3 and increased by anti-α6 (Paulus & Tonn, 1994). Using eight different human astrocytoma cell lines (one of which was a normal astrocytoma cell line and the others were derived from tumours) showed that adhesion to COL, FN and VT was integrin dependent and could be blocked by anti β1 integrin antibodies (Giese et al, 1994). In contrast, attachment to LMN could not be blocked using these antibodies. Tumourigenicity of glioblastoma tumour cells has been observed to be largely dependent on the expression of the αvβ3 integrin (Gladson & Cheresh, 1991).

CD44 is a polymorphic family of membrane glycoproteins expressed in many epithelial tissues and the different forms are thought to be involved in cell-cell and cell-matrix interactions (Haynes et al, 1989) and possibly the local invasion potential of neuroectodermally-derived tumours (Pilkington, 1994). A recent study using eight human glioma cell lines in vitro showed that invasion of Matrigel was inhibited to varying degrees by a CD44 monoclonal antibody (Merzak et al, 1994). Adhesion of glioma cells to FN, LMN, VT and COL I was also inhibited by the CD44 monoclonal antibody (Merzak et al, 1994).

One study reported that oligodendrocytes are able to bind to components of a matrix derived from glial cells in culture via a protein which appeared to have integrin-like binding properties (Cardwell & Rome, 1988). Immunochemical analysis, using antibodies to a number of α and β integrin subunits, observed that oligodendrocytes expressed only one detectable integrin complex (α01β01), whereas astrocytes, the other major type of glial cell in brain expressed multiple integrins including α1β1, α3β1 and α5β1 complexes and these were indistinguishable from integrins expressed by rat fibroblasts (Malek-Hedayat & Rome, 1994).
Chapter 3

Immunocytochemistry

A large body of data has accumulated in the study of structural and surface antigens in human brain and the technology of monoclonal antibodies (MoAbs) has resulted in the production of a large number of antibodies for specific antigenic determinants. This has allowed a precise molecular definition of many normal cell types from the CNS (Eisenbarth et al., 1979; Raff et al., 1979; Franko et al., 1981; Kennedy, 1982; Schachner, 1982).

This has led to the definition of two important different types of antigens, the expression of normal antigens and the production of tumour associated antigens. Expression of normal antigens such as glial fibrillary acidic protein (GFAP) and synaptophysin (SYN) are known as lineage specific markers. For example, neuronal tumours will express normal neuronal markers such as SYN, whereas glial tumours will express normal glial markers such as GFAP. However glial antigens, although expressed usually by glioma cells, can be expressed by reactive astrocytes and non-reactive astrocytes (as in the hemispheric white matter) and other non-neuroectodermal tissues (De Tribolet, 1989). The other type of antigens are known as tumour-associated antigens, since they are not present in normal adult but in some cases have been found in foetal tissues or only in tumours and so may be tumour-specific and include 1H8c12 and 1H8c13 (Wikstrand et al., 1982).

There are a number of other antigenic categories which have been recognised using polyclonal or monoclonal antibodies and include enzymes such as neuron-specific enolase and such antigens are common to various tumours and tissues of neuroectodermal origin including both central and peripheral (Liao et al., 1981; Cairncross et al., 1982; De Tribolet et al., 1984). Finally, a group of antigens includes those that are expressed by circulating lymphocytes and cells of malignant gliomas, the lymphoid differentiation antigens (Greaves et al., 1980; De Tribolet, 1989), e.g., HNK-1 antigen.

Intermediate filaments

In eukaryotic cells the cytoskeleton consists of microtubules with a diameter of 25nm, microfilaments with a diameter of 5-7nm and intermediate filaments (IFs) with a diameter of 10nm (Gordon et al., 1978). A recent review discusses the assembly, dynamic and network aspects of the IFs (Heins & Aebi, 1994). IFs are morphologically similar in most eukaryotic cell types, however they do display a broad heterogeneity in their protein subunits. They are divided into six different classes (I-VI) based on the number and position of their introns (Table
2) (Lazarides, 1982; Steinert et al, 1984). IFs are specifically expressed in restricted tissues, except for the type V lamins which are widely expressed in the nuclei of all eukaryotic cells.

Table 2: Different types of intermediate filament classes

<table>
<thead>
<tr>
<th>IF Class</th>
<th>IF Protein</th>
<th>Location</th>
<th>Cell Type Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Acidic keratins</td>
<td>Cytoplasmic</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>Type II</td>
<td>Basic keratins</td>
<td>Cytoplasmic</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>Type III</td>
<td>Glial fibrillary acidic protein</td>
<td>Cytoplasmic</td>
<td>Astrocytes, Schwann cells</td>
</tr>
<tr>
<td>Type III</td>
<td>Desmin</td>
<td>Cytoplasmic</td>
<td>Muscle cells</td>
</tr>
<tr>
<td>Type III</td>
<td>Vimentin</td>
<td>Cytoplasmic</td>
<td>Mesenchymal cells, other cells, e.g., astrocytes</td>
</tr>
<tr>
<td>Type III</td>
<td>Peripherin</td>
<td>Cytoplasmic</td>
<td>PNS neurons and some CNS</td>
</tr>
<tr>
<td>Type IV</td>
<td>Neurofilament triplet proteins (NF-L, NF-M, &amp; NF-H)</td>
<td>Cytoplasmic</td>
<td>Present in nearly all CNS &amp; PNS neurons</td>
</tr>
<tr>
<td>Type V</td>
<td>Lamins A,B &amp; C</td>
<td>Nuclear</td>
<td>Present in nucleus of many different cell types</td>
</tr>
<tr>
<td>Type VI</td>
<td>Nestin</td>
<td>Cytoplasmic</td>
<td>Neuroepithelial stem cells &amp; subset of other embryonic cells</td>
</tr>
</tbody>
</table>

Tumours originating from different types of tissues generally retain their specific IF proteins (Osborn & Weber, 1983; Ramaekers et al, 1983). Since IF proteins are commonly specific to particular cell types, they have been used as differentiation markers as well as in tumour identification.

Glial fibrillary acidic protein

Glial fibrillary acidic protein (GFAP) was initially purified from multiple sclerosis plaques (Eng et al, 1971) and the protein represents the chemical subunit which characterises gliofilaments. A wide variety of monoclonal and polyclonal antibodies have been raised against this antigen which specifically stains normal astrocytes of the grey and white matter and Bergmann's glia of the cerebellum in man, mammals and other vertebrates (Dahl & Bignami, 1973). However, unexpected staining has been observed outside the CNS, including Schwann cells, enteric cells and cells of the salivary gland tumours (Budka, 1986) which are not of astrocytic lineage and such expression has been recently reviewed (Perentes & Rubinstein, 1987).

A large number of investigators have studied the expression of GFAP in brain tumours (Duffy et al, 1977; Deck et al, 1978; Velasco et al, 1980). It has been found to be expressed in all astrocytic tumours, including pleomorphic xanthoastrocytoma (Kepes et al, 1979) and subependymal giant cell astrocytoma (Bonnin et al, 1984). Positivity has been seen in glial
components of gliosarcomas, gangliogliomas (Lalitha & Rubinstein, 1979) and within ependymomas and oligodendrogliomas (Deck et al, 1978; Duffy et al, 1979; Herpers & Budka, 1984). MBs have also been reported to express GFAP (Palmer et al, 1981; Schindler & Gullotta, 1983; Roessmann et al, 1983).

**Medulloblastomas**

In one immunohistochemical study (Palmer et al, 1981) glial differentiation was observed in 11/13 MBs using GFAP. In contrast, frozen sections (probably resulting in better antigen preservation) yielded 15/15 positive tumours (Bourne et al, 1986). GFAP was expressed in more than 10% of cells in 10/14 MBs and 2/3 supratentorial PNETs (SPNETs) (Zeltzer et al, 1990). The presence of the two divergent cell lineage specific antigens (GFAP and NF) within the same tumour was found in 7/17 tumours (MBs and SPNETs), where more than 10% of cells were positive for each marker (Zeltzer et al, 1990). In another study 15/47 childhood MBs tumours showed positive staining for GFAP (Goldberg-Stern et al, 1991). A study by Mapstone & Galloway (1991), however, showed that two of their MB cell explants showed differing GFAP positivity, one where all cells stained weakly to moderately and the other where only 10% were minimally to moderately stained.

It has been proposed that the positive GFAP observed in MBs does not represent glial differentiation of the tumour cells but trapped normal astrocytes or reactive astrocytes (Camins et al, 1980; Coffin et al, 1983). Schindler & Gullotta (1983) suggested that the positive staining was a consequence of residual GFAP from degenerated astrocytes which were phagocytosed by macrophages. Expression of GFAP seems to be associated with the desmoplastic variant of this tumour type (Szynam et al, 1987; Taomoto et al, 1987), although not observed in all cases (Patt & Zimmer, 1992).

**Ependymomas**

GFAP has been found in the cytoplasm of neoplastic (Deck et al, 1978; Duffy et al, 1979; Velasco et al, 1980) and of reactive ependymal cells (Conley, 1979) but not in mature normal ependyma (Deck et al, 1978; Velasco et al, 1980). Most ependymomas show some GFAP expression, particularly the fibrillary variant where it has been shown to be prominent along the processes forming perivascular pseudorosettes (Duffy et al, 1979; Cruz-Sanchez, 1988; Kaneko et al, 1990). Mannoji & Becker (1988) discovered that a third of their PNETs with ependymal cells and all other ependymal tumours expressed GFAP. Strong positivity in all six ependymomas in a series of 70 CNS tumours removed from children operated on at The Children's Hospital of Philadelphia was observed (Rorke et al, 1992). In a recent report, strong GFAP immunoreactivity was demonstrated in a recurrent infratentorial ependymoma in a 27 month old boy (Twiss et al, 1993). To date no in vitro analysis regarding the expression of GFAP by such cell lines has been reported.
Astrocytomas

Immunocytochemical studies indicate that neoplasms of glial origin exhibit GFAP in the cytoplasm (Deck et al., 1978; Duffy et al., 1977). Trojanowski et al. (1984) found 6/6 GFAP positive tumours and Gottschalk & Szymbas (1987) found all 224 astrocytomas and 105/112 (94%) GBs in adults to be GFAP positive. Molenaar et al. (1989) reported that all astrocytomas from children were positive for GFAP in both frozen and paraffin embedded sections. Bodey et al. (1990) found that in all the 14 childhood astrocytoma cases screened, more than 90% of the cells were GFAP positive on frozen sections. Such positivity was seen in neoplastic cells as well as in normal, fibrillated astrocytes and reactive cells present within the astrocytomas. In another series of 16 different childhood astrocytomas (Bodey et al., 1991) demonstrated that one anaplastic astrocytoma (AA) expressed GFAP in 10-50% of cells, whilst in the remaining 15/16 glial tumours, positivity was seen in more than 50% of cells and in 13 of the 16 in more than 90% of cells. Rorke et al. (1992) in their series observed that all paediatric astrocytomas (15/15) showed strong positivity for GFAP.

Astrocytomas - GFAP in vitro

Many glioma cell lines do not express GFAP (Ponten & Westermark, 1978; Bigner et al., 1981a; Jones et al., 1982; McKeever et al., 1987). GFAP expression in these cell lines is much lower than the number of positive biopsy cases (Jones et al., 1982; McKeever et al., 1987). In vitro studies of human gliomas have shown that primary explants initially expressed GFAP but with repeated passaging, this expression was gradually lost (Yung et al., 1980). GFAP expression was lost as early as passage level 5 or 6 (Lolait et al., 1983), indicating either an overgrowth of primitive tumour cells (glioblasts) or de-differentiation of neoplastic astrocytes had occurred (Paetau et al., 1980). In one series, Kennedy et al. (1987) observed that all the cultured tumour cells from malignant astrocytomas were GFAP positive but the benign were negative. In contrast, Mapstone & Galloway (1991) observed comparable GFAP positivity with both low grade astrocytoma (71%) and high grade astrocytoma (63%) cell lines and also showed that some glioma cells retain GFAP positivity, even up to passage level 11 (Mapstone & Galloway, 1991). The expression of GFAP and FN will be discussed in more detail in the FN section.

External stimuli have been shown to influence also GFAP expression of cells in vitro. For example, the rat glioma C6 expressed more GFAP when cultured in 3-D culture than in monolayer (Bissell et al., 1974). The differentiating agent, retinoic acid produces a 10-fold increase in expression of GFAP by U343MG cells as assessed by enzyme-linked-immunosorbent assays (ELISA) (Rutka et al., 1988b) and is associated with a dose-dependent inhibition of glioma cell proliferation. COL I and IV and leptomeningeal matrix material used as substrates for U343MG cells increased the amount of GFAP as assessed by ELISA between 6-20x (Rutka, 1986; Rutka et al., 1987b).
Neurofilaments

Neurofilament (NF) intermediate filaments are expressed exclusively by neurons and are the major component of the neuronal cytoskeleton (for review, Liem, 1993). They are composed of a triplet of proteins of 68, (NF-L), 150 (NF-M) and 200 kDa (NF-H) molecular weight (for reviews see: Osborn & Weber, 1983; Liem, 1993). The NFs occur in either a phosphorylated or non-phosphorylated form, each of which is distributed differently in axons and cell bodies (Sternberger & Sternberger, 1983). A review discusses in detail the degree of phosphorylation of NFs which appears to be only initiated when the NFs enter axons and carries on throughout axonal transport (Nixon, 1993). NFs appear soon after terminal neuronal differentiation, followed by a gradual decrease during late embryonic and early postnatal development (Yachnis et al, 1993). NF-L and NF-M are expressed prior to NF-H in post mitotic neurons and NF-M attains its mature state of phosphorylation more rapidly than NF-H (Bennett, 1987).

NF expression is observed when ganglion cells or neuronal differentiation is present as in ganglioneuroblastomas, gangliocytomas, gangliogliomas and in phaeochromocytomas (Trojanowski & Lee, 1983; Trojanowski et al, 1984; Mukai et al, 1986). Although NF proteins are markers for differentiated neuronal tumours, they are often expressed in undifferentiated neural tumours such as MBs and NBs (Trojanowski et al, 1984; Mukai et al, 1986). Also recent evidence has indicated that NF-M and NF-L may be expressed in immature Schwann cells (Kelly et al, 1992) and NF-H in T-lymphocytes (Murphy et al, 1993).

Trojanowski and co-workers reported the absence of detectable NF in 10/10 fixed, paraffin embedded cases of MB (1984). Bourne et al (1986) detected only NF-H and that only in 3/10 MBs. In a recent immunocytochemical study, expression of NF proteins on 37 diagnosed PNETs at The Children’s Hospital of Philadelphia, showed that 54% stained for NF proteins (Rorke et al, 1992). Zeltzer and co-workers (1990) observed NF-H was present in 12/14 MBs and in 9 of these more than 10% cells were reactive. NF-M was seen in 5/14 MBs but in only 3 was it present in more than 10% of the cell population. NF-L was expressed in only one MB.

NF proteins have been reported to be co-expressed with a variety of IFs in some CNS tumour cell types. It can be co-expressed with GFAP or with vimentin (VIM) in PNETs (Trojanowski et al, 1984; Tremblay et al, 1985; Katsetos et al, 1988). Trojanowski et al (1984) in an immunocytochemical study on 46 PNET paraffin sections found that 38 cases were positive for GFAP, 15 for VIM but only 3 expressed NF. But in a subsequent study, all 6 cases were positive for GFAP but none for VIM or NF. When immunoblotting was performed on sections adjacent freshly frozen samples of the same cases, GFAP, VIM and NF were expressed in 6, 3, and 4 cases respectively (Tremblay et al, 1985).
Medulloblastomas - in vitro

The pattern of IF expression has been studied in three established MB cell lines, D283 Med, D341 Med and Daoy. The initial biopsy from which the cell line D283 was derived expressed GFAP but this was lost in culture and in subcutaneous xenografts of the cell line. The cell line D341 did not express GFAP but was NF positive. This cell line differs from D283 in that a lower percentage of cells express NF-H or NF-M but no cells expressed NF-L.

Daoy (Jacobsen et al, 1985; He et al, 1989) expresses a rather different phenotype. It expresses neither GFAP or NF (He et al, 1989) but does express a number of other neuroectodermal and glial-associated antigens, indicating that it could be of a glial rather than of neuronal lineage. Immunohistochemical studies showed that two MBs cell lines, ONS-76 and ONS-81 expressed NF-M and NF-H proteins and neuron-specific enolase (NSE) but no GFAP or S-100 was detected (Tamura et al, 1989a). In a recent report, four newly established MB cell lines were found not to express any of the NF proteins (Pietsch et al, 1994).

Astrocytomas

Occasional expression of NF has been reported in astrocytomas, including ependymal giant cell astrocytomas (Bonnin et al, 1984). A recent study of undifferentiated adult brain stem gliomas (Tamura et al, 1989b) has revealed that NF-L positive cells were present in more than 50% of cells in one AA. In contrast, a study with childhood astrocytomas only showed 1-10% of cells positive for NF-L and this in only 4/14 cases (Bodey et al, 1990). Trojanowski et al (1984) found that NF-H proteins were absent from 78 formalin fixed and paraffin embedded glial tumours, while Bodey et al (1990) reported that 4/14 tumours (of which there were 1 GB, 1 AA, and 2 pilocytic astrocytomas) were composed of 10% cells positive for NF-H. Whether this is due to normal, neoplastic derived or in trapped intratumoral neuronal elements is unknown. Simultaneous expression of GFAP and NF triplet proteins was observed in 10-50% of cells of four astrocytomas (Bodey et al, 1990).

Vimentin

Irrespective of cell type, vimentin (VIM) is the first IF to appear in the course of development. In adult cells, it is usually succeeded by the IF characteristic of each cell type but in endothelial cells, fibroblasts, macrophages, chondrocytes and lymphoid cells, it remains the only IF. The appearance of VIM usually precedes that of GFAP in astrocytes during development (Dahl et al, 1981). During the early phases of development of neuroectodermal stem cell maturation, VIM is expressed (Bignami et al, 1982). VIM is weakly positive in normal glia but is strongly positive in reactive astrocytes (Schiffer et al, 1986). In gliomas, expression of VIM has been observed in both endothelial and tumour cells and its distribution is similar to that seen with GFAP (Herpers et al, 1986; Schiffer et al, 1986).
**Medulloblastomas**

VIM expression has been widely reported in MBs *in situ*. Out of 50 cases, 28% were positive (Cruz-Sanchez *et al*, 1989), in another study, 13% of 30 cases (Sime *et al*, 1989) and in 53 MBs (where 91% of patients were less than 20 years old) 20 cases (38%) expressed VIM (Coffin *et al*, 1990). The pattern of VIM immunoreactivity mirrored that of neoplastic cells in both the classic and desmoplastic variant of MBs, where it was diffuse, strong and cytoplasmic. In a recent report positive VIM expression was seen in 21% of childhood cases (Patt & Zimmer, 1992). While in neoplastic astrocytes in gliomas co-expression of GFAP and VIM is common (see below), this is not observed in MBs (Cruz-Sanchez *et al*, 1989; Patt & Zimmer, 1992).

**Astrocytomas**

On frozen sections, Roessmann *et al* (1983) found that neoplastic astrocytes expressed both GFAP and VIM but that VIM was the predominant IF. On formalin fixed tissue (Yung *et al*, 1985) VIM was present in neoplastic astrocytes, vessels and mesenchymal cells. VIM expression has been observed in normal glial cells in tissue culture (Bennett *et al*, 1978) and in human glioma cell lines (Paetau *et al*, 1979; Kashima *et al*, 1993). In certain glial cells, such as immature glia and fibrous astrocytes as well as in cultured glial cells, both GFAP and VIM are synthesised (Quinlan & Franke, 1983). Co-expression of GFAP and VIM has been shown in the human glioma cell line, U-251MG (Paetau *et al*, 1979).

**Cytokeratin**

Although cytokeratins (CYTs) are thought to be specific for epithelial cells and tumours derived from them (Moll *et al*, 1982), expression has been observed in a number of non-epithelial neoplasms (Schiffer *et al*, 1986; Sime *et al*, 1989) as well as in differentiated neuroectodermal tumours (Mork *et al*, 1988; Ng & Lo, 1989).

**Medulloblastomas**

Generally, CYT expression in such tumours has not been observed immunohistochemically (Cruz-Sanchez *et al*, 1989; Sime *et al*, 1989). Grant *et al* (1988) could not demonstrate a positive response in all 10 tumours screened. However, positive CYT expression has been observed in 2 cases in one study and in 1/37 PNETs in another respectively (Mannoji & Becker, 1988; Molenaar *et al*, 1989). Grieshammer *et al* (1991) showed that 3/11 PNET cases were found to express CYT. Positive expression was also seen in a PNET (supratentorial MB) in a 5 month old boy (Zimmer *et al*, 1991) and in another tumour, again of the same age and sex (Patt & Zimmer, 1992). These findings confirm earlier results where during the development of neuroectodermal cells, CYT is occasionally transiently expressed (Bennett, 1987).
**Ependymomas**

Ependymal tumours have been reported not to express CYT with the exception of a proportion of myxopapillary ependymomas (Miettinen et al, 1983; Coakham et al, 1985). Mannoji & Becker (1988) found one out of 17 cases where cells expressed CYT, but such expression was confined to perivascular pseudorosettes.

**Astrocytomas**

Keratin has been reported in some astrocytomas but only in epithelial like areas of very anaplastic tumours (Perentes & Rubinstein, 1987; Mork et al, 1988). Although, two studies have shown that astrocytic tumours have a high incidence of CYT immunoreactivity (Cosgrove et al, 1989; Ng & Lo, 1989).

Bodey et al (1991) showed positive acidic keratin activity in 5/16 using monoclonal antibody (MoAb) AE1 and expression of a keratin pair (acidic and basic) with MoAb AE2 in 5/16 childhood astrocytomas. A second keratin pair, using MoAb AE3 was positive in 14/16 glial tumours. Immunostaining with AE5 defined the expression of another basic keratin (64kDa) and was found to be positive in 9/16 glial tumours. Also another cytokeratin, 51 kDa acidic keratin, detected by the MoAb AE8 was expressed in 14/16 astrocytomas screened. This unexpected expression of some astrocytomas may indicate the degree of anaplasia in poorly differentiated tumours.

Co-expression of VIM, GFAP and several CYTs in glial tumours both in formalin fixed and paraffin embedded tissues has been demonstrated (Cosgrove et al, 1989) and coexpression of GFAP and NF has been found in childhood astrocytomas and PNETs (Bodey et al, 1990). Partial co-expression of at least four IF protein subunits GFAP, VIM, NF (mainly H & M) and CYT subclasses was detected in all 16 childhood astrocytomas (Bodey et al, 1991).

**Desmin**

Dahl et al (1986) suggested that various types of desmins (DES) exist, some of which can be found in astrocytes. However, recent studies using 2-D gel electrophoresis and immunoblotting showed DES-like immunoreactivity of astrocytes was not due to the presence of DES but to the presence of proteins immunologically related to DES (Dahl et al, 1989). One study of 22 human glioma tissues yielded four permanent cell lines (Jacobsen et al, 1987), of which three cell lines were positive for DES to varying degrees. One cell line expressed both DES and myoglobin, another expressed both muscle antigens transiently and the third cell line only expressed DES. All three of these cell lines contained ultrastructural filaments resembling sarcomas of striated muscle (Jacobsen et al, 1987). It has been reported that in human tumour tissue, MB express muscle differentiation more often than gliomas (Bonnin & Rubinstein, 1984). A small number of PNET cases were positive for DES (4/22), where either isolated or cell clusters showed DES expression. The staining for DES however was only seen using immunoblots with 125 I-labelled
secondary antibody (Molenaar et al, 1989). Another study also observed DES expression in PNETs (Gould et al, 1990).

**Fibronectin**

Fibronectin is a protein widely distributed in connective tissue and is not confined to basal membranes as illustrated by its presence in vessel walls of the CNS. The expression of FN in vitro has been associated with cells of mesenchymal origin (Vaheri & Mosher, 1978). Previous studies have been unable to find FN positivity in glial and glioma cells which were GFAP positive (Raff et al, 1979; Paetau et al, 1980; Rutka et al, 1987c). However, there is some immunocytochemical evidence that there are glial cells that express FN (Vaheri et al, 1976; Jones et al, 1982; Mc Keever et al, 1987; Mapstone & Galloway, 1991).

Paetau et al (1980) showed cultured differentiated glial cells as confirmed by GFAP positivity did not express FN and vice versa. Also there was a decrease in the number of GFAP positive cells and increased FN positivity in subcultures, which was possibly due to de-differentiation of subcultured cells (Haugen & Laerum, 1978) or to an overgrowth of mesenchymal entities or both. The presence of FN in astrocytes remains controversial (Paetau et al, 1980; Jones et al, 1982). Lolait et al (1983) confirmed that glioma cells in vitro lost their GFAP expression by the 5th or 6th passage while these cells increased expression of FN. Human gliomas in situ, in xenografts and in cell culture tend to lose glial and develop mesenchymal features, including expression of FN and this is known as "mesenchymal drift" (Lolait et al, 1983; Mc Keever et al, 1987; Rutka et al, 1987b). However, there was no way of identifying the surviving cell population in glioma cultures. It has been postulated that the remaining GFAP-negative cells may be from vascular elements as well as the possible overgrowth of mesenchymal cells to explain the GFAP negative surviving cells (Maunoury, 1977). This was supported by the increased expression of FN as the cells were passaged. Due to diverse morphological forms of cultured glioma cells, the possibility of fibroblast overgrowth has not been confirmed, as one would have expected to find that, with increasing passages in vitro, a similar cell type in all gliomas would be apparent. There is evidence for the cells being neoplastic, in that astrocytomas have very different growth kinetics compared with fibroblasts. Other features include the GFAP negative cells from established human glioma cell lines having many surface microvilli and blebs consistent with tumour cells as well as chromosomal abnormalities (Rankin-Shapiro, 1982). Glioma cell lines frequently display aneuploidy confirming that they are not fibroblasts (Bigner et al, 1981a; Shapiro & Shapiro, 1984).

Kennedy et al (1987) proposed a theory that many gliomas contain two antigenetically distinct populations of transformed cells, a GFAP+/FN- and a GFAP-/FN+ population. In addition, FN+ cells were aneuploid and were contact inhibited in their growth, indicating that these were transformed tumour cells and not fibroblasts (Kennedy et al, 1987). Interestingly, it
has been reported that a rare glioma cell line exists which expresses both GFAP and FN positivity as shown by immunofluorescence (Mc Keever et al, 1989). Such dual expression is unusual and contrasts with mutually exclusive expression of GFAP and FN by non-neoplastic brain cells (Paetau et al, 1980).

Glioma cell lines which have the ability to survive in long-term culture double or nearly double their original ploidy in early passage (Onda et al, 1988). It has been shown that as cells are carried in culture, GFAP expression decreases but the expression of the two IFs, vimentin and FN increases (Bilzer et al, 1991). Reports have also shown that FN⁺ and GFAP⁺ cells express platelet-derived growth factor PDGF-A but not PDGF-B receptors (Bongcam-Rudloff et al, 1991).

Other antigens

Synaptophysin
Synaptophysin (SYN) is a 38 kDa glycoprotein found in the membranes of synaptic vesicles (Jahn et al, 1985; Wiedenmann & Franke, 1985). From studies by Miller et al (1990) it is apparent that it is a reliable and specific marker for neuronal and neuroendocrine tumours in both fixed and paraffin-embedded tissues.

Positive expression has been seen in gangliogliomas, gangliocytomas and PNETs, including MB (Schwechheimer et al, 1987; Coffin et al, 1990; Gould et al, 1990; Diepholder et al, 1991). It is in the ganglion cells of these tumours that positive expression is most prominent. In medulloblastoma, staining is characteristically punctate and is seen in the centre of Homer-Wright rosettes. It is expressed in the vast majority of cases of classic MB (Schwechheimer et al, 1987; Coffin et al, 1990) but less so in the desmoplastic variant (Maraziotis et al, 1992). SYN has been shown to be expressed by most established MB cell lines, including D283, D341, D384, D425 and D458 (Friedman et al, 1991), as well as in MB cell lines, MHH-MED-2, 3 and 4 (Pietsch et al, 1994).

Kemshead and co-workers (Allan et al, 1983; Kemshead et al, 1983) have developed and tested a series of monoclonal antibodies that recognise neuroectodermally associated antigens. A small number of antibodies have been produced; UJ13A (which recognises tenascin), binds to normal and neoplastic neuroectodermal tissue, UJ181.4 shows specificity to oncofetal antigen expressed by foetal brain and neuroblastic tumours and finally, UJ127.11 recognises antigens of normal neuroectodermal tissue, neuronal tumours and schwannomas. These antibodies have been successfully used in the characterisation of surgical material and established cell lines (Kemshead & Coakham, 1983; Bourne et al, 1986; He et al, 1989).
S-100 protein
S-100 protein is a calcium-dependent protein which regulates phosphorylation and microtubule formation (Kligman & Hilt, 1988; Rambotti et al., 1989). Originally thought to be a neuronal marker (Tabuchi & Kirsch, 1975) it now appears to be expressed by a wide variety of cell types in the CNS and PNS including astrocytes (Matus & Mughal, 1975), Schwann cells and satellite cells (Nakajima et al., 1982).

Glioma tissues, including oligodendrogiomas and GB with equivocal or negative reactivity to GFAP may be S-100 positive (Bonnin & Rubinstein, 1984; Perentes & Rubinstein, 1987). It is a less specific glial marker than GFAP (Bonnin & Rubinstein, 1984; Kimura et al., 1986). Data from four studies showed that only 16% of glioma cell cultures are positive for S-100 (Bigner et al., 1981a; McKeever et al., 1981; Liwnicz et al., 1986; Jacobsen et al., 1987). Close linkage of S-100 and GFAP expression was seen in two of these studies (Liwnicz et al., 1986; Jacobsen et al., 1987), but not in the other two (Bigner et al., 1981a; McKeever et al., 1981). Such variation may be due to heterogeneity of glioma cell lines but diversity of S-100 epitopes and assays are also probably responsible (Ponten & Westermark, 1978; McKeever et al., 1981). Different cross-reactive S-100 proteins comprised of homo- and heterodimer domains of α and β chains are present in glial and muscle cells (Kligman & Hilt, 1988). S-100 has been shown to be expressed in MBs (Katsetos et al., 1988) but most commonly in the desmoplastic variant. Of the established cell lines, only Daoy appears to be positive for this antigen, whilst the other cell lines are negative.

Glutamine synthetase
In astrocytomas, glutamine synthetase (GS), is diffusely positive, rarely positive in ependymomas and negative in oligodendrogiomas and meningiomas. Positivity appears to be inversely proportional to anaplasia. Its use in tumour diagnosis is limited for those tumours derived from the CNS, since it has been found to be expressed in other human tissues such as hepatocytes (Pilkington & Lantos, 1982).

Neuron-specific enolase
The enolase isoenzymes are a group of five dimeric proteins comprised of combinations of three different 40-50kD subunits (γ, α and β) (Marangos et al., 1980). They are enzymes of the glycolytic pathway that catalyse the interconversion of 2-phospho-D-glycerate and phosphoenol pyruvate. Neuron-specific enolase (NSE) is a homodimer composed of two γ-subunits. Under normal conditions NSE is confined to CNS and PNS neurons (for review, see Kleihues et al., 1987), however, it has been detected in many small cell neoplasias. Good results have been obtained when applied to glial derived tumours of the CNS (Bonnin & Rubinstein, 1984). Initially, this marker was thought not to be present in MBs (Moss, 1983) but a number of workers have reported otherwise (Perentes & Rubinstein, 1987; Katsetos et al., 1988). In a study
by Coffin et al (1990) of 53 MBs cases 100% expressed NSE. Most of the MB cell lines express NSE except for Daoy.

Tsokos et al (1984) has shown the value of NSE differentiating between NBs and other small round cell tumours in childhood, even though NSE is not usually expressed in neuroblasts of the early embryonic system (Schmechel et al, 1980).

Its use in diagnosing brain tumours is controversial, since, though it may be positive in tumours such as MBs or central neuroblastomas with neuronal differentiation, this enzyme may also be expressed in non-neuronal cells (Haimoto et al, 1985) as well as in reactive astrocytes of malignant glial tumours and in non-neuroepithelial tumours (Bonnin & Rubinstein, 1984; Seshi et al, 1988). Since the γ-subunit of NSE is expressed by non-neuronal and non-neuroendocrine cells, it is no longer thought of as a specific marker for neural or neuroendocrine neoplasms (Gould et al, 1987).

NSE has been useful in predicting not only stages of neuronal development but also the extent to which neuronal metabolic properties have matured (Marangos & Schmechel, 1987). NSE in embryo is found in very low amounts and increases rapidly following birth (Marangos & Schmechel, 1987).

Photoreceptor markers
From immunocytochemical studies, cerebellar MBs may contain varying number of tumour cells which bind mono- and polyclonal antibodies against rod-opsin and S-antigen (arrestin), which are regarded as highly specific markers of retinal photoreceptor cells and pinealocytes (Korf et al, 1987; Bonnin & Perentes, 1988; Czerwionka et al, 1989). Bonnin & Perentes (1988) found 8/16 cases of MBs positive for these two markers and in 19/66 MBs Korf et al (1992) observed immunoreactivity in tumour cells. Such expression indicates that some MBs have the ability to differentiate along the photoreceptor cell lineage, and so a special subtype of MB showing photoreceptor specific characteristics could be designated (Czerwionka et al, 1989).

Nestin: A new VIth class of IF
Nestin is a sixth class of IF protein (Lendahl et al, 1990) and was originally identified by the monoclonal antibody, Rat.401. Using immunohistochemistry (Hockfield & McKay, 1985) and double-label fluorescence-activated cell sorting (Frederiksen & McKay, 1988), nestin was found to be expressed in rat CNS stem cells and more recently in human brain (Dahlstrand et al, 1992; Tohyama et al, 1992). Nestin is expressed in CNS stem cells (Lendahl et al, 1990) and after its
down-regulation, the IFs, GFAP and NFs are expressed in differentiated astrocytes and neurons respectively (for review; Steinert & Liem, 1990; Zehner, 1991).

Valtz et al (1991) showed a nestin positive cell line derived from a rat cerebellum differentiating into multiple fates similar to those seen in MB. Nestin is characteristic of neuroepithelial stem cells (neural precursor cells) (Lendahl et al, 1990) and this has been confirmed in a study where it was found to be expressed by neuroepithelium in the developing CNS (Tohyama et al, 1992). High levels of nestin positivity has been observed in GBs and in PNETs (Dahlstrand et al, 1992; Tohyama et al, 1992). It was reported that GBs expressed higher nestin levels than the less malignant astrocytomas and that this expression may be linked to degree of malignancy within this group of tumours (Dahlstrand et al, 1992). Within the PNET group, both positive and non-positive nestin cells were present (Dahlstrand et al, 1992).

Nestin expression has also been shown in a number of established cell lines (Tohyama et al, 1992). These included two glioma cell lines (U373MG & U251MG) where nestin expression was co-expressed with GFAP. Of the MB cell lines screened, which included D283, D341, D384, D425 and D458, all showed nestin expression, as well as synaptophysin and neurofilament positive cells. Daoy, in contrast did not express nestin but did express glial markers. It has been proposed that Daoy may represent a very immature stem cell tumour prior to nestin expression which is not a true representative of a MB (Tohyama et al, 1992).
Chapter 4

Chemosensitivity

In vitro prediction of chemosensitivity

Because it is impossible to determine on histological or clinical grounds if an individual tumour is going to respond to a particular form of treatment, there has been considerable interest in developing an assay that will quantify drug sensitivity and drug resistance in cultured tumour cells (Puck & Marcus, 1955; Roper & Drewinko, 1976; Hamburger & Salmon, 1977; Von Hoff, 1987). The basic design of such an assay needs to take account of a number of parameters, including the type of cells used, the length of drug exposure, length of recovery following drug exposure as well as the appropriate choice of endpoint. A great number of endpoints can be used but, ultimately, the assay must be designed to accurately determine the number of viable cells remaining at the end of the assay.

Source of material for chemosensitivity assays

i) Directly from tumours

Cells can be isolated from tumours and tested directly for sensitivity without an intervening period of culture. It is important to be able to identify the malignant cells within the cell preparations and to ensure that they are representative of the original tumour biopsy. The advantage with such a system is that no selection pressures are involved and the material on which the assay is carried out is truly representative of the original tumour. Disadvantages include the limited number of cells that are available for the assay, which may limit the type of assay used or the number of drugs which can be tested or the number of replications used.

ii) Short-term cultures

Short-term cultures have been an important source of material from human tumours for chemosensitivity assays. A high percentage of biopsies from human adult malignant gliomas will grow in culture as a monolayer. The disadvantages of these cultures is that they have a short life span and they may be slow growing and unstable in culture.

iii) Long-term cultures

Many tumour types have been established in monolayer culture as continuous cell lines. However, it is important to remember that such cell lines, although extremely useful may not be representative of the complex heterogeneous population of cells present within a tumour in a patient. The cells which establish in vitro may be those with the ability to grow under such conditions. The advantages of these cultures is that a large number of cells are available and so
assays can be reproduced a number of times and such cultures are stable in culture for long periods of time.

**Drug treatment**
The duration of drug exposure and the concentration of drug used in *in vitro* assays are of critical importance. The length of drug exposures can be divided into three groups; short, perhaps only one hour or two hours, intermediate exposure of between 8-24 hours and long-term exposure of 1-3 days.

Other factors which need to be taken into account when designing chemosensitivity assays include *in vivo* exposure levels, *in vivo* activation and drug stability *in vitro*. It is important to determine if all the cells in the assay during the drug exposure period have completed one or more cell cycles. After a short exposure another important issue to be answered is whether the measured *in vitro* sensitivity is stable and whether the drug concentrations needed for these assays are pharmacologically achievable *in vivo*.

**Recovery**
It is important that following drug exposure, a period of recovery is allowed so that residual viability can be determined. Different recovery periods can be employed;

i) short (1-4 hours) - allowing for equilibrium of acid-soluble pools and the efflux of unbound drug from the cell surface.

ii) intermediate (2-3 days or 1-3 cell population doublings) - providing sufficient time for cells to recover from sub-lethal damage and reversible metabolic effects.

iii) long (14-21 days or seven population doublings) - time necessary for a single cell to produce a colony of over 100 cells in the clonogenic assay.

The major differences between various assay techniques mainly lie in the determination of the residual viability.

**Measurement of endpoint**

**Assays based on morphological damage**
In these assays tumour cells were dispersed and plated into monolayer culture and following a period of attachment and short-term growth in culture, drugs were added and the resulting damage recorded subjectively under the microscope. This early type of *in vitro* predictive assay illustrated a number of drawbacks including difficulty in distinguishing between fibroblasts, normal epithelial cells and adherent macrophages; secondly interpretation and thirdly, difficulty in quantification. This system has been shown frequently to give false positive results (Hurley & Yount, 1965), although others have found it to be an accurate indicator of drug...
sensitivity (Limburg & Heckmann, 1968). Using this type of assay it has been possible to demonstrate that patients with ovarian cancers who followed the prediction of the assay had longer short-term survival times than patients who did not follow the test results (Wheeler et al, 1974; Dendy et al, 1981).

Brain tumours have been assessed for chemosensitivity using morphological analysis and have shown a marked range in the response of cultures to single agents, including vinblastine (Wilson & Barker, 1965), mithramycin, (Wilson & Barker, 1967), actinomycin C and D (Gazso & Afra, 1969).

**Assays based on cell counts**

Endeavours to quantify the drug effects observed in morphological assays led researchers to employ a range of cell-counting methods based on intact monolayers, fixed and stained monolayers and Coulter counting. The number of cells remaining on glass coverslips after drug treatment per unit area determined with a calibrated eyepiece graticule was used to screen 12 glioma cultures against a variety of chemotherapeutic agents and considerable heterogeneity in response was noted (Easty & Wylie, 1963). A test in which cells were treated for 72 hours with drug prior to being trypsinised and counted with a Coulter counter was described by Holmes & Little (1974). Accurate retrospective predictions were seen in 11/12 patients.

Using a similar approach based on Coulter counting, other workers have not been able to confirm this relationship (Berry et al, 1975). By measuring the number of cells remaining adherent to Terasaki plates after drug treatment, a marked variation in the response of malignant gliomas to BCNU (Mealey et al, 1974; Kornblith & Szypko, 1978; Kornblith et al, 1981) and radiation (Mealey et al, 1974) was observed. Kornblith et al (1981) using this type of assay were able to show correlation in the responses of 10/13 patients with malignant glioma to BCNU or CCNU *in vitro*. There were five true positives and five true negatives. Three false positives were also observed and there were no false negatives.

**Assays based on dye exclusion**

This type of assay was based on the assumption that viable cells remain unstained by actively excluding a dye. Dead cells which could not maintain membrane integrity stained with the dye (Phillips, 1973). The test was quick and could be employed for cells which grew in suspension or which could readily be disaggregated by enzyme treatment. The degree to which cells took up dye was pH dependent; at pH 7.5, maximum uptake to trypan blue occurs, whereas for erythrosine B it is pH 7.0. The dye exclusion characteristics of the cell may well be influenced by the environmental conditions used in the assay. Uptake of trypan blue was also affected by the presence of serum in the culture medium, for which it has a strong affinity.
In both trypan blue and erythrosine B dye exclusion assays, $^{51}$Cr release gave similar results for drugs which affected membrane integrity. However, other authors have questioned the reliability of such dyes as indicators of drug-induced cytotoxicity (Roper & Drewinko, 1976). Dye exclusion assays seemed to be incapable of detecting cell death when applied immediately after drug exposure. Weisenthal & Lippman (1985), in a subsequent study, showed that for promyelocytic leukaemic cells (HL60), the viability of cells when assayed 2, 4 and 6 days after drug exposure was seen to fall from 38% to 17% to 0% respectively. This emphasises the importance of allowing sufficient time prior to taking measurements following drug exposures.

A 4-day dye exclusion assay (also known as differential staining cytotoxicity (DiSC) assay) based on fast green staining was shown to correlate well with a clonogenic assay (Weisenthal et al, 1983). It has been possible to predict correctly the response in 19/21 patients with chronic lymphoblastic leukaemia (CLL) cases (Bosanquet et al, 1983). *In vitro* chemosensitivity of tumour cells from 44 patients with CLL and non-Hodgkin’s lymphoma were assessed using the DiSC assay which correctly predicted seven sensitive and 30 resistant tumours, an overall positive correlation of 84% (Bird et al, 1988). In 8 patients with CLL who had tumour samples available or underwent repeated testing a significant and progressive development of drug resistance was observed, whereas in those patients with CLL who were untreated, their corresponding results did not change.

The DiSC assay results were correlated with clinical response in patients with haematological malignancies (Bosanquet, 1991). In approx. 18% (22/119) of cases *in vitro* results correlated with clinical outcome, where extreme drug resistance was observed. The number of patients responding to therapy and 50% patient survival increased with corresponding *in vitro* drug-sensitivity. In another set of experiments where eleven samples showed extreme resistance to anticancer drugs prescribed, these samples were also tested with unprescribed drugs *in vitro*. Results showed that patients showing some drug sensitivity to drugs used tended to have a longer survival than those patients whose tumours were extremely drug resistant to all drugs tested *in vitro* (Bosanquet, 1991).

**Assays based on biochemical determinations**

Inhibition of a single biochemical pathway or the overall inhibition of cellular metabolism in tumour cell suspensions or monolayer cultures are involved in these assays (for review see Hall, 1974). The earliest work documenting a correlation between patient response and *in vitro* results from an assay of this type was provided by Black & Speer (1954). In their assays, slices of fresh human tumours were incubated with and without drugs to which a tetrazolium dye was added. This dye was reduced by succinate dehydrogenase to a brown colour which could be measured spectrophotometrically. Di Paolo & Dowd (1961) modified the Black & Speer assay by suspending tumour cells in agar and the cell suspension was overlaid with disks containing
test drugs analogous to bacterial tests. Cellular dehydrogenase activity was measured by reduction of methylene blue dye and the area of unreduced dye showed inhibition of dehydrogenase activity. Although, the assay was 90% predictive for resistance, only 50% of patients whose tumours were responsive in vitro were so in vivo.

This idea was further investigated by Kondo et al (1966) and Kondo (1971) who measured succinic dehydrogenase activity in suspensions of fresh tumour cells by recording the colour change resulting from the reduction of 2,3,4-triphenyltetrazolium chloride. These results showed a good correlation between the in vitro data and the clinical outcome of 15/18 patients screened.

Radioactive precursor uptake assays

Short-term assays

The use of thymidine incorporation into DNA for in vitro predictive testing was first reported by Bickis et al (1966). The assay involved incubating slices of fresh tumours in the presence of radioactive tracers and test drugs. Volm et al (1979) and Silvestrini (1981) employed short-term assays that measured the inhibition incorporation of thymidine and uridine into drug treated suspension of human cells. Good in vivo: in vitro correlations were observed.

Another type of assay (Sanfilippo et al, 1981) used cell suspensions or tissue fragments prepared from tumours which were treated with drugs for two hours. A further incubation of one hour followed the addition of 3H-thymidine and 3H-uridine. Owing to the presence of free precursors trapped in the intercellular spaces of tissue fragments, these preparations were chased with 100-fold concentrations of unlabelled precursors. The nucleic acids were extracted from the cells and the proportion of incorporated label into RNA and DNA measured.

This assay has been employed for a number of tumour types, including non-Hodgkin's lymphoma and testicular cancer and results have shown a true positive rate of 83% and a true negative rate of 63% (Silvestrini et al, 1983). These authors have focused on determining the sensitivity of tumours that responded well to chemotherapy and may explain the high true positive rate observed. In another study (Sanfilippo et al, 1986) the chemosensitivity of germ cell testicular tumours was determined using the incorporation of both 3H-thymidine and 3H-uridine following a 3 hour exposure to a number of drugs (CDDP, vinblastine, bleomycin, doxorubicin and dactinomycin). A significant correlation was observed between in vitro and clinical sensitivity to the same drugs (p=0.026) and an overall agreement of 92% when the tumour metastases were tested in vitro. However, when the primary tumour was tested no significant association was found and there was an agreement of only 62%.

Twenty seven tumour specimens from patients with Merkel cell carcinoma (MCC) were tested against 9 drugs in a short-term assay, measuring the inhibition of thymidine
incorporation (Kearsley et al, 1993). The assay showed sensitive tumours in 19/20 specimens and 16/17 patients had a tumour that was sensitive to at least one drug used in the assay. Specimens were considered sensitive to a drug, if at approximately peak plasma concentrations (PPC), the inhibition of thymidine was more than 50% when compared with untreated controls. The highest sensitivity to drugs in order of frequency was observed to be to doxorubicin, epirubicin, cyclophosphamide, etoposide and cisplatin. At least 40% of tumours were sensitive to these 5 drugs. Previous studies on ovarian cancer (Khoo et al, 1988; Hayward et al, 1992) were carried out but the success rate of the assay was greater in the study using MCC.

Intermediate duration tests

Radioisotope-uptake potentially can be employed in assays of a relatively long duration (e.g., 3-10 days) where it is possible to produce monolayer cultures which retain characteristics of the tumour of origin. These assays use microtitration plates which make it easier to handle a large number of replicate cultures. This in turn allows the analysis of multiple drug concentrations in vitro as well as in combination and, since radioactive isotopes are used, a degree of automation can be introduced. It is also possible to produce sensitivity readings after drug exposure of various time periods (e.g., 24, 48, 72, hours or longer) to determine the rate at which drug sensitivity develops over the exposure period as well as to measuring the maximum sensitivity expressed which may be a stable figure if a plateau is reached.

i) Using radiolabelled amino acids

Freshney and colleagues (1975) employed $^3$H-leucine incorporation to measure residual protein synthesis in HeLa cells treated with drugs. The endpoint was measured by extracting the acid insoluble residue in sodium hydroxide solution and measuring $^3$H-leucine uptake by scintillation spectrometry. Results indicated that misleading sensitivities were observed with short drug exposure times, i.e., less than one cell cycle and concluded that extended drug exposures and recovery times were needed for reproducible and stable ID$_{50}$'s.

The microtitration assay was found to correlate closely with a monolayer cloning assay (Morgan et al, 1983), provided the assay was continued after drug exposure until just before density-dependent inhibition of growth was evident. The use of $^{35}$S-methionine, a higher energy $\beta$-emitting amino-acid, made it possible to treat radio-isotopically labelled cells in situ with a fluorescent material (organic solvent-based scintillation fluid; Carlsson et al, 1976; or salicyclic acid, Chamberlain, 1979) and so carry out scintillation autofluorography. The $\beta$-particles, instead of interacting directly with the photographic emulsion as in conventional autoradiography, interact with the fluor to produce multiple photons of visible light which could be detected on blue-sensitive X-ray film. In the chemosensitivity assay, the X-ray film was placed against the underside of the microtitration plate and held tightly against the well bases with a polythene sponge and aluminium pressure plate. The X-ray film was developed, fixed and scanned with a densitometer. A permanent record of each experiment was produced.
Thomas et al (1985) using a microtitration assay with $^{35}$S-methionine, screened cell cultures from 40 patients against three drugs (CCNU, PCB and VCR). They demonstrated a correlation between the *in vitro* PCB and CCNU sensitivity of biopsied glioma tissue and the relapse free interval (RFI) of patients from whom the tumours were derived, where twenty-two of the forty (55%) patients responded to PCB and/or CCNU in the assay and their sensitivity to these drugs was associated with an increased RFI. In contrast, the sensitivity to VCR was not linked to increased RFI. In addition, other factors such as grade of tumour resulted in different sensitivities *in vitro*, where the grade III tumours were found to be more sensitive than grade IV tumours. Also, the more chemosensitive tumours *in vitro* were found to be derived from younger patients than those patients with less sensitive tumours *in vitro*.

The *in vitro* sensitivity of ovarian carcinoma against a number of drugs was reported (Wilson & Neal, 1981). Tumour cells were exposed to drugs for 48 hours and then a recovery period of 24 hours used. Following a three hour pulse of $^3$H-leucine, the endpoint was measured by scintillation spectrometry (Freshney et al, 1975). The tumour samples showed marked heterogeneity in their response to the drugs. In 15 patients, who all had stage III or IV ovarian carcinoma, it was possible to compare their *in vitro* sensitivity data with their clinical response. The patients were divided into a further two groups, where 8 patients had received only first-line chemotherapy and the remaining 7, who had relapsed on first-line chemotherapy, were undergoing a second-line chemotherapy. Two of the three patients who received first-line chemotherapy and were sensitive *in vitro* but did not respond clinically were said to be equivocal cases because combinations of drugs with differing *in vitro* activities were used in their treatment. There was increased drug resistance in tumour cells taken from treated patients compared to those who were previously not treated.

*ii) Using radiolabelled nucleic acid precursors*

The uptake of radiolabelled DNA precursors can also be used as an index of chemosensitivity. One problem associated with these types of assays was the lack of incorporation of DNA precursors by tumour cells with long cycle times. Additionally, due to damage in the isolation process, precursor incorporation into cells may be temporarily arrested. The decreased estimate of DNA synthesis may be due to drug-induced depression of labelled nucleoside transport or changes in nucleotide pools. Alternatively, an increased estimate of DNA synthesis may be caused by increased use of salvage pathways in the presence of drugs, such as 5-FU or methotrexate which may affect the *de novo* pathways (Wolberg, 1972).

A close correlation was seen with *in vitro* results and clinical outcome for patients with acute leukaemia by performing a dual assay measuring RNA and DNA synthesis in cells (Cline & Rosenbaum, 1968). Raich (1978) in 26 patients, observed correlation between the *in vitro* results and clinical outcome. Complete or partial remission was seen in 13/14 patients when *in*
inhibition was seen to be 85% or greater, whereas this was not the case in 10/12 patients where the in vitro inhibition was less than 85%.

Sondak et al (1985) improved the isotope uptake assay by reducing the time required to obtain an answer from 2-3 weeks to 5 days. With such a rapid assay, 819 tumours of various histologies were processed with an overall success rate of 59.3% which compared favourably to the overall success rate of 58.2% for colony-formation assays (Bertelsen et al, 1984). Sixty-five correlations of in vitro and in vivo responses were made. None of the 30 tumours predicted to be resistant in vitro responded to chemotherapy clinically. However, patients whose tumours were predicted to be sensitive in vitro, had a 43% clinical response rate (Sondak et al, 1985).

Kern et al (1985), improved the nucleic acid precursor incorporation assay by plating tumour cells into a double-layer agarose, impregnated with drugs. After 72 hrs incubation, $^3$H thymidine was added for 24 hours and then counted by liquid scintillation spectrometry. It was noted that 280 out of 351 (80%) human solid tumours of various histologies gave evaluable chemosensitivity results. Compared with the colony-forming assay this assay was shorter in duration, fewer cells were needed and ease of quantification as well as applicability to testing human tumours made this assay more acceptable than the colony-forming assay. In addition, artefacts such as cell clumps, debris and clots were not problems.

Zaffaroni et al (1988) using human tumours (11 ovarian cancers and 22 malignant melanomas) compared an antimetabolite assay (Silvestrini et al, 1983) with an antiproliferative assay (Tanigawa et al, 1982) against drugs (doxorubicin, CDDP, bleomycin and VCR). They found both assays to be in good agreement (100%) for in vitro sensitivity or resistance for ovarian cancer. For melanoma, however, 97% of samples were resistant in both assays but only 45% of the samples which were sensitive in the antimetabolite assay were so in the antiproliferative assay.

Akiyoshi et al (1990) showed that 15/17 (88%) patients with various carcinomas gave evaluable chemosensitivity results in a simplified tritiated thymidine incorporation assay with four drugs (ADR, MMC, 5-FU and CDDP). All 15 patients were evaluable for in vitro-in vivo correlations. An accuracy of 75% for prediction of response (3 of 4) and an accuracy of 82% for prediction of resistance (9 of 11) when the peak achievable plasma levels were selected as concentrations used in in vitro sensitivity testing (a 80% inhibition of thymidine incorporation as cut-off level was adopted) was observed.

The chemosensitivities of 29 primary tumours (11 gastric, 8 colorectal, 4 breast and 6 other cancers) and simultaneous metastatic lymph nodes were tested against 6 drugs (MMC, 5-FU, ADR, doxorubicin (DOX), carboquone (CQ) or CDDP) (Nio et al, 1990). Differences between the chemosensitivities of the primary tumour and metastases were observed. This observation
has been previously documented by other researchers (Schlag & Schreml, 1982; Fidler & Poste, 1985). It was also noted that metastases appeared to be more sensitive to drugs compared with the primary tumour and this heterogeneity extended to which type of drugs the two samples were sensitive to in vitro. For example, the primary samples were sensitive to 5-FU, whilst the metastatic samples were sensitive to CDDP (Nio et al, 1990).

Clonogenic assays for human tumour cells

In 1955, Puck & Marcus produced colonies from HeLa cells and found that nearly all the HeLa cells were clonogenic. They used this method to measure the X-ray sensitivity of HeLa cells (Puck & Marcus, 1955). However, under certain circumstances, it has been shown that normal cells as well as transformed ones produce colonies when plated out (Macpherson & Montagnier, 1964). In order to overcome this problem, Macpherson & Montagnier (1964) cloned cells in medium set with 0.3% agar which permitted the selective growth of both human (HeLa, Hep 2) and animal (BH21/13, L cells) transformed cell lines.

Simple cloning assays like this have proved difficult to adapt for cells derived from human brain tumours. This has led to the development of more complex cloning assays for these cells. There are two main types of colony-formation assays which have been used for human tumours, the Hamburger-Salmon assay and the Courtenay-Mills assay.

i) Hamburger-Salmon assay

In this assay tumour cells are plated in an enriched semi-solid medium in petri dishes, over a base layer of agar incorporating a complex range of growth factors (including media conditioned by adherent spleen cells from BALB/C mice) (Hamburger & Salmon, 1977; Hamburger, 1983). Subsequent studies showed that many tumours do not require such a complex medium and that conditioned medium seemed to be a requirement for human myeloma cells only (Hamburger, 1983). Colonies formed between 14-21 days of incubation. Studies characterised the cells that produced these colonies and have confirmed their tumour cell origin (Hamburger & Salmon, 1977; Salmon, 1980).

Clinical correlations with the Hamburger-Salmon assay

A large number of clinical correlations have been cited in the literature using this assay (for reviews: Salmon, 1980; Salmon & Von Hoff, 1981). For three tumour types, ovarian carcinoma, myeloma and melanoma, the true positive rate of this assay was 67% and the true negative rate was 93% (Salmon, 1983). Durie et al (1983) found a relationship between survival time and in vitro chemosensitivity in patients with multiple myeloma using the Hamburger-Salmon assay (H-S assay). A median survival of 48.5 months was observed in those patients who responded to melphalan in vitro and who were treated with this drug as compared to a median survival time of only 9 months in those patients where no response was seen in vitro but received melphalan as part of their treatment program.
Clinical response of high-dose intraarterial chemotherapy (HDIAL) was accurately predicted by the colony forming assay in 11/12 cases in patients with colorectal liver metastases and in a subsequent series of 30 patients with liver metastases. Twenty-one of these patients whose tumours were sensitive in vitro had clinical response to HDIAL, whilst 6/9 patients predicted by the assay to be resistant had no clinical response (Link et al, 1986).

Experiments aimed at comparing the colony formation efficiency and chemosensitivities of different areas of the same primary tumour, the primary tumour versus its metastasis and two different metastases in the same patient have produced a number of interesting results. Chemosensitivity profiles for the same specimen processed on separate occasions showed good agreement as did the sensitivity profiles from two different areas of the same primary tumour. However, when sensitivity profiles of primary samples and their metastases were sampled in a number of different cancers, there was poor agreement (Meyskens et al, 1981; Schlag & Schreml, 1982; Von Hoff et al, 1986).

ii) Courtenay & Mills colony formation assay
A number of studies have shown that the addition of red blood cells (RBCs) and reducing the oxygen tension increased the colony formation of mouse marrow cells in vitro (Bradley et al, 1971). This has led to the development of an in vitro assay of clonogenic cells from animal tumours such as Lewis lung tumour and B-16 melanoma by Courtenay (1976) and human tumour cells (Courtenay & Mills, 1978; Courtenay, 1983). The technique was performed in tubes with a replenishable fluid phase, with the cells suspended in a soft agar plug at the bottom of the tube. RBCs, derived from August rats added to the agar which released growth factors during the assay (by lysis) and appeared to be important for tumour cell growth. Low levels of oxygen (less than 5%) were also introduced by means of gassing mixtures.

The colony forming efficiency of cells derived from most tumours is higher in the Courtenay assay than in the H-S assay, including melanomas, ovarian carcinomas, gliomas and breast tumours (Tveit, 1983; Ottestad et al, 1989; Tveit et al, 1989). Recently, Wilks & West (1991) modified the Courtenay-Mills assay (C-M assay) to support the clonogenic growth in soft agar of human cervical carcinoma cells lines xenografted into nude mice in serum-free conditions with comparable results to those obtained with the traditional C-M assay.

Clinical correlations with the Courtenay assay
Tveit et al (1982) using fresh melanoma cells produced dose response curves and determined the ID$_{50}$ (i.e., dose of drug that inhibited colony-forming efficiency (CFE) by 50%) and, using calibration curves of 1/ID$_{50}$, against growth delay of xenografts in vivo (obtained from previous experiments, Tveit et al, 1980), were able to determine the “expected growth delay” for each human tumour. Results showed that patients whose biopsy cells were sensitive (“expected growth delay” greater than 2) were more likely to respond to those drugs clinically. Ahman et al
(1982) showed using 4-(9-acridinylamino)methanesulphon-m-anisidide (m-AMSA) in a prospective *in vitro* Phase II trial that 3/3 patients who were sensitive *in vitro* responded clinically and 5/5 patients whose *in vitro* results were insensitive failed to respond clinically.

**Clonogenic assays for brain tumours**

Monolayer clonogenic assays have been developed for both animal (Rosenblum *et al*, 1975) and human (Rosenblum *et al*, 1978) brain tumours. In this assay single cell suspensions were treated with drugs and plated with heavily X-irradiated 9L gliosarcoma feeder cells. The colonies were composed of cells with both glial and malignant cell characteristics (Rosenblum *et al*, 1983). Although 95% of surgical specimens produced viable colonies, the colony-forming efficiencies were in the region of 0.1% (Rosenblum *et al*, 1980). Other workers have reported that between 60 and 80% of samples from human malignant gliomas can be successfully assayed for drugs using clonogenic techniques (Georges *et al*, 1984; Mulne *et al*, 1984).

There is a relationship between resistance *in vitro* and resistance clinically (Rosenblum *et al*, 1980). In a study of six patients, only one tumour displayed a cell kill greater than 50% against BCNU and this patient responded clinically to this drug. The remaining 5 patients had maximum cells kills in the range of 0-42% and none of them responded clinically (Rosenblum *et al*, 1980). It was later discovered in a larger series of patients that the clonogenic assay is inclined to overpredict clinical sensitivity (Rosenblum *et al*, 1983).

**MTT assay**

MTT (3-(4,5-dimethylthiazol-2-yl).2,5-diphenyltetrazolium bromide) is a tetrazolium compound which is taken up by living cells and reduced by the mitochondrial enzymes to a blue formazan product. This is insoluble in aqueous solution but can be dissolved to produce a blue coloured solution by a variety of polar solvents (Mosmann, 1983). Optical density of the resultant solution is measured at a wavelength between 540-560nm (Denizot & Lang, 1986; Carmichael *et al*, 1987).

Since the original description of the assay, a number of changes for use on different cell lines has been cited in the literature and it is clear that the MTT assay must be optimised for seeding conditions and assay duration to obtain satisfactory results. Its sensitivity is dependent on the cell type used (including adherent (A) and non-adherent (NA), see table), their ability to metabolise MTT and the method selected for dissolving the formazan crystals (Cole, 1986; Denizot & Lang, 1986; Carmichael *et al*, 1987; Twentyman & Luscombe, 1987). Table 3 summarises some of the variations of the MTT assay, proposed by various authors.
### Table 3: Modifications of the MTT assay

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<tbody>
<tr>
<td>Concentration</td>
<td>5 mg/ml</td>
<td>5 mg/ml</td>
<td>5 mg/ml</td>
<td>5 mg/ml</td>
<td>2 mg/ml</td>
</tr>
<tr>
<td>MTT incubation</td>
<td>4 hours</td>
<td>3 hours</td>
<td>4.5 hours</td>
<td>2 hours</td>
<td>4 hours</td>
</tr>
<tr>
<td>Formazan solubilisation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent</td>
<td>Iso-propanol/ HCl</td>
<td>Iso-propanol</td>
<td>10% SDS in 0.01M HCl</td>
<td>20% SDS in 50% DMF, pH 4.7</td>
<td>Mineral oil</td>
</tr>
<tr>
<td>Incubation</td>
<td>5 mins</td>
<td>1 min with shaking</td>
<td>overnight at 37°C</td>
<td>overnight at 37°C</td>
<td>overnight at 40°C</td>
</tr>
<tr>
<td>Reading wavelength</td>
<td>570-630 nm</td>
<td>560-690 nm</td>
<td>590 nm</td>
<td>570 nm</td>
<td>565 nm</td>
</tr>
</tbody>
</table>

A problem with the MTT assay is the difficulty of dissolving the formazan crystals without precipitation of serum proteins. Such precipitated proteins and cellular debris are a potential source of experimental error since they interfere with the optical density measurements. However, dual wavelength photometry, that is reading plates at a principal and reference wavelength, can eliminate the effect of debris.

Chemosensitivity testing with a wide range of cytotoxic drugs against cell lines derived from a variety of different tumours with this assay has shown good correlation with other assays (Carmichael et al, 1987; Alley et al, 1988; Kohnoe et al, 1991; Vertosick et al, 1994). Using glioma-derived cell lines a good correlation between the MTT assay and the 35S-methionine uptake assay has been documented (Nikkhah et al, 1989). Kuratsu & Ushio (1990) have used the MTT assay to examine the antiproliferative effect of trapidil, a platelet-derived growth factor (PDGF) antagonist in two established glioma cell lines and observed that trapidil increased the cytotoxic effect of ACNU in vitro. Jordan et al (1992) examined the use of the MTT assay in detecting the chemosensitivity of established glioma cell lines against a panel of 10 drugs. One cell line (U138MG) failed to produce the formazan product and so its drug sensitivity profile could not be determined, otherwise the remainder were evaluable.

Another compound, XTT is metabolised by viable cells to a water-soluble formazan, thereby permitting the direct reading of optical density in culture wells without further processing. However, XTT gives higher background readings than MTT.
Clinical correlations with non-clonogenic assays

It has been shown that the MTT assay can be employed as a reliable and simple procedure for the rapid chemosensitivity testing of human brain tumours giving comparable results with those obtained with the $^{35}$S-methionine assay (Nikkhah et al., 1992b). A good correlation between the MTT assay and the colony forming assay (CFA) in drug concentration range used was observed in one study on adult brain tumours (Nikkhah et al., 1992a). The nitrosoureas ACNU and BCNU showed a mild to moderate in vitro effect. Nikkhah et al. (1992a) observed that younger patients (< 40 years) appeared to have a higher sensitivity to mitoxantrone (0.35μg/ml), the mean resistance being 38% in the younger group compared with 50% in the older group) in the MTT assay, whereas in the CFA assay, it was the older patients (60-90 years) who had a better in vitro response.

In retrospective studies, the MTT assay has accurately predicted the initial response to chemotherapy in acute leukaemia (Santini et al., 1989; Sargent & Taylor, 1989), ovarian malignancy (Wilson et al., 1990) including long-term clinical outcome (Pieters et al., 1991) and also gastric cancer (Yamaue et al., 1992).

The MTT assay may also predict drug sensitivity profiles according to histological grade of tumour. For example, Kusumoto et al. (1992) using tumours derived from 40 patients with poorly differentiated adenocarcinoma found that sensitivity of stage III group to cisplatin was higher than that of stage IV group. This indicated that tumours with infiltrative growth, lymphatic invasion in gastric wall or lymph node metastasis were resistant to drugs ADR, MMC, CDDP and CQ.

Protein assays

Assays have been developed which rely on staining of total cellular protein with dyes like sulphurhodamine (SRB). Although these assays typically involve more experimental steps, a major advantage is the stable endpoint and this maybe more applicable for high volume drug screening. There appears to be good agreement between the SRB assays and the MTT assays for a variety of drugs when used in both established cell lines and short-term lines derived from human gliomas (Rubinstein et al., 1990; Pizao et al., 1992; Haselberger et al., 1994).

Radiosensitivity testing of gliomas in vitro

As adjuvant radiation produces only a modest increase in survival with patients with malignant glioma, there has been considerable interest in examining the cause of radiation resistance in these tumours. It has been suggested that the major cause of this is the low intrinsic radiation sensitivity of gliomas (Davis, 1989). This has been investigated by a number of workers and a number of glioma cell lines have been used in such investigations (Weichselbaum et al., 1976; Gerweck et al., 1977; Masuda et al., 1983; Schultz & Geard, 1990; Yang et al., 1990). These studies have tended to confirm that glioma cells are intrinsically resistant to
radiation and typically have surviving fraction (SF₂) values (clonogenic surviving fractions following 2Gy of irradiation) >0.4. More recently, attempts have been made to correlate cellular radiosensitivity and clinical outcome in patients with malignant glioma (Allalunis-Turner et al, 1992; Taghian et al, 1992). Taghian et al (1993) has summarised much of this data and presented radiation sensitivity information in a panel of 85 glioma cell lines. The results showed a broad distribution of SF₂ values and differences were observed in sensitivity between grade III and grade IV tumours. However, there was not a strong correlation between the SF₂ values and clinical outcome in these patients (Taghian et al, 1993), in contrast to similar studies using squamous cell carcinoma of the uterine cervix where an association was found (Davidson et al, 1990).

Experimental chemosensitivity testing of human brain tumours

In vivo experiments
The chemosensitivity of human brain tumours can be determined in athymic mice or rats bearing either subcutaneous or intracranial xenografts which can be exposed to cytotoxic drugs via a variety of different routes of administration, including intraperitoneal, intracarotid, intravenous and intrathecal administration (Bullard et al, 1981; Friedman et al, 1984; for review, Peterson et al, 1994). The response of subcutaneous tumours can be determined by tumour growth delay and that of intracranial tumours by prolongation of survival or time taken to becoming moribund.

Drugs which have been found to be effective against xenografted glioma cell lines include nitrosoureas and PCB as might be expected and more surprisingly drugs like melphalan, cyclophosphamide and fludarabine (Schold & Bigner, 1983; Schold et al, 1984; Schold et al, 1987). Subsequent Phase II clinical trials with cyclophosphamide of gliomas were based on the experimental data from xenografted glioma studies (Schold et al, 1987) and for MBs with melphalan (Friedman et al, 1989). Similar studies with a panel of MB cell lines (D283 Med, D341 Med, D425 Med, D384 Med and Daoy) growing intracerebrally and subcutaneously in nude mice showed extended median survival times and growth delays when tumour bearing mice were treated with melphalan, cyclophosphamide, ifosphamide and thiotepa but not with CCNU and bultuban (Friedman et al, 1988b).

Xenografted human brain tumours have provided a model system to test novel treatments for these tumours. The cytotoxic activity of melphalan and cyclophosphamide has been shown to be enhanced in both intracranial MB and glioma xenografts following glutathione depletion by buthionine sulfoximine (Skapek et al, 1988a, b; Friedman et al, 1989; 1992a). Another sensitizer, metoclopramide enhanced the toxicity of BCNU against brain tumours in an in vivo rat model (Salford et al, 1992).
Other studies have investigated the efficacy of radio-iodinated anti-tenascin monoclonal antibody in both subcutaneous and intracranial glioma xenografts (Lee et al, 1988) which subsequently underwent clinical trials (Zalutsky et al, 1989) as well as the efficacy of immunotoxins targeted to the transferrin receptor (Martell et al, 1993).

More recently, using the 9L gliosarcoma model, Culver et al (1992) performed transfections of glial proliferative cells by intratumoral injection of a retroviral recombinant vector containing the herpes simplex virus thymidine kinase gene designed to infect only cells in rapid division. The thymidine kinase-containing cells thus conferred sensitivity to ganciclovir on the infected cells. Eleven of 14 brain glioma-grafted animals exhibited a total regression of tumours when treated with ganciclovir, while this drug failed to control tumour growth in animals whose tumours were treated either with saline alone or with control vector producing fibroblasts.

**In vitro experiments**

Friedman et al (1988b) found the MBs cell lines, D283 and Daoy sensitive to active cyclophosphamide analogues. The ID$_{90}$'s of D283 and Daoy to 4-hydroperoxycyclophosphamide were 22.47mM and 29.90mM for 1 hour respectively. Tomlinson et al (1991) found that BCNU and an analogue of cyclophosphamide, mafosfamide (MFs), used in the treatment of MB clinically were active against early passage MB cell lines. However, other agents such as cisplatin and hydroxyurea, VCR and cytosine arabinoside were ineffective. Nine out of ten tumours screened were sensitive to MFs, 7/12 to BCNU, 12/13 to teniposide (VM-26) and 7/13 to etoposide (VP16-213). Sensitivity to BCNU in early passage cultures have been demonstrated in both gliomas and MBs (Kornblith et al, 1981; Rosenblum et al, 1983; Tomlinson et al, 1991).

Taxol is a relatively new antineoplastic drug and its efficacy in a number of human tumours has been established, particularly in breast. However, a recent report has documented the in vitro chemosensitivity (using the clonogenic assay) of 5 human glioblastoma cell lines and the 6C rat glioma to taxol, together with an affect of this drug on glioblastoma cell locomotion (Silbergeld et al, 1995). All 6 cell lines showed sensitivity to taxol with ID$_{50}$ values between 1nM and 250nM, and a fraction of cells were found to be resistant to taxol (Silbergeld et al, 1995).
Problems with chemosensitivity testing in vitro

Tumour heterogeneity and cell selection

In vitro chemosensitivity assays provide a rapid means by which drug sensitivity and the prediction of clinical response may be evaluated. However, there are associated with this technique a number of problems.

Studies on both cell lines and clones derived from human malignant gliomas have demonstrated genotypic and phenotypic heterogeneity of human gliomas within a single tumour and between tumours of the same histological group (Bigner, 1981; Shapiro & Shapiro, 1984). Such heterogeneity has also been observed with regard to in vitro chemosensitivity to drugs such as BCNU and cisplatin (Shapiro et al, 1981b; Yung et al, 1982).

Another theoretical problem as a consequence of growing tumour cells in culture are the differences in chemosensitivity between primary and secondary cultures, and changes in the DNA profiles of cells during long-term passaging and this has been reported in culture of human gliomas (Shapiro & Shapiro, 1985). Difficulties may therefore arise when interpreting results from different passage levels. One way to overcome this is to carry out assays on early passaged cells to ensure the cells are as near to the original tumour as possible. It has been observed that sensitivity of short-term cultures changes during passaging as the number of near-diploid BCNU resistant cells are replaced over time with more sensitive 3n± and 4n± cells (Shapiro et al, 1993), thus confirming the differences in the cytogenetic profiles of cells between different passage levels.

In addition, the correlation between in vivo response and in vitro sensitivity is higher for metastatic lesions than for primary tumours (Von Hoff et al, 1981; Sanfilippo et al, 1986) probably indicating that cell selection has taken place during the metastatic process. This may tend to limit the use of material from primary tumours when planning chemosensitivity for disseminated disease.

Methodological considerations

A major consideration when planning an in vitro clinical correlation with patients is the question of how many tumours can be successfully cultured and assayed in vitro. Not all tumour samples from patients will grow in vitro. Some groups of tumours, for example cancers of the ovary, uterus, kidney, brain and mesothelioma appear to grow well in clonogenic assays as well as monolayer cultures in vitro (Von Hoff, 1990). However, Von Hoff (1990), reviewed the results using the clonogenic assay in 14,000 tumours from patients with a range of neoplasms and found that it was possible to produce assay results in only about 30% of cases.
For simplicity, in chemosensitivity assays, drugs are prepared in solution in advance and stored diluted prior to usage (for reviews Bosanquet, 1985, 1986, 1989). Recently, the biological activity of the most commonly used drugs were investigated (Hunter et al, 1994). Remarkably, most drugs, including 4-HC, CDDP, carboplatin, ADM, epirubicin, MMC, mitoxantrone, 5-FU, methotrexate, VCR and etoposide were found to be stable when aliquoted and frozen at -20°C and few drugs showed any significant decline in activity over periods of up to a year. These results are important for in vitro chemosensitivity testing, given the expense of making up drugs freshly at low concentrations or on rare occasions for just a few wells of a microplate. Use of frozen stock therefore adds much to the practicability of these assays (Hunter et al, 1994).
Chapter 5

Materials and methods

Materials

Source of material
A total of 103 brain tumour samples were collected from The National Hospital for Neurology and Neurosurgery, Queen Square; The Hospital for Sick Children, Great Ormond Street and from The Queen's Medical Centre, Nottingham. Samples received that were contaminated on arrival or within one week of culturing have not been included (21/103, 20%).

Initially, diagnoses were taken from routine neuropathology reports prepared at the time of diagnosis and recorded in the patients' hospital notes. However, as samples used in this study were collected over a number of years, from a number of different neurosurgical units and screened by a number of different consultant and trainee neuropathologists, it was impossible to ensure that there was consistency in the criteria used in preparing the initial reports. Similarly, refinements in the pathological classification of these tumours had occurred over the period of this study, culminating in the revised WHO report (Kleihues et al, 1993). Therefore, consultant neuropathologists at Great Ormond Street Hospital (Dr B. Harding), the National Hospital for Neurology and Neurosurgery (Dr T. Revesz) and the Queen's University Medical Centre, Nottingham (Professor J. Lowe) were asked to review the slides of the cases included in this study to ensure diagnostic consistency and to reclassify the tumours in relation to the most recent WHO classification. All diagnoses presented in this thesis are those provided following this review procedure. The full diagnosis of the tumour samples received is summarised in Appendix I.

Low grade astrocytomas were divided into pilocytic and fibrillary astrocytomas and grades III and IV were designated as "high grade" astrocytomas. Medulloblastomas located in the posterior fossa were termed MBs and those located elsewhere as PNETs. Tumours received comprised of 26 low grade astrocytomas (16 pilocytic and 10 fibrillary), high grade astrocytomas (8), ependymomas (7, one of which is an ependymoblastoma; a PNET with ependymal differentiation), medulloblastomas (21) and PNETs (3). In addition, there were 8 miscellaneous tumours which comprised of choroid plexus tumours (3), dysembryoplastic tumours (3), germinomas (2) and one each of a craniopharyngioma, ganglioglioma, malignant fibrous histiocytoma, meningioma, pineoblastoma and primitive neuroepithelial tumour.
Some of the tumour samples received were from patients with either a history of CNS neoplasms or a genetic disorder. One fibrillary astrocytoma (IN2003) was from a patient with a family history of retinoblastoma, another (IN1837) from a patient with neurofibromatosis, and a third patient (IN2012) had previously an optic nerve glioma. One high grade astrocytoma sample was from a patient with Turner's syndrome and another patient with high grade astrocytoma had previously a PNET but no other indication of underlying genetic disease. In addition one MB sample was from a recurrent tumour, as was one from an ependymoma. Table 4 summarises the types and location of the tumour received.

Two established cell lines were also used in the present study for the chemosensitivity comparison between these cell lines and the short-term cell lines derived from the paediatric brain tumours and included, Daoy and TE671. Daoy was derived from a child with medulloblastoma located in the cerebellum, and the biopsy was disaggregated mechanically without enzymatic digestion (Jacobsen et al, 1985). TE671 was one of the earliest described medulloblastoma cell lines (Mc Allister et al, 1977), but has subsequently been shown to be identical to the RD cell line, originally derived from a rhabdomyosarcoma (Stratton et al, 1989b). A number of experiments had already been performed prior to the knowledge that TE671 was a rhabdomyosarcoma cell line and not in fact a medulloblastoma cell line, which is why these results are included in the present thesis.

Table 4: Summary of tumour types received

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<tr>
<th>Tumour type</th>
<th>Number</th>
<th>Location</th>
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<tbody>
<tr>
<td></td>
<td>Primary</td>
<td>Recurrent</td>
</tr>
<tr>
<td>Pilocytic astrocytoma</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>Fibrillary astrocytoma</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>High grade astrocytoma</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>PNET</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Ependymoma*</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Choroid plexus tumour</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Dysembryoplastic tumour</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Germomas</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Craniopharyngioma</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ganglioglioma</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Malignant fibrous histiocytoma</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Meningioma</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pineoblastoma</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Primitive neuroepithelial tumour</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>2</td>
</tr>
</tbody>
</table>

*includes an ependymoblastoma
Of the tumour samples received, the majority of the MBs, low grade pilocytic astrocytomas and ependymomas were infratentorially located and fibrillar astrocytomas, high grade astrocytomas and neuroblastomas were supratentorial (Fig. 1). The sex distribution of tumours was different; a greater proportion of medulloblastomas, ependymomas and neuroblastomas were received from male patients whilst a higher proportion of astrocytomas received were from female patients (Fig. 2). However, fibrillary astrocytomas were more likely to be from a female patients.

The age distribution of the various tumour types is summarised in Figs 3 a-e. Medulloblastomas and PNETs (Fig 3a) display a normal distribution, peaking between 6 and 8 years of age. In ependymomas (Fig 3b) and high grade astrocytomas (Fig 3e) there did not appear to be any relationship with age, although, this is likely to be because of the small number of tumours received. The majority of ependymoma samples were received from patients in the 0-2 and 9-11 age groups. The pilocytic astrocytomas appeared to occur most commonly in younger children with the incidence between 3 and 5 years of age (Fig 3c). In contrast, the peak age for the low grade fibrillary astrocytomas, was 6-8 years with the distribution spread over a wider range (Fig 3d). As with the ependymomas, the high grade astrocytomas, age did not seem to fall into any particular pattern.
Fig 1: Site of tumours

Fig 2: Sex distribution of tumour samples
Media and reagents for cell culture

Agar
Agar (either Noble or Bacto) was freshly prepared as required as a 10% w/v solution in sterile tissue-culture grade water.
Supplier: Difco Laboratories, East Molesey, Surrey.

Amphotericin B
A sterile solution (250μg/ml) stored frozen at -20°C in 1ml aliquots.
Supplier: ICN Biomedicals Ltd., Thame, Oxfordshire
Code: 16-723-46

bisBenzimide (Hoechst dye, No.33258)
(2'-[4-hydroxyphenyl]-5-[4-methyl-1 piperazinyl]-2,5'-bi-1H-benzimidazole)
Supplier: Sigma Chemical Company Ltd., Poole, Dorset
Code: B1155

Collagenase
Grade 1A collagenase was dissolved in HBSS to give a concentration of 2000 units/ml, sterilised by filtration and stored frozen at -20°C in 1ml aliquots.
Supplier: Sigma Chemical Company Ltd.
Code: C2674

Dimethylsulphoxide (DMSO)
Grade I, used as a cryoprotectant when freezing down cells and for the solubilization of formazan crystals in the MTT assay.
Supplier: Sigma Chemical Company Ltd.
Code: D5879

Glycerol
A mounting mixture of glycerol/PBS, for use in mycoplasma screening.
Supplier: Sigma Chemical Company Ltd.
Code: G2025

Ham's F-10 medium
Nutrient mixture, with L-glutamine and 25mM HEPES supplied as a X1 concentrate liquid and stored at +4°C.
Supplier: Gibco BRL, Paisley, Scotland
Code: 041-02390M

Hanks' balanced salt solution (HBSS)
A sterile X1 concentrate of modified Hanks' balanced salt solution, with phenol red.
Supplier: Gibco BRL
**Kanamycin**
A sterile solution (5000μg/ml) and stored frozen at -20°C.
Supplier: ICN Biomedicals Ltd.
Code: 16-720-48

**Phosphate buffered saline (PBS)**
Prepared from phosphate buffered saline tablets (modified Dulbecco’s formula, without calcium and magnesium). Each tablet was dissolved in 100mls of de-ionised water and filtered if sterility was required. Stored at +4°C if not needed immediately.
Supplier: Sigma
Code: P4417

**Penicillin and streptomycin**
A mixed, sterile solution of penicillin (5000 iu/ml) and streptomycin (5000μg/ml) and stored frozen at -20°C until use.
Supplier: Gibco BRL
Code: 043-05070H

**Serum**
Foetal calf serum (FCS) supplied sterile and free of mycoplasma and bovine adventitious agents. All batches used were tested for their ability to support the growth of both established glioma cell lines and short-term cell lines in monolayer culture.
Supplier: Gibco BRL
Code: 01106290M

**Silicone grease**
Aliquots were transferred into 5ml glass screw-capped bottles and autoclaved at 121°C before use.
Supplier: ICI Ltd., Organic Division, Ayrshire

**Trypsin solution**
A 0.25% w/v solution of trypsin prepared as a 1X concentrate liquid, in Gibco solution A (i.e., 0.4g/L KCl, 2.2g/L NaHCO₃, 6.8g/L NaCl, 1.0g/L Glucose, 0.005g/L Phenol red). Aliquoted in 10-20ml lots into plastic universal containers and stored frozen at -20°C.
Supplier: Gibco BRL
Code: 043-05050M
Media and balanced salt solution formulations

**Biopsy collection medium (CM)**
Ham's F-10 medium, supplemented with penicillin, 100 iu/ml, streptomycin, 100μg/ml, kanamycin, 100μg/ml and amphotericin B, 20μg/ml.

**Growth medium (GM)**
Ham's F-10 medium with 50 iu/ml penicillin and 50μg/ml streptomycin, buffered with 25mM HEPES and supplemented with 10% v/v selected FCS.

**Plastic- and glass- ware for cell culture**
Nunclon™ (Gibco) or Falcon tissue culture flasks were used throughout. Falcon tissue-culture-treated petri-dishes (50mm) were used for the dissection of surgical biopsies while plastic 30ml universal containers were employed for washing biopsies and tissue fragments. For chemosensitivity assays, rigid 96-well polystyrene, flat-bottom-well microtitration plates (Nunclon™, Gibco BRL). Preparation of drug dilutions was carried out in 60ml sterile pots. For coverslip cultures, 24-well plates and for initiation of cultures on different coated-surfaces, 12-well plates were used (Falcon). All multiple well plates were sealed with clear, Titertek, non-toxic (Mylar) plate sealers (ICN Biomedicals Ltd.). For immunocytochemistry, 8-slide sterile Permanex plastic slide chambers were used (Nunc, Gibco BRL), or glass coverslips (13mm) which were flamed for sterility.

Routine manipulation of liquids for cell culture and drug experiments were carried out with 1, 5 and 10 ml plastic pipettes (Falcon). Manipulation of small volumes (10-200μl) of liquid were carried out with Finnpipettes with sterile plastic tips (Alpha Labs., Eastleigh, Hants). For chemosensitivity experiments a Finnpipette Stepette (Labsystems) was employed for repetitive dispensing of reagents. Cells were frozen in polypropylene self-standing screw-capped cryotubes, 1.2ml capacity (Gibco BRL). Glass coverslips 9 x 22mm or 18 x 18mm, no.1 thickness were supplied by Marathon Laboratory Supplies, London. Glass flasks were cleaned, dried and autoclaved at 121°C.

All Falcon products were supplied by Marathon Laboratory Supplies, London. All universals and various sized pots (60-200ml) were supplied by Central Laboratory Supplies, Basingstoke, Hampshire.

**Materials and reagents for culture of cells on different coated surfaces**

*Cell-tak®*
A sterile 1mg/ml solution in 5% acetic acid and stored at +4°C.
Supplier: Stratech Scientific Ltd., Luton, Beds.
Code: 40240
Dispase
Supplied frozen in HBSS pH 7.4. To recover cells from Matrigel; 50 caseinolytic units/ml was used. Aliquoted as 1ml aliquots in cryotubes and stored frozen at -20°C.
Supplier: Stratech Scientific Ltd.
Code: 40235

Fibronectin (from human plasma)
Each mg of fibronectin (FN) had been lyophilised from 1.0ml NaCl, 0.05M Tris/HCl, pH 7.5. The fibronectin was reconstituted as directed according to manufacturers instructions; to each mg of the protein, 1ml of sterile water was carefully added and allowed to stand for 30 minutes to dissolve (without agitation). So that repeated freezing and thawing could be avoided, a working solution was prepared by further diluting the stock solution in sterile culture medium, aliquoted and stored at -20°C.
Supplier: Sigma Chemical Company Ltd.
Code: F2006

Laminin
Laminin (LMN), was received as a sterile frozen solution in a vial containing 1mg in a [0.05M] tris-buffered [0.15M] sodium chloride solution. The source of the laminin was extracted from the basement membrane of Engelbreth-Holm-Swarm (EHS) mouse sarcoma. To prevent refreezing and thawing, laminin was thawed slowly overnight at +4°C (to prevent formation of a gel) and later diluted with serum free media and transferred into 30ml universal containers and then stored frozen at -70°C.
Supplier: Sigma Chemical Company Ltd.
Code: L2020

Matrigel
Matrigel is a solubilized basement membrane preparation extracted from EHS mouse sarcoma and supplied as a 10ml solution in Dulbecco's modified Eagle's medium with 10µg/ml gentamycin. Liquid Matrigel rapidly formed a gel at 22°C- 35°C and so had to be thawed overnight at 4°C in the refrigerator. To avoid repeated freezing and thawing, it was aliquoted and stored at -20°C. It was imperative that all procedures associated with Matrigel were carried out on ice at all times so as to prevent formation of gel.
Supplier: Stratech Scientific Ltd.
Code: 40234

Poly-L-lysine (PLL)
Supplied as a non-sterile solution, Mr 150,000-300,000. A stock solution of 4mg/ml was prepared with deionised water and 150µl aliquots were made in cryotubes and stored at -20°C.
Supplier: Sigma Chemical Company Ltd.
Code: P1399
**Vitrogen 100 collagen**

A sterile solution of purified, pepsin-solubilized bovine dermal collagen dissolved in 0.012N HCl. The collagen concentration of vitrogen was approximately 3mg/ml and was stored at +4°C.

Supplier: Imperial Laboratories Ltd., Andover, Hants.

**Materials and reagents for chemosensitivity**

**MTT**

(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazoyl blue), was prepared as a solution of 1mg/ml, in Ham’s F-10 as and when required and filter sterilised with a 0.22µm filter (Millipore).

Supplier: Sigma Chemical Company Ltd.

Code: M2128

**Drugs**

Three drugs were used to screen a number of short-term cultures derived from childhood brain tumours. These included CCNU (lomustine); procarbazine, (Natulan, PCB) and vincristine (“Oncovin”, VCR). The range and dilution of drugs is summarised in Table 5. No drug was used after its expiration date.

**Table 5: Range of drug concentrations**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Stock solution</th>
<th>Initial dilution</th>
<th>Subsequent dilution</th>
<th>96-well plate concentration range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNU</td>
<td>4mg/ml</td>
<td>1/3</td>
<td>1/2</td>
<td>0.08 - 30 µg/ml</td>
</tr>
<tr>
<td>PCB</td>
<td>10mg/ml</td>
<td>1/2</td>
<td>1/2</td>
<td>0.3125 - 10 mg/ml</td>
</tr>
<tr>
<td>VCR</td>
<td>100µg/ml</td>
<td>1/10</td>
<td>1/4</td>
<td>9.8 x 10^{-5} - 0.1 µg/ml</td>
</tr>
</tbody>
</table>

**PCB**

The contents of capsules (50mg procarbazine), as PCB hydrochloride (USP) were dissolved in 10mls of Ham’s F-10. Solutions made from capsules were centrifuged at 3000 rpm to remove insoluble material in the pharmaceutical preparation. It was necessary to adjust the pH of the drug solution to pH 7.2-7.4 by the addition of 250-500µl of sterile 1M sodium hydroxide solution (BDH). The drug solution was then sterilised by passage through a 0.22µm filter.

Supplier: Roche Products Ltd., Welwyn Garden City, Herts.
CCNU
The contents of one 40mg capsule were dissolved in 10mls of absolute ethanol and centrifuged to remove insoluble material from the pharmaceutical preparation. The resulting stock solution (4mg/ml) was transferred into a 30ml universal and stored at -70°C.
Supplier: Lundbeck Ltd., Milton Keynes, Bucks.

VCR
Sealed vials (containing vincristine sulphate) were stored at +4°C. For use, 1ml of Ham's F-10 was introduced into the vial via a sterile hypodermic needle and syringe. The stock solution was stored frozen at -70°C.
Supplier: Eli Lilly Company Ltd., Basingstoke.
For each set of experiments, drug solutions were made up freshly from the stock solution.

Materials and reagents for immunocytochemistry

Antibodies
Intermediate filament primary antibodies were received on dry ice and upon thawing were aliquoted into 100µl Eppendorf tubes and stored at -70°C. Secondary and tertiary antibodies were stored at +4°C.
Supplier: Amersham International plc., Bucks.
Polyclonal primary antibodies were stored at +4°C. Both mouse and rabbit sera were stored at +4°C.
Supplier: Dako Ltd., High Wycombe, Bucks.

Citifluor
To prevent loss of fluorescence intensity on visualisation, an antifadent mountant was used, which was prepared in a glycerol/PBS solution.
Supplier: Agar Scientific Ltd., Stansted, Essex.
Code: R1320 (AF1)

Propidium Iodide
A stock solution of propidium iodide (40µg/ml) was made up in HBSS without phenol red and stored in the refrigerator, with the pot surrounded by tin foil to protect from the light.
Supplier: Sigma Chemical Company Ltd./ Code: P4170

Methods

Primary cultures
Preparation of disaggregated surgical specimens
Biopsy samples taken at surgery were immediately placed in collection medium (CM) and transported to the laboratory. In most cases, the samples were received within 1-4 hours of
removal. The biopsy samples were transferred to a plastic petri dish, together with some fresh biopsy collection medium and any non-tumour material was removed by dissection. The tissue fragments were then weighed and chopped into 1-2mm pieces using crossed scalpels. The blades were changed between biopsies to ensure that sharpness was maintained and the biopsy was cleanly cut rather than mangled. The fragments were then transferred to a 30ml universal container and resuspended in complete growth medium (GM) (Ham's F-10, buffered with 25mM HEPES and supplemented with 10% v/v selected foetal calf serum and 100 units/ml penicillin and 100mg/ml streptomycin). A volume of 1ml crude collagenase solution was added to 2ml of complete GM, to give a final working concentration of = 666 units/ml.

The tissue was incubated for between 1-4 hours at 37°C. When the tissue was reduced to small aggregates rather than a single cell suspension, a further 7mls of complete GM was added and the preparation agitated several times. This tissue suspension was then centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and cells were resuspended in fresh complete GM and plated into a small (25cm²) flask. The flask was incubated at 37°C overnight, after which the medium and the non-adherent material were removed. The culture was re-fed with fresh medium and examined twice a week and re-fed as required.

Preparation of explant culture for small surgical biopsies
This technique was developed for small biopsy samples taken during stereotactic neurosurgical operations (Thomas et al, 1984). This procedure was also carried out if the tumour specimen was less than 5mm in size. The biopsy samples were collected as mentioned previously, weighed and washed with GM. Using a sterile 1ml pipette, two small spots of silicone grease (Edwards high vacuum grease) were placed approximately 1cm apart on the base of a 25cm² plastic cell culture flask. Using sterile forceps, the fragment was placed between the spots of silicone grease in a drop of biopsy collection medium. A sterile glass coverslip (9 x 22mm, No.1 thickness, Marathon Laboratory Supplies, London) was placed over the biopsy and spots of silicone grease. The coverslip was pressed down using the sterile forceps until the biopsy sample was held securely down against the plastic surface and the coverslip was firmly anchored by the silicone grease in such a way that the biopsy was half under the coverslip and half out of it, so that the exposed half of the tumour biopsy was in direct contact with the medium. Five ml of complete GM was added carefully down the side of the flask, taking care not to dislodge the coverslip or explant. The flask was then incubated at 37°C. When significant cell growth from the biopsy was evident, the coverslip and the remains of the biopsy were removed with sterile forceps and the culture refed with fresh complete GM.

Preparation of tumours for growth as floating aggregate cultures

Preparation of plasticware
In order to prevent adherence of cells to the base of the tissue culture treated flasks, Noble or Bacto agar solutions (10% w/v) were made when required and diluted in prewarmed GM to
give a 0.5% w/v solution. This solution was immediately used to coat the base of 25cm² flasks (5ml/flask). The flasks were allowed to stand for about 30 minutes so that the agar solidified.

Maintenance of floating aggregate cultures

Tumour biopsies were collected and disaggregated as described previously. Generally only samples that were thought to be medulloblastomas were additionally prepared in this way. If sufficient material was received, 5mls of cell suspension (containing approximately $5 \times 10^5$ cells) in GM was introduced to a 25cm² agar-based flask. After 2-3 days in culture, the GM and floating aggregates were removed, using a 10ml pipette, and transferred into a new agar-coated flask. This was a necessary procedure since cells were still able to migrate under the agar to the base of the flask where cells would grow as a monolayer, even if detachment of the agar did not occur. On re-feeding, the spent GM and the floating aggregates were transferred to a 30ml universal container and were then allowed to settle under gravity. The spent medium was carefully removed (care being taken not to lose any floating aggregates) and new medium introduced, after which the entire contents were transferred into a new flask.

Re-feeding

Cultures were re-fed on the first day after plating following enzymatic disaggregation, to remove any dead cells, residual enzymes or red blood cells. Refeeding was performed every 2-3 days, or when the pH changed from neutral to acidic. Slow growing cultures were fed at either weekly or fortnightly intervals. The process of re-feeding involved removing the old medium and replacing it with an equal volume of fresh complete GM. Generally, small (25cm²) and medium (75cm²) flasks required 5ml and 10-20ml of medium respectively.

Passaging cultures (subculturing)

Generally, cultures were passaged once they reached confluency. For primary cultures, this varied from between 2-5 weeks. Explant cultures, however, did not reach confluency as such and would rather grow as islands of heaped, non-contact inhibited cells and thus were passaged prior to entering the plateau phase of growth.

The depleted medium was aspirated and the cells were rinsed twice with 10mls of HBSS. The monolayer was then rinsed with 1ml of trypsin solution (0.25% trypsin, Gibco BRL). A further aliquot of 3ml trypsin was added to the flask, which was then incubated for between 10-15 minutes, or until such time that the cells detached from the plastic. In some instances, scraping of cells with a rubber cell scraper was needed where a sub-population of cells had to be removed by mechanical means since they were still strongly attached to the base of the flask. The cell scraper was introduced into the flask and the adherent cells gently scraped off the base of the flask. This enabled the harvesting of the majority of cells present in the flask. Seven ml of complete GM was added to the flask and the cell suspension transferred to a sterile universal container and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and the
cells resuspended in 10ml of fresh complete GM. The cells were counted and either prepared for cell freezing or further passage. The total contents of the primary cultures were passaged from a 25cm² flask into a 75cm² flask (i.e., 1:3 split).

**Cell counting using a ZM Coulter counter**

When the cells were resuspended with fresh complete GM, following centrifugation, 0.4ml of the cell suspension was pipetted into a pot containing 19.6ml Isoton II. This was counted using a ZM Coulter Counter, giving a reading, which was then multiplied by 1000, to give the actual total cell count in the 10ml suspension.

**Cell freezing**

The procedure was the same as that for passaging cells, to the point where a cell count was carried out, after which cells were recentrifuged to remove the supernatant. The cell pellet was then gently resuspended in a 10% solution of DMSO in complete GM, at a density of $1 \times 10^6$ cells/ml. The DMSO acted as a cryoprotectant. One millilitre aliquots containing $1 \times 10^6$ cells were transferred into a freezing vial (cryotubes, Nunc). The vial was sealed and placed either in a polystyrene box which was placed in a -70°C freezer for a minimum of 8 hours before being transferred to the liquid phase (-196°C) of liquid nitrogen freezer, or in a programmable controlled rate freezer (Planer Biomedical Ltd) set to produce a cooling rate of 1°C a minute, for optimum preservation. Cultures were frozen down routinely between passage level 2 and 3.

To revive the cells from the liquid nitrogen bank, the vial was removed from the liquid phase and quickly transferred to a covered plastic container with water at 37°C (a precaution to avoid an explosion caused by any liquid nitrogen trapped within an incompletely sealed vial). Once thawed, the contents were removed from the vial and then slowly diluted into 10mls of complete GM in a 75cm² flask and incubated overnight. The following day the cells were re-fed.

**Population doubling times and saturation cell densities**

The population doubling time (PDT) is defined as the time taken for the cell population to increase 2-fold during exponential growth. Population doubling times were calculated for a small number of short-term cultures from childhood brain tumours. Cells were seeded into thirty 25cm² culture flasks at an initial density of $5 \times 10^5$ cells/ml in 5mls complete GM per flask. The flasks were incubated at 37°C under standard conditions. At each time point, two flasks were removed from the incubator, the monolayers washed with HBSS and the cells detached with trypsin, centrifuged and resuspended in HBSS. The cells were counted using a ZM Coulter Counter and the number of cells/cm² was calculated. PDTs were calculated from
Fig 4: Derivation of doubling time and saturation density from growth curves

Saturation density (cells/cm² growth substrate)

Doubling time (hours)

Days post plating

Cells/cm² growth substrate
the exponential portion of the growth curve for each cell line as illustrated in Fig. 4. The time in hours taken for the population of cells in logarithmic phase to double was extrapolated from the graph. It should be noted that in some instances, the exponential phase of growth of some short-term cell lines was comprised of only two or three points on the curves, whilst in others >3 points were available. The saturation densities were determined by extrapolation of the horizontal portion of the growth curve (plateau phase) to the y-axis as illustrated in Fig. 4.

**Mycoplasma screening using DNA staining**

All cultures were routinely screened for mycoplasma infection. This was carried out in a 24-well microtitration plate. A sterile 13mm diameter coverslip was placed into each well and approximately 0.5ml of poly-L-lysine (PLL) solution (13.1mg/ml in deionised water) was added. This was left for 45 minutes, after which the PLL solution was removed from the wells and the plate was allowed to dry for a few minutes. About 50 μl of cell suspension was placed into each of two wells per cell line using a sterile Finn tip. The cultures were then fed with 0.5ml complete GM and sealed with a Mylar sheet.

When the culture reached 60% confluency (after 3-4 days incubation), the coverslip was removed and placed with the cells uppermost on a holding tray. They were rinsed (3x) with HBSS without phenol red (Gibco BRL), fixed with 100% methanol at -20°C for 5-10 minutes in a freezer and then washed with HBSS (3x). The cells were incubated with a Hoechst stain (Hoechst 33258, bisbenzimide, 0.25mg/ml in HBSS without phenol red) for 30 minutes at room temperature. Three more washes followed with HBSS and twice with deionised water. The coverslip was then mounted in a glycerol/PBS solution with the cells facing downwards, blotted with tissue paper and sealed with clear nail varnish.

The stained preparations were examined using either a 40x or 100x oil immersion objective for evidence of extranuclear staining, under a Zeiss microscope. A reflected light fluorescence was incorporated, fitted with a LP 440nm barrier and a 330/380 nm exciter filter.

**Covering cell culture surfaces with components of the ECM**

The effect of ECM components was tested on a number of biopsies, to try to enhance the initial attachment of childhood primary brain tumours *in vitro*. The biopsies were disaggregated as mentioned in the preparation of primary cultures section but following collagenase digestion the tumour suspension was distributed into microtitre wells coated with either Cell-tak (polyphenolic attachment proteins from marine mussel *Mytilus edulis*), FN, LMN, Matrigel (synthetic basement membrane), PLL and vitrogen (bovine dermal collagen). One well (untreated) served as a control. Cells were additionally cultured on glass. When the wells became confluent, the cells were trypsinised and plated onto 25cm² flasks, also coated with the relevant attachment factor.
Preparation of Cell-tak-coated culture dishes

A sterile buffer solution of 0.1M sodium bicarbonate, pH 8.0, was prepared prior to coating. The appropriate amount of Cell-tak required for a density of 3.5mg/cm² was calculated. The stock solution was then diluted 10:1 with sterile tissue culture grade water. Only the amount required for that day was prepared. A sufficient volume of buffer was dispensed to cover the bottom of the dish. The diluted Cell-tak was then dispensed onto the dish containing the buffer, the solution pipetted up and down twice and the well swirled immediately to disperse the Cell-tak. The pH of the buffer dropped to between 7.3 - 7.8. The 12-well microtitration plate was set to one side. Similarly, Cell-tak was added to further wells. The wells were allowed to adsorb for 20 minutes at room temperature, after which the Cell-tak/buffer solution was aspirated and the wells washed twice with sterile water. The wells were then ready for immediate use or the culture dish could be stored at 4°C, after having been air-dried.

Preparation of fibronectin-coated culture dishes

For cell attachment, a 3μg/cm² density was required. The frozen stock was diluted in sterile culture medium to the appropriate concentration and the culture surface was coated with a minimal volume (e.g., 1ml/25cm² and 2ml/75cm²). The culture dish was rocked gently to ensure even coating of the culture surface and allowed to air dry in a laminar flow hood for at least 45 minutes at room temperature. Excess fibronectin could have been aspirated, although this was not necessary.

Preparation of laminin-coated culture dishes

For cell attachment a density of 2μg/cm² was used. After having prepared the appropriate dilution for the culture dish, the surface of which was coated with a minimum volume (as for fibronectin). The solution was again gently rocked to ensure even distribution throughout the surface of the flask or well. Before introducing cells and medium the culture dish was allowed to air dry for at least 45 minutes.

Preparation of Matrigel-coated culture dishes

The thawed vial was swirled to ensure even dispersion of Matrigel, taking care to prevent warming of the solution and an appropriate amount was pipetted using a pre-cooled pipette into a culture dish (well or small flask) which had also been pre-cooled in the refrigerator. The amount of Matrigel pipetted into the culture dish was sufficient to produce a thin film on top of which cells were introduced following gelation. For each well of a 12-well microtitration plate, 0.25ml was added and 1.0ml for 25cm² flask.

Preparation of poly-L-lysine-coated dishes

A further dilution of 1 in 300 of the stock solution prior to use was made to give a final concentration of 13.3μg/ml. Aseptically the culture surface was coated with 0.5ml/25cm² of solution. Again the culture dish was rocked gently to ensure even distribution of the PLL. After about 5 minutes the solution was aspirated and the surface thoroughly rinsed with sterile
water. Before introducing cells and medium, the culture surface was allowed to dry for between 1-2 hours.

Preparation of vitrogen 100 collagen films
A sufficient amount of volume to cover the base of the culture dish was pipetted into the well (0.5ml) or 25cm² flask (1ml). The culture dish was left uncovered in a laminar flow hood for the entire day or overnight to allow for normal evaporation. The dish was then rinsed with media to remove any residual acid and to rehydrate the collagen prior to use. The collagen coatings prepared in this manner were nonfibrillar in nature.

Determining the maximum passage level
The maximum passage level attainable was determined for a number of cultures derived from different types of childhood brain tumours when grown on plastic-, FN- and LMN-coated flasks. Routine culturing was carried out as described in the previous section but on passaging, the culture was split (1:3), with one third used for further culturing and the remaining two-thirds were frozen down. This procedure continued until the culture ceased proliferating.

Population doubling times
The population doubling times (PDTs) of cultures grown on the three surfaces were also determined. It was decided that the cells would be grown on 96-well microtitration plates. Again, the wells were plated with the appropriate attachment factor (i.e., FN or LMN). Cells were seeded at an initial density of 2 x 10³ cells in 0.1ml complete GM per well. The 96-well microtitration plates were incubated at 37°C under routine conditions and cell counts were performed at regular intervals. Briefly at each time point medium was aspirated from 6 wells and the cells washed twice with 0.1ml HBSS and once with 0.1ml trypsin. The cells were then incubated with 0.1ml fresh trypsin solution, resuspended in 20mls of Isoton II (Coulter) isotonic saline and counted in a precalibrated Model ZM Coulter counter fitted with a 100μm orifice tube. From these recorded cell counts, the growth curves were plotted using a computer software programme, from which a graph could be charted on a logarithmic scale.

Immunocytochemical analysis of cultures in vitro
Antigenic profiles of primary cultures of childhood brain tumours
Specific antibodies were used as markers for distinguishing different cell types using immunocytochemical techniques. Early passage cell lines were used to screen the antigenic expression by cells derived from childhood brain tumours. Additionally, the antigenic profiles of some cultures grown on different attachment factors were also screened.

These studies concentrated on intermediate filaments, including GFAP, vimentin, neurofilament, cytokeratin and fibronectin. Synaptophysin as an additional marker was used to
screen the medulloblastoma cultures. A small number of cultures were screened for desmin. The species of origin, working dilution and supplier are summarised in Table 6.

Table 6: Type and working dilution of antibodies

<table>
<thead>
<tr>
<th>Antibody Class</th>
<th>Antibody</th>
<th>Source</th>
<th>Working Dilution</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>Primary</td>
<td>GFAP</td>
<td>mouse (mono)</td>
<td>1:10</td>
<td>Amersham RPN 1106</td>
</tr>
<tr>
<td></td>
<td>NF (160kD)</td>
<td>mouse (mono)</td>
<td>1:10</td>
<td>Amersham RPN 1104</td>
</tr>
<tr>
<td></td>
<td>VIM</td>
<td>mouse (mono)</td>
<td>1:10</td>
<td>Amersham RPN 1102</td>
</tr>
<tr>
<td></td>
<td>CYT</td>
<td>mouse (mono)</td>
<td>1:10</td>
<td>Amersham RPN 1100</td>
</tr>
<tr>
<td></td>
<td>DES</td>
<td>mouse (mono)</td>
<td>1:10</td>
<td>Amersham RPN 1101</td>
</tr>
<tr>
<td></td>
<td>FN</td>
<td>rabbit (poly)</td>
<td>1:200</td>
<td>Dako A245</td>
</tr>
<tr>
<td></td>
<td>SYN</td>
<td>rabbit (poly)</td>
<td>1:25</td>
<td>Dako A010</td>
</tr>
<tr>
<td>Secondary</td>
<td>Anti-mouse Ig</td>
<td>sheep (mono)</td>
<td>1:50</td>
<td>Amersham RPN 1001</td>
</tr>
<tr>
<td></td>
<td>Anti-rabbit Ig</td>
<td>donkey (poly)</td>
<td>1:50</td>
<td>Amersham RPN 1004</td>
</tr>
<tr>
<td>Staining</td>
<td>Fluorescein</td>
<td></td>
<td>1:50</td>
<td>Amersham RPN 1232</td>
</tr>
</tbody>
</table>

Preparation of early passage cultures for immunocytochemistry

Under aseptic conditions, cells were prepared for immunocytochemical staining, either on 13mm diameter coverslips (glass) or on multi-chamber slides (Nunc). Coverslips were plated with 5 x 10^4 cells in 0.5ml medium and the 8-slide chambers were seeded with 1 x 10^5 cells in a total volume of 4ml. The cells were incubated at 37°C for 2-4 days, until 60-85% confluency was reached. This was important because, firstly, the cells would have reached a stage whereby the morphology seen would be that of cells during the exponential phase of growth, secondly, the cells would not be too confluent to interfere with interpretation of the results and, thirdly, piling up of cells would have been kept to a minimum. In addition, the passage level at which these cultures was screened was not beyond level five in order to be as close to the original tumour specimen as was possible.
Preparation of ECM passaged cultures for immunocytochemistry

The same procedure was carried out as that for cultures grown on different attachment factors. However, since earlier attempts showed that coating the 8-slide chambers with the appropriate attachment factor seemed to interfere with the final results when viewed under the microscope, it was decided to initially grow the cultures on the relevant attachment factor and then on reaching exponential growth to seed the cells and then plate them onto the multi-chamber slides without the attachment factor. The cultures were then treated as described above.

The Biotin-Streptavidin system

The biotin-streptavidin technique was employed in detecting and screening the antibody-antigen immunocomplexes (Amersham). Based on the biotin-avidin immunoperoxidase method (Amersham), this technique has been enhanced, combining the specificity of highly purified antibody probes with high affinity binding of the small water soluble vitamin biotin to the bacterial protein streptavidin. Species-specific secondary antibodies against the primary immunoglobulin were covalently linked to biotin. A tertiary, non-immunological reagent composed of biotin-streptavidin complex covalently bound to a fluorochrome such as fluorescein isothiocyanate (FITC) was used. A much improved signal, identifying the primary antibody-antigen interaction, was therefore generated.

Immunofluorescence

Cell monolayers were washed with HBSS or PBS (3x) and then fixed with 1% HCl in 70% ethanol for 10 minutes. Following neutralisation with a further three washes with HBSS, the primary antibody was applied for 1-2 hours at room temperature. Slides were kept in a humidified environment to prevent evaporation. The cells were washed three times with either HBSS or PBS and a species-specific biotinylated anti-immunoglobulin was then added (1:50 dilution) for 30 minutes. The cells were washed again x3 with HBSS/PBS, and a fluorochrome biotin-streptavidin complex was finally (1:50 dilution) added for 15 minutes. A further three washes followed with HBSS or PBS. Fluorescence-labelled cells were additionally very briefly immersed in propidium iodide dissolved in HBSS (40µg/ml) between the final washes to give the nuclei a red counterstain. The cells were mounted in Citifluor aqueous mountant and examined using a Zeiss Axiostop routine microscope incorporating reflected light fluorescence with appropriate excitation and barrier filters. The cells were examined at either x40 or x100 magnification, under oil immersion.
Chemosensitivity assay

Principle
The principle of the assay will be briefly explained as follows: cells in exponential growth are treated with cytotoxic drugs. The length of exposure to drugs is the time taken for maximal damage to occur (but, this is also governed by the stability of the drugs). The drugs are then removed and the cells are allowed to recover (e.g., cells are allowed to grow for 2-3 doubling times, so that cells that remain viable and capable of proliferation are distinguished from those cells that are merely viable, but unable to proliferate). The surviving cell fraction is then determined indirectly by MTT dye reduction. The amount of MTT-formazan produced is then measured spectrophotometrically following solubilization of the crystals in a suitable solvent.

Preparation of cells and determination of PDT in vitro
Cell cultures in exponential phase of growth were trypsinised and diluted in complete GM to give a cell suspension with a total cell count of between 1-2 x 10^4 cells/ml. Using a Finnpipette Stepette (size 3 tip), 1-2 x 10^3 cells in 0.1ml cell suspension was added to each well of a 96-well microtitration plate with repeating dispenser. Plates were sealed with Titertek non-toxic sealer and incubated at 37°C for 72 hours.

For each cell line tested, a separate plate was prepared and daily cell counts carried out to determine the PDT of each cell line, in addition to ensuring that the cultures were in the exponential phase of growth during the time course of the assay. This was important because the drugs tested act against rapidly dividing cells and not quiescent cells. In addition, the vinca alkaloids (eg., VCR) and antimetabolites are most effective during a particular phase of the cell cycle. Even drugs which are not cycle specific act more effectively against dividing cells rather than slowly or quiescent cells. At daily intervals, these plates were re-fed with fresh GM and at each time point medium was aspirated from 6 wells and the cells washed twice with 0.1ml HBSS and once with 0.1ml trypsin. The cells were then incubated with 0.1ml fresh trypsin solution, resuspended in 20mls of Isoton II (Coulter) isotonic saline and counted in a precalibrated Model ZM Coulter counter fitted with a 100μm orifice tube. From these recorded cell counts, the growth curves were plotted using a computer software programme, from which a graph could be charted on a logarithmic scale. Growth was considered to be exponential if a plot of cell numbers against time was linear.

Drug treatment and recovery
Stock solutions of drugs were made according to the methods described above and were diluted in GM for use in drug assays. Following 72 hours incubation at 37°C, the GM was aspirated from each well and replaced with 0.1ml of the appropriate drug dilution. Two wells in each column were re-fed with GM, which acted as a control. Each plate was then resealed with a Titertek non toxic sheet and returned to the incubator. Drug solutions were renewed at
Fig 5: Derivation of the ID$_{50}$ from a dose-response curve

Proportion of cells surviving

Drug dose (µg/ml)

ID$_{50} = 4.5$ µg/ml
24 and 48 hours, resulting in cells having been exposed to drugs for a total of 72 hours.

Following drug exposure of 72 hours, the cells were washed twice with warmed HBSS (so as to remove residual drug) and fresh GM was added so that culturing could continue, in the form of a "recovery period". This medium was replaced at 24 hours. The cells were then left for a further 48 hours.

**Addition of MTT**

After a 72 hours recovery period, the GM was aspirated, and 0.1ml of MTT (1mg/ml) was added to each well. The MTT was made in Ham's F-10 without FCS and then filter sterilised, using a 0.22μm filter, to remove any spontaneously formed formazan. After a 4 hour incubation at 37°C, the Titertek non toxic sheet was removed and the spent MTT solution was carefully removed. Care was taken not to dislodge or aspirate any formed formazan crystals. To each well, 0.1ml of DMSO was then added to dissolve any formazan crystals, producing a violet solution. Each plate was then shaken for a 2-3 minutes on a plate shaker to ensure even dissolution of the formazan crystals.

**Determination of sensitivity from densitometer readings**

Absorbence readings were performed using a MR600 microplate reader at a wavelength of 570nm. The microplate reader was initially blanked against wells which contained medium, MTT but no cells. The printed values from the densitometer were manually transferred to a computer into a spread sheet programme which calculated the mean absorbance values for each drug concentration (done in quadruplicate) and then calculated each mean value as a percentage of the control values. A dose response curve was plotted and the ID$_{50}$ (dose of drug which inhibits MTT conversion to formazan product) was determined as illustrated in Fig. 5.
Chapter 6

Results

Cell culture of primary childhood brain tumours

Primary culture

A total of 82 childhood brain tumours were collected and prepared as primary cultures. In the majority of cases, the samples were prepared using collagenase digestion and the remainder where tissue fragments received were too small for digestion as explant cultures.

<table>
<thead>
<tr>
<th>Preparation method</th>
<th>Number of cultures</th>
<th>Growth success</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digestion</td>
<td>75/82 (91.5%)</td>
<td>74/75 (99%)</td>
</tr>
<tr>
<td>Coverslip (explant)</td>
<td>7/82 (8.5%)</td>
<td>5/7 (71%)</td>
</tr>
</tbody>
</table>

In total, 96% (79/82) of the samples grew successfully. Of the tumour types received all 21 medulloblastomas, 3 PNETs, 7 ependymomas (including an ependymoblastoma), 3 neuroblastomas, 3 choroid plexus tumours, 2 germinomas and one each of craniopharyngioma, gangliogioma, histiocytoma, meningioma and a primitive neuroepithelial tumour produced primary cultures. In addition, 25/26 low (16/16 pilocytic and 9/10 fibrillary) and 7/8 high grade astrocytomas and 2/3 dysembryoplastic tumours successfully produced a primary culture. The four cultures which resulted in no growth were derived from a fibrillary astrocytoma (IN1882), a dysembryoplastic neuroectodermal tumour (IN2089), a high grade astrocytoma (IN2041) and a pineoblastoma (IN1861) respectively. Two of these cultures (IN1882 and IN1861) were small samples from stereotactic burr-hole biopsies. Even though the other tumour samples received were from larger resections, these cultures also could have contained necrotic or non-tumour material. From the two preparative techniques, 99% of samples prepared using digestion and 71% of those samples prepared as explant cultures were successful in producing a primary culture.

Nine MBs & PNETs (9/24, 37.5%), three pilocytic astrocytomas (3/16, 19%), two fibrillary astrocytomas (2/9, 22%) and one ependymoma (14%) culture produced large quantities of floating aggregates on initial plating. These aggregates were separated from adherent cells and placed into agar-coated flasks. Unfortunately, none of these floating aggregate cultures survived long-term passaging. The aggregates either clumped together or attached (despite the agar) to the surface of the flask and cells grew out as an explant culture.
All tumour samples were plated out on conventional cell culture plastic-ware and, if sufficient material was available, they were also plated on different substrates (see below). A difference between the tumour groups in the time taken to reach passage level 1 on plastic was observed and summarised in Table 7. Cultures derived from high grade and pilocytic astrocytomas, ependymomas and neuroblastomas tended to reach passage level 1 more quickly than those derived from MBs, PNETs and low grade fibrillary astrocytomas. Analysis using the Student's t-test showed significant differences between a small number of groups, namely, between, neuroblastomas and MBs (p=0.0076) and fibrillary astrocytomas (p=0.043) in the time taken to reach passage level 1. Interestingly, there was nearly a significant difference between the neuroblastomas and the pilocytic astrocytomas (p=0.068). The MBs on average took the longest time (2.7 weeks), although some individual cultures took much less time to reach confluency. Neuroblastoma cultures took the shortest time, taking less than 2 weeks to reach passage level one. High grade and pilocytic astrocytomas, ependymomas and neuroblastomas tended to produce fast, vigorous cultures overall, whilst MBs and low grade fibrillary astrocytomas grew more slowly. Interestingly, there was a marked difference between the pilocytic and fibrillary astrocytomas in terms of the time taken to reach passage level 1, however, was found not to be significant.

Table 7: Summary of time taken to reach passage level 1

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Number</th>
<th>Time taken to reach passage level 1 (in weeks)</th>
<th>Mean (in weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilocytic astrocytomas</td>
<td>N=16</td>
<td>1 - 5.5</td>
<td>2.31</td>
</tr>
<tr>
<td>Fibrillary astrocytomas</td>
<td>N=9</td>
<td>1 - 5.0</td>
<td>2.78</td>
</tr>
<tr>
<td>High grade astrocytomas</td>
<td>N=7</td>
<td>1 - 5.0</td>
<td>2.29</td>
</tr>
<tr>
<td>Medulloblastomas</td>
<td>N=21</td>
<td>1 - 7.5</td>
<td>2.70</td>
</tr>
<tr>
<td>PNETs</td>
<td>N=3</td>
<td>1 - 4.0</td>
<td>2.33</td>
</tr>
<tr>
<td>Ependymomas*</td>
<td>N=7</td>
<td>1 - 3.5</td>
<td>2.07</td>
</tr>
<tr>
<td>Neuroblastomas</td>
<td>N=3</td>
<td>1.5-2.0</td>
<td>1.66</td>
</tr>
</tbody>
</table>

(* including the ependymoblastoma)

Initial growth on different substrates
In attempting to increase the success rate of establishment of primary cultures, particularly from medulloblastomas, a number of cultures were grown on different attachment factors. In most cases there was "good" (defined as initial plating, attachment of floating cells and the early signs of growth of more than half the cell population within 24 hours) initial attachment in primary culture in tumour types on most substrates. However, there was
heterogeneity within tumour groups and between the different groups. Overall, it was
observed that at least 75% of cultures derived from medulloblastomas attached well to
plastic, FN, LMN and vitrogen. In contrast, PLL, Matrigel and Cell-tak were found to be less
effective and only 50% of cultures on these surfaces attached well. However, glass was found
to be a very poor substrate for this group of cultures, where although spheroids attached quite
strongly to the glass base, there was little further growth. For cultures derived from both low
and high grade astrocytomas, the number of substrates that resulted in good attachment was
greater than compared to medulloblastomas, in that 75% of these cultures were observed to
attach well on plastic, FN, LMN, PLL, vitrogen, Cell-tak and glass. Possibly the electrostatic
nature of PLL and glass is important in the attachment of cells present in this group of
tumours. The only difference between the low and high grades was that the higher grade
astrocytoma cells grew well on Matrigel, unlike those from lower grade tumours. For cultures
derived from ependymomas, more than 75% of cultures attached well on plastic, FN, LMN
and occasionally on glass. However, less than 30% of cultures derived from these tumours
attached well on vitrogen, Matrigel and Cell-tak. Thus overall, Matrigel, vitrogen and glass
were the least effective of substrates overall for all the tumours studied. All cultures grew on
at least three other surfaces, indicating that viable cells must have been present in the
original cell suspension. Most of the cultures also maintained growth to at least passage level
2 on three or more surfaces.

Sustained growth on different substrates
The results of growing cells derived from 8 medulloblastomas, 6 low grade astrocytomas (4
pilocytic & 2 fibrillary), 5 high grade astrocytomas, 3 ependymomas and 1 each of a
malignant fibrous histiocytoma, craniopharyngioma and neuroblastoma on different
substrates is summarised in Fig 6. It is apparent that there were marked differences in the
ability of different substrates to maintain the growth of particular tumour types beyond
passage level 3.

Plastic was consistently able to maintain growth of nearly all tumour types. FN was
found to sustain growth to the same degree for both low pilocytic and fibrillary astrocytomas
but only supported the growth of 88% (7/8) medulloblastomas, 67% (2/3) ependymomas, 80%
(4/5) high grade astrocytomas and a single case of neuroblastoma, craniopharyngioma and a
malignant fibrous histiocytoma. LMN supported the growth of 75% (6/8) medulloblastomas,
67% (2/3) ependymomas, 83% (5/6) low (3/4, 75% pilocytic and 2/2 100% fibrillary) and 80%
(4/5) high grade astrocytomas and the same tumours from the miscellaneous group (as for FN
above). Although LMN was not capable of supporting growth to the same extent as plastic or
FN, there was little evidence of this being influenced by tumour histology.

Overall, PLL was found not to be an appropriate substrate for sustained growth of
these tumours in culture, but there were a few exceptions. PLL appeared to support growth of
Fig 6
Percentage growth success of paediatric short-term cultures beyond passage level 3 when grown on different substrates.
both low (83%, all 4 pilocytic tumours, but only one of the two fibrillary) and high (60%) grade astrocytomas but to a lesser extent than plastic or FN. The growth of medulloblastomas (50%) and ependymomas (33%) was less successful. It appeared that low grade pilocytic astrocytoma cultures did well on this substrate. For example, four such cultures (IN1520, IN1524, IN1533 & IN1591) grew to passage levels 7, 7, 11 and 10 respectively. A culture derived from each of a MB and a craniopharyngioma both grew to passage level 8. In contrast, the neuroblastoma culture (IN1634) did not grow on this substrate.

Cell-tak proved a poor substrate for the growth of most kinds of childhood brain tumours, although a small proportion managed to grow beyond passage level 3. However, the growth was slow in these cases. On Cell-tak, cultures IN1594 (ependymoma), IN1601 (medulloblastoma) and IN1634 (neuroblastoma) grew to passage level 7, 9 and 6 respectively giving rise to vigorous growth and relatively high yields on seeding.

A small number of cultures grew well on vitrogen including IN1566 (HGA), IN1591 and IN1533 (LGA, pilocytic) and IN1601 (MB), where each grew to passage level 8, 10, 4 and 6 respectively. A single ependymoma culture (IN1497) grew to passage level 4 on vitrogen.

There were a number of technical difficulties using these growth substrates, particularly Matrigel and vitrogen. With Matrigel, prevention of a gel forming on thawing was difficult as well as keeping other cell culture disposables at 0°C (such as pipettes and flasks). Another problem with Matrigel was that during routine passaging it was often difficult to retrieve the cells from the gel even using the recommended enzyme dispase. Consequently, this resulted in cell yields which were 1.3 - 2.9-fold less, with a median of 1.5-fold on Matrigel, compared to plastic. Vitrogen was an adequate substrate only if it was completely dry but this was sometimes difficult to achieve. In addition, as with agar (probably because vitrogen film had not dried efficiently), cells had a tendency to grow underneath the film rather than on it. Again there was often a reduced harvest of cells on passaging, so that cell yields were in the order of 3.5x less on vitrogen than the same culture grown on plastic. The difference in cell yield ranged between 1.3-18-fold less, with a median of 1.9-fold on vitrogen compared with plastic. However, the three surfaces plastic, FN and LMN increased initial growth and promoted sustained growth with higher cell yields on seeding and consequently were used in subsequent culturing of new tumours. For most of the cultures from the four major groups, there was little difference in cell yields between these three surfaces, although there were individual cultures that had higher cell yields on one particular substrate. For example, 50% of high grade astrocytoma-derived cultures had a 2-fold higher cell yield on plastic compared with that on FN and LMN in the first three passage levels, but in later passages this difference was no longer apparent. Less than 50% of ependymoma cultures on FN and LMN produced cell yields between 2-4-fold and 2.5-3-fold greater compared with plastic respectively. For the low grade astrocytoma and medulloblastoma cultures less than 20% produced greater numbers of cells on FN and LMN.
compared with plastic. As with the high grade astrocytoma and ependymoma cultures this difference was no longer evident at later passages. In summary, the overall success rate of sustained growth of cultures on Matrigel, PLL and vitrogen was poor, but was much better on plastic, FN and LMN. The other substrates, glass and Cell-tak, appeared to be of intermediate benefit.

Morphology of cultures

A variety of cell sizes and morphologies was displayed both in primary and low passage and sometimes in higher passage level cell cultures. The morphology displayed included spindle-shaped (bi-polar) and stellate (multi-polar), epithelial-like cells, multi-processed cells as well as small and large flat cells and polygonal cells of various sizes. All cultures displayed at least one or more of these morphologies.

Astrocytomas

Most low and high grade astrocytoma cultures showed a great diversity of cell types in short-term culture and included thin spindle-shaped cells, with both long and short processes, and large flat cells with processes as shown in Figs 7a, 8a and 9a respectively. The only slight difference between the low and high grade tumours was that the cells derived from the higher grade tumours had a tendency to grow in clusters of tightly packed cells. In cultures derived from pilocytic astrocytomas, a striking feature was the presence of cells with very long processes which resembled oligodendrocytes (Figs 7e & 8c respectively) which were infrequent in cultures derived from high grade astrocytomas. However, there did not appear to be differences between the pilocytic and fibrillary tumours in terms of cell types.

Medulloblastomas & PNETs

The most common feature of cultures derived from these tumours was the presence of floating aggregates. Such cultures also displayed heterogeneity in cell types present and included round, small and flat polygonal cells, both bi- and tri-polar cells as shown in Figs 10a and 11a respectively. Fascicular cells were displayed by a number of MB cultures and are illustrated in Fig 11e. In one PNET, (not shown) the culture displayed a varied morphology together with a "Homer-Wright" rosette pattern of growth.

Ependymomas

The majority of ependymoma cultures comprised various cell types, including large flat and polygonal cells with frequent nucleoli present in many of the cells as illustrated in Figs 12a-c and 13a-c. In some large flat cells, individual fibres were visible as shown in Figs 12b and 13c. As these cultures were passaged, the morphology tended to change to a pattern of uniform
sheets of bi-polar cells. Although cell lines grown on plastic are not illustrated, the morphology of these cultures was similar to those seen on other substrates.

**Morphology on different substrates**

When the same culture was grown on different substrates, a different type of morphology or the predominance of a particular type of cell was observed in some cases. However, common cell types were seen in all tumour groups on different substrates. For all tumour histologies and substrates investigated, bi-polar cells were the most frequent cell type observed. Tri-polar morphology was seen mainly in low grade astrocytomas, medulloblastomas and ependymomas, but were not a prominent feature of high grade astrocytoma cultures. For ependymomas, growth on plastic, FN and LMN and vitrogen produced cell cultures with pleomorphism more marked than that observed on PLL, Cell-tak, Matrigel and glass. On the former surfaces, a number of different cell types were present in these cultures, including small and large polygonal cells (including flat and round), bi- and tri-polar cells with many nucleoli, while on PLL, Cell-tak, Matrigel and glass, a more homogeneous cell population was observed, where, on a particular substrate, a certain cell type would dominate. For example, on Matrigel, large fascicular cells with visible striated fibres were seen, while on glass or PLL, cells would grow as sheets of bi-polar cells. Cultures produced from medulloblastomas displayed considerable heterogeneity in morphology *in vitro*.

The different growth patterns seen in within each of the histological groups grown on a variety of substrates are illustrated in Figs 7-13 and are described below.

**Low grade pilocytic astrocytoma culture (IN1520) grown on plastic, LMN, Cell-tak, Matrigel, glass and vitrogen is illustrated in Figs 7a-f.** A similar type of morphology was seen on plastic, LMN and vitrogen, i.e., bi- and tri-polar cells with short processes with the occasional flat polygonal cell. However, on surfaces Cell-tak, Matrigel and glass, a much more varied morphology was displayed, including fascicular cells (on Cell-tak) and large flat cells (Cell-tak and Matrigel). Also of interest was the presence of cells with very long processes which resembled oligodendrocytes displayed on these three surfaces, which did not appear in cultures grown on plastic, LMN and vitrogen. This suggests that Cell-tak, Matrigel and glass promoted the adherence and growth of such cells.

Ependymoma cultures (IN1497 and IN1594) also showed a varied morphology depending on substrate. For example, IN1594 displayed similar cell types when grown on FN, LMN and Cell-tak (Figs 13a-c), i.e., flat polygonal and fascicular cells. However, cells on Cell-tak were much larger with few processes, whilst those grown on FN and LMN were smaller, with a rather larger number of cells with short processes. In addition, on LMN a more compact growth was observed.
**Low grade pilocytic astrocytoma**

**Fig 7a:** IN1520P2 on plastic showing mainly spindle-shaped and stellate cells with small processes.

**Fig 7b:** IN1520P3 on LMN displaying a similar morphology to that found on plastic.

**Fig 7c:** IN1520P2 on Cell-tak where the majority of cells are fascicular with many nucleoli and individual cells with long processes present, resembling oligodendrocytes.

**Fig 7d:** IN1520P1 on Matrigel showing a greater number of cells that resemble the morphology of oligodendrocytes as well as round small cells.

**Fig 7e:** IN1520P1 on glass with the majority of cells being spindle-shaped and multi-processed.

**Fig 7f:** IN1520P1 on vitrogen showing bi- and tri-polar cells.
Low grade pilocytic astrocytoma

**Fig 8a:** IN1524P2 on plastic showing spindle-shaped and stellate cells and large flat cells with small processes.

**Fig 8b:** IN1524P3 on FN displaying fascicular cells and large flat polygonal cells with very long processes.

**Fig 8c:** IN1524P1 on glass showing a much more varied morphology of large flat polygonal cells and cells with very long processes, resembling oligodendrocytes.

**Fig 8d:** IN1524P3 on PLL showing similar morphology as seen on glass but less confluent.

**Fig 8e:** IN1524P1 on Cell-tak, showing small and large polygonal cells with long and short processes, together with phylopodia.

**Fig 8f:** IN1524P1 on Matrigel showing bi- and tri-polar and fascicular cells and again some phylopodia.
High grade astrocytoma

**Fig 9a**: IN1419P2 on plastic showing mainly spindle-shaped and stellate cells with small processes and some phylopoidia present.

**Fig 9b**: IN1419P3 on FN displaying small and large polygonal cells.

**Fig 9c**: IN1419P3 on LMN showing a mixed morphology of spindle-shaped, stellate and flat cells with short processes.

**Fig 9d**: IN1419P3 on PLL showing fascicular cells with many nucleoli present.

**Fig 9e**: IN1419P2 on Cell-tak illustrating heterogeneity of cell types, including spindle-shaped cells with short and long processes, stellate cells and fascicular cells.
Medulloblastoma

**Fig 10a:** IN1352 on plastic showing compact fascicular cells with many nucleoli present.

**Fig 10b:** IN1352 on FN displaying small and large polygonal cells, together with bi- and tri-polar cells.

**Fig 10c:** IN1352 on LMN showing fascicular cells.

**Fig 10d:** IN1352 on PLL showing small round cells and flat polygonal cells.
Medulloblastoma

**Fig 11a:** IN1367 on plastic showing fascicular cells mainly with many nucleoli and some flat polygonal cells.

**Fig 11b:** IN1367 on FN displaying small and large polygonal cells, together with bi- and tri-polar cells.

**Fig 11c:** IN1367 on LMN showing large flat polygonal and stellate cells.

**Fig 11d:** IN1367 on PLL showing fascicular cells with many nucleoli present.

**Fig 11e:** IN1367 on Cell-tak illustrating fascicular cells and phylopodia.
Ependymoma

Fig 12a: IN1497P3 on LMN showing fascicular cells with many nucleoli present.

Fig 12b: IN1497P1 on Matrigel displaying fascicular cells with visible striated fibres.

Fig 12c: IN1497P3 on vitrogen with large polygonal cells and stellate cells.
**Ependymoma**

**Fig 13a:** IN1594P2 on FN showing fascicular cells with many nucleoli present.

**Fig 13b:** IN1594P4 on LMN displaying compact fascicular cells.

**Fig 13c:** IN1594P2 on Cell-tak with large polygonal cells showing the presence of striated fibres in some cells.
MB cultures (IN1352 and IN1367) illustrate a varied morphology on the different substrates (Figs 10a-d and 11a-e respectively). For example, IN1352 on plastic (Fig 10a) displayed fascicular and bi-polar cells with compact growth, whilst on the other three surfaces, growth was less compact on FN (Fig 10b), with pleomorphism in evidence, including bi- and tri-polar cells and small flat polygonal cells with short processes. In contrast, on PLL (Fig 10d), a different type of cell predominated small round cells with no processes. The occasional presence of large flat cells was also seen.

A high grade astrocytoma (IN1419) is shown when grown on five substrates (Figs 9a-e). Although a similar morphology is illustrated on all surfaces, on Cell-tak (Fig 9e) there are more spindle-shaped cells compared with the other surfaces, the cells on FN (Fig 9b) are much larger and more polygonal in shape and the cells on PLL (Fig 9d) are more compact.

**Passaging of primary cultures**

All cultures managed to grow to passage level (PL) 2 on plastic and on every other substrate used. A sub-group of 42 cultures derived from the four tumour groups, medulloblastomas, ependymomas and both low and high grade astrocytomas were passaged for an extended period of time on three substrates, plastic, FN and LMN, in order to determine if a particular surface tended towards establishment of a cell line. All of these grew to at least passage level 4. The results of these studies are illustrated in Figs 14a-e. These cultures were passaged until they ceased proliferating.

For the MBs (Fig 14a), it appeared that plastic and LMN substrates resulted in cell lines growing to a higher passage level than FN. For example, 6/15 (40%) cultures reached a higher passage level on plastic and 6/15 grew better on LMN and only 1/15 (=7%) grew to a higher PL on FN. Most striking was the enhanced growth of cell lines IN2022, IN2072 and IN1352 which reached PL 29, 17 and 16 on LMN compared with PL, 25, 9 and 8 on plastic and 15, 9 and 9 on FN respectively.

A similar trend was displayed by the low grade astrocytoma cultures (Fig 14b, pilocytic and 14c, fibrillary), where plastic and LMN surfaces were better than FN. Of the pilocytic astrocytomas, 7/10 (70%) cultures did better on these two substrates compared with only 2/10 (20%) cultures favouring FN with regard to enhanced and sustained growth. One pilocytic culture (IN1591) reached the same passage level on both LMN and FN. Looking at individual cell lines, LMN again appears to have greatly enhanced the growth of cell line IN1533 (pilocytic). Although the culture grew well on all three surfaces, sustained growth appeared to have been achieved, with passage levels of 18 observed on LMN compared to 14
**Figs 14 a-e**

Histograms showing the maximum passage level reached of cultures derived from medulloblastomas, low and high grade astrocytomas and ependymomas on plastic, laminin and fibronectin.

**KEY**

- Plastic
- Fibronectin
- Laminin
on plastic and 13 on FN respectively. Culture IN1591 did very well on both FN and LMN, reaching PL 25 on both substrates but very poorly on plastic (PL10). Four out of ten (40%) pilocytic astrocytomas grew better on plastic.

Of the 6 fibrillary derived cultures, 2/6 (=33%) faired better on plastic and LMN (IN2003 and IN2044) compared to FN. For example, good sustained growth was seen on plastic and LMN with cultures achieving PLs of 23 and 21 on plastic compared to 20 and 13 on LMN and 14 and 11 on FN respectively. Three out of six (50%) cultures grew better on plastic and included IN2003, IN2044 and IN2012. One culture in this group of tumours grew consistently well on all 3 surfaces; IN1751 reached PL 21, 22 and 29 on plastic, FN and LMN respectively. This was not observed in the pilocytic astrocytomas. However, one such culture IN1591, did reach PL 25 on both FN and LMN but only PL 10 on plastic.

Interestingly, the trend for the high grade astrocytoma groups (Fig 14d) showed a slightly better growth on FN compared with plastic and LMN, where 3/6 (50%) cultures grew to a higher PL on LMN, followed by 2/6 (33%) on FN and only 1/6 (=17%) doing better on plastic. For one culture, IN2080, growth on plastic was very poor and only to PL 2, but growing to PL 7 on FN.

Ependymomas (Fig 14e) showed a mixed reaction to the substrates and growth was not specifically enhanced by any attachment factor. Nevertheless, 3/6 cultures grew slightly better on LMN, 2/6 better on FN and only 1/6 grew to a higher PL on plastic compared with LMN and FN. There were two exceptions; IN1932 grew much better on plastic reaching PL 20 compared to PL 11 and 16 on FN and LMN respectively, and IN1594, where the maximum PL attained was 21 on LMN, followed by 18 on FN and finally 13 on plastic.

The generation number which may be defined as the number of population doubling times (estimated from dilution at subculture) that a culture has undergone since explantation, represents an approximation of the number of generations in primary culture. Despite this being an important parameter, given that high cell loss may have occurred at subculture in some cases (and maybe in all), such an calculation is very difficult to determine and therefore not included. The missing data on some of these cultures on any particular surface was the result of either that factor not being available at time of setting up primary culture or contamination.

Cell kinetics

The population doubling times (PDTs) of a number of cultures was determined in plastic flasks and the data shown overleaf. These include cultures derived from low fibrillary (Fig 15c), pilocytic (Fig 15d) and high (Fig 15e) grade astrocytomas, MBs (Fig 15a), ependymomas (Fig
Figs 15a-15b

These graphs show the growth curves of a number cultures derived from seven medulloblastoma/PNETs and four ependymomas. Each point represents the mean of two cell count readings taken at regular intervals when grown in 25cm³ flasks for up to 25 days. The population doubling times and saturation densities were determined as described in Chapter 5 (materials & methods).
Fig 15a: Growth curves derived from medulloblastoma and PNET cultures

Fig 15b: Growth curves derived from ependymoma cultures
Figs 15c-15d

These graphs show the growth curves of a number cultures derived from one fibrillary and four pilocytic astrocytomas respectively. Each point represents the mean of two cell count readings taken at regular intervals when grown in 25cm$^3$ flasks for up to 25 days. The population doubling times and saturation densities were determined as described in Chapter 5 (materials & methods).
Fig 15c: Growth curve derived from a fibrillary astrocytoma

Fig 15d: Growth curves derived from pilocytic astrocytoma cultures
Fig 15e-15f

These graphs show the growth curves of a number cultures derived from four high grade astrocytomas and various brain tumours respectively. Each point represents the mean of two cell count readings taken at regular intervals when grown in 25cm³ flasks for up to 25 days. The population doubling times and saturation densities were determined as described in Chapter 5 (materials & methods).
Fig 15e: Growth curves derived from high grade astrocytomas

Fig 15f: Growth curves derived from other brain tumour cultures
15b) and a number of miscellaneous tumours (Fig 15f): a primitive neuroepithelial tumour (IN1637), a choroid plexus carcinoma (IN1863) and finally a germinoma (IN1857). Cultures were passaged only when they had reached confluency. PDTs were calculated from the exponential portion of the growth curve for each cell line as illustrated in Fig. 4 (in Materials & Methods). The time in hours taken for the population of cells in logarithmic phase to double was extrapolated from the graph. It should be noted that in some instances, the exponential phase of growth of some short-term cell lines was comprised of only two or three points on the curves, whilst in others >3 points were available.

The widest range of PDTs was observed in the MB cultures (24-120 hrs), where 4/7 (57%) cultures had individual PDTs less than 36 hrs and 3/7 (43%) with greater than 50 hrs. The shortest PDT was 24 hrs (IN1527). Low grade astrocytoma (two of pilocytic and one fibrillary) cell lines had longer PDT than MBs, where 4/7 had PDTs of 60 or more hours. The high grade astrocytomas tended to have shorter PDTs than cultures derived from lower grade tumours, where 1/4 (36%) and 3/4 (75%) cultures had PDTs of 36 hours and between 45 and 48 hours respectively.

Low grade pilocytic and fibrillary astrocytomas and medulloblastomas were slower growing than high grade astrocytomas and took longer to reach plateau phase (Figs 15c, 15d & 15a respectively). In contrast, the other groups of tumours reached their cell densities relatively quickly. A high saturation density (>1x10^5 cells/cm^2) was reached by one culture derived from an ependymoma (IN1594). However the majority of all cultures studied had saturation densities of between 2.3x10^4 - 4x10^5 cells/cm^2.

It appears from Table 8, that high grade astrocytomas and pilocytic astrocytomas reached similar saturation densities (in the region of 1x10^5 cells/cm^2). Interestingly, cultures derived from various tumour histologies had similar values to the MBs. There was a marked difference between the pilocytic and fibrillary variants, where the former had a mean saturation density which was 5.2-fold greater than that found for the fibrillary variant. Low grade fibrillary astrocytomas reached relatively low cell densities in vitro (= 1x10^6 cells/cm^2). In contrast, ependymomas reached high cell densities, nearly double that of the low grade pilocytic astrocytomas and 10.5-fold greater than the only fibrillary astrocytoma. One ependymoma cell line had an extremely high saturation density (14x10^5 cells/cm^2, IN1594).
Table 8: Cell kinetics of short-term paediatric brain tumour cultures

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Number</th>
<th>Range of population doubling times (hours)</th>
<th>Mean &amp; median population doubling times (hours)</th>
<th>Mean &amp; median saturation densities (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low grade pilocytic astrocytomas</td>
<td>N = 4</td>
<td>36 - 60</td>
<td>Mean = 51, Median = 54</td>
<td>Mean = 100000, Median = 92000</td>
</tr>
<tr>
<td>Low grade fibrillary astrocytomas</td>
<td>N = 1</td>
<td>67</td>
<td></td>
<td>19000</td>
</tr>
<tr>
<td>High grade astrocytomas</td>
<td>N = 4</td>
<td>36 - 48</td>
<td>Mean = 44, Median = 47</td>
<td>Mean = 110000, Median = 110000</td>
</tr>
<tr>
<td>Medulloblastomas &amp; PNET</td>
<td>N = 6</td>
<td>24 - 120</td>
<td>Mean = 59, Median = 36</td>
<td>Mean = 94157, Median = 110000</td>
</tr>
<tr>
<td>N = 1</td>
<td></td>
<td></td>
<td>Mean = 68, Median = 70</td>
<td>Mean = 200000, Median = 120000</td>
</tr>
<tr>
<td>Ependymomas</td>
<td>N = 4</td>
<td>36 - 96</td>
<td>Mean = 44, Median = 47</td>
<td>Mean = 110000, Median = 110000</td>
</tr>
<tr>
<td>Primitive neuroepithelial tumour</td>
<td>N = 1</td>
<td>48</td>
<td></td>
<td>73300</td>
</tr>
<tr>
<td>Choroid plexus carcinoma</td>
<td>N = 1</td>
<td>38</td>
<td></td>
<td>207000</td>
</tr>
<tr>
<td>Germinoma</td>
<td>N = 1</td>
<td>48</td>
<td></td>
<td>96700</td>
</tr>
</tbody>
</table>

There were individual cultures, including those derived from MBs/PNETs, ependymomas and a primitive neuroepithelial tumour (IN1562, IN1583, IN1563 & IN1637) respectively, that had very low saturation density values of less than 8x10⁴ cells/cm². Cultures IN1562 and IN1583 had the longest PDTs of 118 hrs and 120 hrs respectively but IN1637 had a fairly short PDT of 48 hrs. PDTs did appear to reflect the ability of a culture to reach high saturation density. One ependymoma culture (IN1563) had the lowest saturation density of only 2x10⁴ cells/cm² and a rather long PDT of 96 hrs. However, one primitive neuroepithelial tumour (IN1637), with a short PDT of 48 hours (half that of the ependymoma culture), managed to reach a saturation density of around 7.3x10⁴ cells/cm², nearly 3x that of the ependymoma culture.

Cell kinetics on substrates

Population doubling times

For a small number of cultures the PDTs and saturation densities of the same culture were determined when grown on plastic, FN and LMN in 96-well microtitration plates. It should be noted that cultures used in this experiment were different from those studied in the previous section with small flasks. However, there were two of cell lines which were the same, a low fibrillar (IN2003) and a high (IN1566) grade astrocytoma.
Table 9: Range of population doubling times of cultures grown on 3 surfaces

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Range of population doubling times (hours)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number Plastic</td>
<td>Number Fibronectin</td>
</tr>
<tr>
<td>Low grade pilocytic astrocytomas</td>
<td>3 34 - 43</td>
<td>3 48 - 135</td>
</tr>
<tr>
<td></td>
<td>mean = 34</td>
<td>mean = 85</td>
</tr>
<tr>
<td></td>
<td>median = 36</td>
<td>median = 72</td>
</tr>
<tr>
<td>Low grade fibrillary astrocytomas</td>
<td>3 58 - 108</td>
<td>3 48 - 96</td>
</tr>
<tr>
<td></td>
<td>mean = 79</td>
<td>mean = 72</td>
</tr>
<tr>
<td></td>
<td>median = 72</td>
<td>median = 73</td>
</tr>
<tr>
<td>High grade astrocytomas</td>
<td>3 28 - 46</td>
<td>3 26 - 48</td>
</tr>
<tr>
<td></td>
<td>mean = 37</td>
<td>mean = 40</td>
</tr>
<tr>
<td></td>
<td>median = 36</td>
<td>median = 47</td>
</tr>
<tr>
<td>Medulloblastomas</td>
<td>5 30 - 84</td>
<td>5 21 - 75</td>
</tr>
<tr>
<td></td>
<td>mean = 58</td>
<td>mean = 47</td>
</tr>
<tr>
<td></td>
<td>median = 54</td>
<td>median = 49</td>
</tr>
<tr>
<td>Ependymomas</td>
<td>5 30 - 70</td>
<td>5 28 - 96</td>
</tr>
<tr>
<td></td>
<td>mean = 41</td>
<td>mean = 61</td>
</tr>
<tr>
<td></td>
<td>median = 32</td>
<td>median = 58</td>
</tr>
</tbody>
</table>

Similarities in the mean population doubling times for a given substrate and tumour group were evident (Table 9). For the pilocytic astrocytomas, there appeared to be a distinct difference in PDTs on all 3 surfaces tested. For example, the mean PDT on plastic, FN and LMN were 34, 85 and 44 hrs respectively, clearly showing a wide range of values. Also, the range in PDTs was greatest on FN, between 48 - 135 hrs, and this was reflected in the highest mean PDT of 85 hrs. In contrast, the fibrillary astrocytomas had different characteristics, with very similar PDTs on all three surfaces. For example, 79, 72 and 65 hrs on plastic, FN and LMN respectively. The only similarity found between the pilocytic and the fibrillary variants was the median PDT values for cultures when grown on FN, 79 and 72 and on LMN, 50 respectively. Interestingly, the fold difference in doubling times between the pilocytic and the fibrillary astrocytomas on all 3 surfaces was comparable, e.g., for the former, the fold differences were 1.3, 2.8 and 3.8 on plastic, FN and LMN, whilst for the fibrillary types the values were 1.9, 2 and 3.8 respectively.
MBs had similar mean PDTs for plastic and LMN, but a shorter PDT on FN (47 hrs). In contrast, ependymomas and high grade astrocytomas had different PDTs for all three surfaces, but the overall PDTs were shorter on plastic compared to FN or LMN.

Differences and similarities between the different tumour groups were also evident. A similar mean PDT for tumour groups, HGA, MBs and ependymomas was observed for cultures grown on LMN, i.e., around 56 hrs. The pilocytic astrocytomas were nearly of the same order, with a mean PDT of 47 hrs on LMN. In contrast, the fibrillary astrocytomas had a much higher mean value, that of 65 hrs. An interesting finding was that the low grade pilocytic astrocytomas had similar values to their higher grade counterparts, particularly on plastic and LMN. The mean PDTs for the pilocytic tumours were 34 and 47 hrs, whilst for the high grade astrocytomas, 37 and 52 hrs, on plastic and LMN respectively. In contrast, the fibrillary astrocytomas consistently had higher mean PDTs compared to the pilocytic variant, as well as the high grade astrocytomas. One would possibly have expected to observe a closer partnership between the fibrillary and the higher graded astrocytomas, since both of these groups are of the more malignant type, whilst the pilocytic are regarded as the more benign tumour type. On the other hand, some tumour groups appeared to have similar mean PDTs, where for plastic, low grade pilocytic astrocytomas, high grade astrocytomas and ependymomas were in the region of ≈ 37 hrs. The widest range of mean PDTs appeared to be when cultures were grown on FN, but no particular pattern was evident. It is therefore clear that substrate did seem to have an effect on the growth kinetics of some cultures derived from childhood brain tumours. This suggests that those cell populations that grew on any one of the three substrates included a population of cells that adapted well to the in vitro conditions and also had similar PDTs.

*Saturation densities*

The saturation densities obtained by cultures also appeared to differ according to the substrate used in Table 10 overleaf. The saturation densities were determined by extrapolation of the horizontal portion of the growth curve (plateau phase) to the y-axis as illustrated in Fig. 4 (Materials and Methods).
Table 10: Range of saturation densities of cultures grown on 3 surfaces

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Range of saturation densities (cells/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
</tr>
<tr>
<td>Low grade pilocytic astrocytomas</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>mean = 64961</td>
</tr>
<tr>
<td>Low grade fibrillary astrocytomas</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>mean = 56437</td>
</tr>
<tr>
<td>High grade astrocytomas</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>mean = 56040</td>
</tr>
<tr>
<td>Medulloblastomas</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>mean = 42944</td>
</tr>
<tr>
<td>Ependymomas</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>mean = 53629</td>
</tr>
</tbody>
</table>

On plastic, all cultures derived from childhood brain tumours reached similar cell saturation densities, although as a group of tumours, medulloblastomas had the lowest value on this substrate. However, on FN, there was a marked difference in the saturation densities reached between the different tumour groups. For example, in order of ascending saturation densities, cultures derived from ependymomas achieved the highest value, followed by medulloblastomas, high grade astrocytomas, and lastly, the low grade astrocytomas (the fibrillary variant had the lowest values) which produced the lowest saturation cell densities. It appeared that for the low grade fibrillary astrocytomas, the substrate appeared not to influence the saturation densities attained. Although for the pilocytic astrocytomas a slight difference was observed, where the saturation density increased in value in ascending order when grown on FN, plastic and LMN respectively. In contrast, high grade astrocytomas appeared to be markedly inhibited on LMN. They reached only very low cell densities and had the longest doubling times on this substrate. On LMN, this group reached a mean saturation density value of only 2.3x10⁴ cells/cm², while on plastic the mean value was 2.5x greater than that reached on LMN (5.6x10⁴ cells/cm²). An even greater differential was seen between FN and LMN, where the mean saturation density was 3.5x greater on FN compared to LMN. For MBs and ependymomas mean values reached were = 2-fold less on plastic and LMN.
than those values observed on FN. For both these histologies, a similar saturation density value was reached on both plastic and LMN. A most striking observation was the fold-difference between the lowest and highest saturation densities attained by the fibrillary compared to the pilocytic astrocytomas. For example, the mean-fold difference in range of saturation densities attained on plastic, FN and LMN for the fibrillary variant was 7.1-, 11.8- and 35-fold respectively, whilst for the pilocytic it was 1.8-, 3.5- and 2.8-fold respectively. Clearly, as a group, the fibrillary astrocytomas showed greater heterogeneity. This is in contrast to the doubling times values, which were similar for both groups on all 3 surfaces. Overall, the fibrillary variant derived cultures had the greatest-fold difference on the 3 surfaces compared to the other tumour groups. The pilocytic astrocytomas, had similar fold-differences to their higher counterparts, the HGAs, e.g., fold-differences of 2.5-, 4- and 1.4-fold on plastic, FN and LMN respectively. This again reflects the same observation as was found with the population doubling times.

Figs 16a-d illustrate the growth patterns of cultures from each tumour group on the three attachment factors, plastic, FN and LMN. Differences in growth patterns were apparent for the same culture when grown on different substrates, and these illustrate not only the overall behaviour of cultures derived from any one tumour group, but the heterogeneity between different tumour groups and within the same tumour type. For example, faster growth was seen with FN and plastic and slower growth on LMN for a high grade astrocytoma, IN1566, showing that growth was inhibited on LMN (Fig 16d). Cultures derived from medulloblastomas, ependymomas and low grade astrocytomas reached similar saturation densities when grown on plastic (Figs 16a, 16b and 16c). These findings are in agreement with the overall behaviour of these tumour groups. Conversely, for cell lines derived from a low grade fibrillary astrocytoma and MB (IN2003 and IN2077), growth on plastic was more vigorous (as shown in Figs 16c and 16a respectively) than the corresponding culture on FN and LMN. Although this pattern of growth was similar to the overall behaviour for the low grade astrocytomas (Fig 16c), the medulloblastoma culture (Fig 16a) behaved differently. The growth on FN and LMN of the two tumour types was also similar for the same culture and between the two tumour types. In contrast, the growth curves for cultures on each of the substrates derived from an ependymoma and a high grade astrocytoma were different (Figs 16b and 16d respectively).
These graphs show the growth curves derived from a medulloblastoma (IN2077) and an ependymoma (IN1932) culture respectively, when grown on three different surfaces (plastic (PL), fibronectin (FN) and laminin (LMN) in a 96-well microtitre plate, at regular intervals over a period of about 25 days. The points represent the mean cell count obtained from harvesting cells from 6 wells. The population doubling times and the saturation densities were determined as described in Chapter 5 (materials & methods).
Fig 16a: Growth curves of a MB culture on three surfaces

Fig 16b: Growth curves of an ependymoma culture on three surfaces
Fig 16c-16d

These graphs show the growth curves derived from a fibrillary (IN2003) and a high grade astrocytoma (IN1566) culture respectively, when grown on three different surfaces (plastic (PL), fibronectin (FN) and laminin (LMN) in a 96-well microtitre plate, at regular intervals over a period of about 25 days. The points represent the mean cell count obtained from harvesting cells from 6 wells. The population doubling times and the saturation densities were determined as described in Chapter 5 (materials & methods).
16c: Growth curves of a fibrillary astrocytoma culture on three surfaces

![Growth curve of a fibrillary astrocytoma culture on three surfaces](image)

Fig 16d: Growth curve of a HGA culture on three surfaces

![Growth curve of a HGA culture on three surfaces](image)
Immunocytochemistry

A number of short-term cell lines were screened for intermediate filaments and fibronectin expression, both on plastic and a smaller number when grown on different substrates. Detailed results are shown in Tables 11a-f. The data is summarised in Tables 12a and 12b.

GFAP

This IF is expressed by glial tumours and such expression was maintained by some of the short-term cell lines derived from MBs, ependymomas, low and high grade astrocytomas and neuroblastomas in low passage cultures (e.g., Figs 17a, 17d, 17f and 17h). The staining pattern of GFAP varied, so that in some cultures expression was found only in isolated cells and was bright and uniform with the staining pattern clearly defined throughout the cytoplasm and in the processes (as illustrated in Figs 17a and 17f), whilst in others (where the expression was seen in more than 50% of cells) the intensity was very weak and not clearly demarcated.

In each of the tumour groups a small number of cultures were positive for GFAP to varying degrees on different substrates (Tables 11a-f). GFAP positivity was seen in all (6/6) high grade astrocytoma (Table 11e) and in 7/11 (64%) low grade astrocytoma (Table 11c and 11d) cell lines on at least one substrate, as might be expected. Of the pilocytic cultures, 5/8 (62.5%) and 2/3 (66.7%) of the fibrillary cultures were positive on at least one surface. However, in the latter group, a greater number of cells were positive for GFAP (i.e., > 10% of cells) compared to the pilocytic-derived cultures (< 10%). This was also observed in 3 fibrillary astrocytoma-derived cultures, when grown on plastic and FN expressed GFAP in >50% of cells compared to the pilocytic astrocytomas, where no such expression was noted. One culture IN1567 (a fibrillary astrocytoma) was consistently negative for GFAP on all surfaces. Of the MB and PNET group (Table 11a), only 50% of cultures screened were positive for GFAP (8/16), suggesting that these cells were derived from MBs with glial rather than neuronal differentiation, or that the cultures were composed of both glial and neuronal cells. All the ependymoma (Table 11b) cultures (7/7) showed positivity for GFAP. Two out of the three NB cultures were found to express this antigen and the primitive neuroepithelial tumour was negative.

However, some of the cultures did not express GFAP even in the first one or two passages, indicating that either there was a down-regulation of this IF upon transfer to culture, or a poor ability for GFAP-positive cells under certain circumstances, to grow well in culture. Alternatively, the absence of expression may be due either to the overgrowth of non-GFAP-expressing cells present within the culture, such as poorly differentiated tumour cells.

The substrate appeared to have an influence on some cultures with regard to GFAP expression. For example, IN1520 (pilocytic astrocytoma) expressed in less than 10% of cells
Table 11a: Immunocytochemistry results of medulloblastoma and PNET cultures

<table>
<thead>
<tr>
<th>PLASTIC</th>
<th>GFAP</th>
<th>Neurofilament</th>
<th>Vimentin</th>
<th>Cytokeratin</th>
<th>Synaptophysin</th>
<th>Fibronectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN1352</td>
<td>-</td>
<td>-</td>
<td>++++</td>
<td>++ weak</td>
<td>ND</td>
<td>++++</td>
</tr>
<tr>
<td>IN1367</td>
<td>-</td>
<td>-</td>
<td>++++</td>
<td>++</td>
<td>+++ weak</td>
<td>++++</td>
</tr>
<tr>
<td>IN1438</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>ND</td>
<td>++++</td>
</tr>
<tr>
<td>IN1482</td>
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<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>IN1527</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>IN1545</td>
<td>+++ weak</td>
<td>-</td>
<td>++++</td>
<td>-</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>IN1562</td>
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<td>+++ weak</td>
<td>++++</td>
<td>+</td>
<td>+++ weak</td>
<td>++++</td>
</tr>
<tr>
<td>IN1583</td>
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* = Ependymoblastoma
Table 11c: Immunocytochemistry results of pilocytic astrocytoma cultures

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### Table 11d: Immunocytochemistry results of fibrillary astrocytoma cultures

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<th>Neurofilament</th>
<th>Vimentin</th>
<th>Cytokeratin</th>
<th>Synaptophysin</th>
<th>Fibronectin</th>
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</tr>
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<td>-</td>
<td>++++</td>
<td>-</td>
<td>ND</td>
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**FIBRONECTIN**

| IN1567        | -    | -             | ++++     | ++          | ND            | -           |
| IN1751        | ++   | -             | ++++     | -           | ND            | ++++        |
| IN1930        | +++  | -             | ++++     | -           | ND            | ++++        |

**LAMININ**

| IN1567        | -    | -             | ++++     | -           | ND            | -           |
| IN1751        | ++   | -             | ++++     | -           | ND            | ++++        |
| IN1930        | ++   | -             | ++++     | -           | ND            | ++++        |

**POLY-L-LYSINE**

| IN1567        | -    | -             | -        | -           | ND            | -           |
| IN1751        | ++   | -             | ++++     | -           | ND            | ++++        |

**CELL-TAK**

| IN1567        | -    | -             | -        | -           | ND            | -           |
| IN1751        | ++   | -             | ++++     | -           | ND            | ++++        |

**VITROGEN**

| IN1567        | -    | -             | ++++     | -           | ND            | -           |

**GLASS**

| IN1567        | -    | -             | -        | -           | ND            | -           |
### Table 11e: Immunocytochemistry results of high grade astrocytoma cultures

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Table 11f: Immunocytochemistry results of neuroblastoma & primitive neuroepithelial tumour cultures

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<td>-</td>
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<td>ND</td>
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</tbody>
</table>

Key

++++ = 100% of cells
++++ = > 50% of cells
++ = < 50% of cells
+ = < 10% of cells
- = 0% of cells
GFAP on Cell-tak and Matrigel but showed weak expression in as many as 50% of cells on plastic. However, when grown on FN, LMN, PLL and glass all cells were negative. One high grade astrocytoma, IN1495, was found to be negative for GFAP on plastic, FN, PLL, Cell-tak and glass but >50% of cells were weakly positive on Matrigel.

One of the ependymoma cultures, IN1497, showed weak GFAP expression in a higher percentage of cells on plastic, Cell-tak, Matrigel, vitrogen and glass but was negative on FN and LMN. For culture IN1563, the presence of GFAP-positive cells is illustrated in Fig 17d when grown on Matrigel and Fig 17c when on Cell-tak. The frequency of GFAP positive cells was greater on Cell-tak compared to Matrigel, suggesting that Cell-tak may promote the growth of such cells. Unexpected expression was seen in two neuroblastomas (IN1921 and IN2059) on plastic and FN (Table II). Positivity is illustrated by IN1921 on plastic in Fig 17g. It is clear that although the majority of cells are GFAP-negative, nevertheless a small proportion of cells growing on top of the GFAP-negative cells are GFAP-positive. These cells are composed of a number of cell types including spindle-shaped and stellate-shaped cells as well as very large flat cells where the individual fibres are clearly visible.

The pattern of expression was also sometimes affected by the substrate. For example, the MB culture, IN1405, is shown in Figs 17a and 17b when grown on Cell-tak and FN respectively, in both of which there is a single large cell showing GFAP positive expression. However, the cell on Cell-tak is slightly smaller with longer processes, whilst the cell on FN substrate has a greater cytoplasm ratio with few processes and the staining is far more fibrous than on Cell-tak. The pattern of expression and the effect of substrate is further illustrated in the low grade fibrillary astrocytoma culture, IN1751 (Figs 17e and 17f). On plastic the GFAP-positive cells are more frequent and include expression in the cytoplasm which extends along the processes of these stellate-shaped cells. On the other hand, on PLL, there are only three GFAP-positive cells which are much smaller with no processes and no individual fibres are visible. On plastic the majority of cells are GFAP-positive, whilst on PLL a much smaller proportion of such cells are present.

**Neurofilament**

Expression of NF-M was detected in a number of cultures. Positivity was mainly confined to the nucleus and on the periphery, with occasional cytoplasmic expression, in some cases which extended to the processes (as illustrated in Figs 18a-c).

The frequency of MB cultures expressing this IF was high, 12/16 (75%) expressed it, even though the range of positive expression was wide; from less than 10% of cells to weak expression in more than 50% of cells (Table 11a). This is interesting, since most of the established cell lines derived from such tumours do indeed express NFs. As mentioned above, 50% of MBs and PNETs were positive for GFAP and here 75% were positive for NF-M, thus
confirming the bi-differentiation nature of this group of tumours. All 3 neuroblastoma cultures expressed NF-M to varying degrees as did the one primitive neuroepithelial tumour (Table 11f). The majority of astrocytomas were negative for this IF, except for 3/8 (37.5%) pilocytic and 2/6 (33%) high grade astrocytomas where weak positivity in less than 10% of cells in situ (Tables 11c & 11e) was observed. Such expression is normally not present in these tumours and could be accounted for the cross-reactivity of the antibodies which recognise this antigen with other intermediate filaments. However, no such expression was found for the 3 fibrillary astrocytoma cultures screened. These results suggest strongly that there is marked difference in the in vitro phenotypic characteristics between the pilocytic and fibrillary astrocytomas. Only one (1/7) ependymoma (IN1563) showed apparent weak expression of NF-M.

Vimentin
With a few exceptions, VIM was strongly expressed by all cells from all the different groups of tumour (as shown in Table 12a and b). However, one culture (IN1567) from the fibrillary group of astrocytomas was found to be negative for VIM when grown on plastic, PLL, CT and glass, but positive on FN, LMN and VT. This was the only culture out of both the fibrillary, as well as the pilocytic astrocytomas where a difference in VIM expression was noted. For example, all cultures derived from pilocytic astrocytomas were positive on all surfaces, in contrast to the fibrillary variant where cultures were either positive in >50% of cells or expression was absent (see Table 11c). These results clearly show a difference in behaviour between these two variants, despite the fact that a small number of cultures were screened.

Expression was seen to vary with regard to degrees of intensity and was distributed throughout the cytoplasm, with some cells showing dense VIM filaments. Such expression was also extended to processes. The pattern of expression is elegantly illustrated in Fig 19a, where cells of various sizes and shapes are expressing this antigen and the individual fibres are visible. Figs 19b and 19c show a NB with cells positive for VIM on plastic and FN respectively. The effect of substrate is again visible in that on plastic the cell staining is well demarcated and smoother and the fibres more prominent, whereas staining in cells when grown on FN are less well defined and more granular.

Cytokeratin
Most of the cultures were negative for this IF, although occasionally weak expression was detected in some cells in a number of tumour types. Cytokeratin staining was seen in isolated cells, which was bright and in some individual cells the fibres were visible. When expression was present in the majority of cells this staining was weak. All (7/7) ependymomas expressed this IF, followed by 67% of high grade astrocytomas (4/6) and neuroblastomas (2/3), 50% of MBs (8/16) and only 1/3 of low grade fibrillary astrocytomas and that on only one surface
(FN). In contrast, 4/8 (50%) of the pilocytic variant expressed this antigen to a degree and the expression was widely distributed, unlike in the fibrillary variant, where there was no expression observed (with the exception of IN1567, where some expression was observed only when grown on FN). The only primitive neuroepithelial tumour was negative.

Interestingly, vitrogen, Cell-tak and Matrigel did not appear to support cytokeratin-positive cell growth, whereas LMN, FN and plastic did. Figs 20a and 20b show a low grade pilocytic astrocytoma and medulloblastoma respectively with cytokeratin positive cells, both on a LMN substrate. Expression in IN2055 is far less well demarcated, with only just visible fibres and incorporating two nuclei. In contrast, the MB culture (Fig 20b), despite high background staining, shows a large positive cell in the middle which is clear with visible fibres.

**Desmin**

This IF was only screened in a small number of cultures and such expression was seen in MBs but in less than 5% of cells. Such expression is not normally a feature of these tumours, and this positivity was most probably due to cross-reacting epitopes in GFAP. A medulloblastoma culture, IN2088, is shown in Fig 21, with three distinct spindle-shaped cells positive for desmin amongst a large population of desmin-negative cells. The expression is very intense and clearly defined.

**Fibronectin**

This cell surface glycoprotein was screened on unfixed (unpermeabilized) cells and strong positivity was observed in all cultures (as shown in Fig 22/Tables 12a and 12b). Expression was bright throughout the cytoplasm and extended to the processes. In some cases, intense perinuclear expression was observed (Fig 22). There was a marked difference in FN expression between the pilocytic and fibrillary astrocytomas, where essentially, the pilocytic tumours were found to be 100% FN positive on any surface, whilst the fibrillary tumours displayed both positive and negative expression depending on the substrate used. However, as with the other intermediate filaments expression, this study was able to screen only a small number of tumours from either group. Nevertheless, these results certainly do illustrate a trend, that of a marked difference between these two types of astrocytomas. The substrate again appeared to have an influence on expression so that a low grade fibrillary astrocytoma, IN1567, was positive for FN only on plastic and negative on other substrates. This again could reflect the tumour's location in the brain (cerebellum, it being unusual). A high grade astrocytoma, IN1566, was positive for FN on LMN, PLL and FN but negative on plastic, Cell-tak, Matrigel and vitrogen. Also both low and high grade astrocytomas were the only tumour groups which had a proportion of cells which were negative for this antigen.
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<th>Vimentin</th>
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### Table 12b: Summary of immunocytochemistry results

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Table 12a: * = Ependymoblastoma
**Synaptophysin**

Synaptophysin is a neuronal marker and positivity for this antigen is observed with tumours derived from such a lineage. Therefore only cultures derived from MBs and PNETs were screened, although after receiving confirmation of diagnosis, an ependymoblastoma (IN2030) and a neuroblastoma (IN2059) had also been selected (Table 12a and b). Expression for this antigen was only determined for the cultures when grown on plastic and 73% (11/15) MB cultures were positive for this antigen, as was one ependymoblastoma and one neuroblastoma culture.

Positive expression was seen either in a perinuclear fashion or would sometimes extend into the cytoplasm and occasionally into the processes if present. There appeared to be a nucleolar reaction in all the cultures screened and this type of expression was also sometimes seen with NF-M (Fig 18c). A range of positive expression was observed in 10/15 MB cultures tested. In the majority of cases, expression was weak and seen in more than 50% of cells, e.g., in Fig 23b, where the majority of cells are weakly positive and expression seen in the cytoplasm and processes. The nucleolar reaction is also clearly visible. In the centre of the photograph there is a single cell which is highly positive for this antigen, similar to that observed with IN2030 (Fig 23a). One neuroblastoma, IN2059, was also found to be positive for SYN, but in less than 50% of cells.
Fig 17a: A medulloblastoma culture (IN1405) showing a large GFAP positive cell when grown on a Cell-tak substrate.

Fig 17b: A medulloblastoma culture (IN1405) showing a large GFAP positive cell when grown on a fibronectin substrate.

Fig 17c: An ependymoma culture (IN1563) showing GFAP positive cells when grown on a Cell-tak substrate.

Fig 17d: An ependymoma culture (IN1563) showing GFAP positive cells when grown on a Matrigel substrate.

Fig 17e: A low grade fibrillary astrocytoma culture (IN1751) showing GFAP positive cells (multi-polar) when grown on a plastic substrate.

Fig 17f: A low grade fibrillary astrocytoma culture (IN1751) showing GFAP positive cells with no processes when grown on PLL substrate.

Magnification: X1000
Fig 17g: A neuroblastoma culture (IN1921) showing very large GFAP positive cells when grown on a plastic substrate.

Magnification: X400

Fig 17h: A high grade astrocytoma culture (IN2080) showing a very large GFAP positive cell when grown on a plastic substrate.

Magnification: X1000
**Fig 18a:** A neuroepithelial tumour culture (IN1637) showing several neurofilament positive cells, together with nucleolar reaction when grown on a plastic substrate.

Magnification: X400

**Fig 18b:** A neuroblastoma culture (IN2059) showing positivity for neurofilament when grown on a plastic substrate.

Magnification: X1000

**Fig 18c:** A medulloblastoma culture (IN2088) showing two neurofilament positive cells, together with a nucleolar reaction of the remaining cells when grown on a plastic substrate.

Magnification: X400

**Fig 19a:** A medulloblastoma culture (IN1601) showing positive cells for vimentin (some showing individual fibres) when grown on a Cell-tak substrate.

Magnification: X400

**Fig 19b:** A neuroblastoma culture (IN2059) showing vimentin positive cells when grown on a plastic substrate.

Magnification: X1000

**Fig 19c:** A neuroblastoma culture (IN2059) showing vimentin positive cells (less fibrous) when grown on a fibronectin substrate.

Magnification: X1000
Fig 20a: A low grade pilocytic astrocytoma culture (IN2055) showing a large cytokeratin positive cell when grown on a laminin substrate.

Magnification: X1000

Fig 20b: A medulloblastoma culture (IN2139) showing a large cytokeratin positive cell when grown on a laminin substrate.

Magnification: X1000

Fig 21: A medulloblastoma culture (IN2088) showing a few desmin positive cells (bipolar) when grown on a plastic substrate.

Magnification: X400

Fig 22: A medulloblastoma culture (IN1838) showing a large population of fibronectin positive cells when grown on a plastic substrate.

Magnification: X400
Fig 23a: An ependymoblastoma culture (IN2030) showing a small cell positive for synaptophysin when grown on a plastic substrate.
Magnification: X1000

Fig 23b: A medulloblastoma culture (IN2085) showing one strongly synaptophysin positive cell, with the remaining cells showing a nucleolar reaction when grown on a plastic substrate.
Magnification: X1000
Chemosensitivity

A number of cultures were screened using the MTT assay against three drugs, CCNU, PCB and VCR. As well as the short-term cultures from the tumour biopsies, two established cell lines were used, Daoy and TE671 (the latter at the time of use was thought to be a MB, but has since been shown to be a rhabdomyosarcoma, Stratton et al., 1989). Details of these assays are shown in Table 13 (ID$_{50}$ concentrations) and Table 14 (number of assays), and a summary of the data is provided in Table 15. Assays were carried out at various passage levels, although attempts were made, where possible, to keep this variation to a minimum. In some cases, however, the same passage level was used. All the low grade astrocytomas tested for chemosensitivity were of the pilocytic type. One of the ependymoma derived cultures was later found to be an ependymoblastoma.

Reproducibility of the MTT assay is illustrated in Figs 24a-c. The graphs show a dose response curve of a pilocytic astrocytoma culture performed on two separate occasions, against CCNU, PCB and VCR. Clearly, the MTT assay is shown to be very reproducible. In addition, the difference in the passage level had no affect on the ID$_{50}$. Figs 25-27a-c show dose response curves of a range of paediatric brain tumour derived cultures to the three drugs screened. In some cases, the reproducibility of the assay is very close (ie., Figs 26a, 26b & 27a). In other instances a greater range in the ID$_{50}$ values and the type of dose response curve was observed (Figs 25a-c, 26c & 27b-c). The type of dose response curve appeared not to be dependent on the drug, since this sort of pattern was observed for all three drugs screened. It did however, appear to be cell culture specific, where for example, cultures IN1495, IN1591 and IN1759 were found to have virtually the same dose response curves for VCR and PCB respectively (Figs 27a, 26a & 26b). The passage level again appeared not to influence the ID$_{50}$ value. In contrast, there were cultures that showed a greater variation in their dose response curves (e.g., Figs 25a-c for CCNU, Figs 26a & 26c for PCB and Figs 27b & 27c for VCR).

Chemosensitivity data for each group of childhood brain tumour are additionally presented as a cumulative proportion plot (e.g., Figs 29a-c). Each symbol represents the ID$_{50}$ (i.e., ug/ml) of a single culture. The cultures are ranked in order of ascending ID$_{50}$ values and the vertical displacement of each of the symbols are a function of the number of cultures examined. For example, if 10 cultures were plotted, the vertical displacement would be 10% between symbols. It is assumed that the experimentally derived range of ID$_{50}$'s represents the distribution that would be found in the whole population of cultures derived from these tumours. Graphical representation in this way, therefore clearly shows the distribution of ID$_{50}$s and reduces the "bunching" of symbols which is common on scatter plots, when used to compare sets of experimental data which have broadly similar values. Such representation also allows the median and the inter-quartile values to be readily determined form the graph without the need for further calculation.
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<td>RMS</td>
<td>14.79</td>
<td>4.91</td>
<td>5486</td>
</tr>
</tbody>
</table>

**N.B.**

Missing data, where for example some cultures have values only for one drug was unavoidable. Unfortunately, even though these cultures were screened for the drugs, the assay was not successful in producing ID<sub>50</sub> values or contamination during the course of the assay occurred, and in some cases it was not possible to carry out any further assays. This also applies to the number of replications performed.
### Table 14: Replication of assays

<table>
<thead>
<tr>
<th>Culture</th>
<th>Tumour type</th>
<th>Number of replications</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CCNU</td>
<td>PCB</td>
</tr>
<tr>
<td>IN1352</td>
<td>MB</td>
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<td></td>
</tr>
<tr>
<td>IN1367</td>
<td>MB</td>
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</tr>
<tr>
<td>IN1405</td>
<td>MB</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>IN1419</td>
<td>HGA</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>IN1438</td>
<td>MB</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>IN1482</td>
<td>MB</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>IN1495</td>
<td>HGA</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>IN1497</td>
<td>EP</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>IN1523</td>
<td>HGA</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>IN1524</td>
<td>LGA</td>
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<td>3</td>
</tr>
<tr>
<td>IN1527</td>
<td>MB</td>
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<td>5</td>
</tr>
<tr>
<td>IN1533</td>
<td>LGA</td>
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<td>7</td>
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</tr>
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<td>IN1932</td>
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<td>IN1950</td>
<td>LGA</td>
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<td>3</td>
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<td>IN2030</td>
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<tr>
<td>IN2139</td>
<td>MB</td>
<td>2</td>
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</tr>
<tr>
<td>Daoy</td>
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<tr>
<td>TE671</td>
<td>RMS</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
Figs 24a-24c

These graphs show the dose response curves of a culture derived from a pilocytic astrocytoma. The MTT assay was carried out twice on separate occasions and at different passage levels. These graphs illustrate the reproducibility of the MTT assay, as well as showing that the passage level has little or no effect on the ID$_{50}$ value.
Fig 24a Dose response curves to CCNU for a pilocytic astrocytoma culture

Fig 24b Dose response curves to PCB for a pilocytic astrocytoma culture

Fig 24c Dose response curves to VCR for a pilocytic astrocytoma culture
These graphs show the dose response curves of three different cultures derived from three different tumour types to CCNU. The cultures are derived from a pilocytic astrocytoma (IN1591), medulloblastoma (IN1438) and an ependymoma (IN1638) respectively. The reproducibility of the MTT assay is illustrated, as well as some variation in the ID$_{50}$ values obtained when the assay is performed several times.
Fig 25a
Dose response curves to CCNU for a pilocytic astrocytoma culture

Fig 25b
Dose response curves to CCNU for a medulloblastoma culture

Fig 25c
Dose response curves to CCNU for an ependymoma culture
These graphs show the dose response curves of three different cultures derived from three different tumour types to PCB. The cultures are derived from an ependymoma (IN1759), pilocytic (IN1591) and high grade (IN2087) astrocytoma respectively. The reproducibility of the MTT assay is illustrated in the ID$_{50}$ values obtained when the assay is performed several times. The graphs are much closer than those obtained for CCNU.
Fig 26a
Dose response curves to PCB for an ependymoma culture

Fig 26b
Dose response curves to PCB for a pilocytic astrocytoma culture

Fig 26c
Dose response curves to PCB for a high grade astrocytoma culture

Drug dose (ug/ml)

% cell survival

100 1000 10000

100000

Drug dose (ug/ml)

% cell survival

100 1000 10000

100000

Drug dose (ug/ml)

% cell survival

100 1000 10000

100000
These graphs show the dose response curves of three different cultures derived from two different tumour types to VCR. The cultures are derived from a high grade (IN1495) astrocytoma and two medulloblastoma (IN1405 & IN1482) respectively. The reproducibility of the MTT assay is illustrated in the ID$_{50}$ values obtained when the assay is performed several times. Fig 29a shows very tight ID$_{50}$ values, whilst the cultures derived from the two medulloblastoma cultures show a variation in the ID$_{50}$ values to VCR.
Fig 27a
Dose response curves to VCR for a high grade astrocytoma culture

Fig 27b
Dose response curves to VCR for a medulloblastoma culture

Fig 27c
Dose response curves to VCR for a medulloblastoma culture
One phenomenon that was observed in these assays was the slight "kink" upwards of the dose response curves at the highest concentration. This is clearly an artefact, as on inspection there were no viable cells remaining in the wells. One explanation could be that the cell debris of these dead cells could have absorbed some of the MTT dye and therefore resulted in elevated reading levels. Also the position of this drug concentration on the 96-microtitre plate (which was consistent throughout the research) may have resulted in the evaporation of the drug medium (despite the fact that the wells were sealed with non-toxic plate sealers).

Table 15: Summary of chemosensitivity results

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Drug</th>
<th>Number of cultures</th>
<th>Mean ID$_{50}$ (µg/ml)</th>
<th>Median ID$_{50}$ (µg/ml)</th>
<th>Standard deviation</th>
<th>Standard error of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medulloblastoma*</td>
<td>PCB</td>
<td>13</td>
<td>3272</td>
<td>2468</td>
<td>3198</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>VCR</td>
<td>12</td>
<td>0.00238</td>
<td>0.00192</td>
<td>0.00179</td>
<td>0.00027</td>
</tr>
<tr>
<td></td>
<td>CCNU</td>
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<td>8.39</td>
<td>6.37</td>
<td>6.47</td>
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<td>Medulloblastoma</td>
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<td></td>
<td>VCR</td>
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<td>CCNU</td>
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<td>8.39</td>
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<td>1.88</td>
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<td>139</td>
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<tr>
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<td>VCR</td>
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<td>8.23</td>
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<td>Low grade pilocytic astrocytoma</td>
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<td>2740</td>
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<tr>
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<td>6</td>
<td>0.0037</td>
<td>0.0015</td>
<td>0.007</td>
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<td></td>
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<td>8.2</td>
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<td>High grade astrocytoma</td>
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<tr>
<td></td>
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<td>0.0022</td>
<td>0.0019</td>
<td>0.0005</td>
</tr>
<tr>
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<td>CCNU</td>
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<td>8.5</td>
<td>8.05</td>
<td>4.87</td>
<td>1.22</td>
</tr>
<tr>
<td>Neuroblastoma</td>
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<td>3110</td>
<td>3582</td>
<td>1269</td>
<td>634</td>
</tr>
<tr>
<td></td>
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<td>0.0014</td>
<td>0.001</td>
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<td>CCNU</td>
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<td>0.0004</td>
<td>0.0002</td>
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<tr>
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<td>CCNU</td>
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<td>14.79</td>
<td>14.79</td>
<td>4.91</td>
<td>3.47</td>
</tr>
</tbody>
</table>

* The data for Daoy are included as part of the medulloblastoma group.
CCNU

Fig 28a shows the meaned dose response curves for the different tumour types studied. The number of cultures screened comprise of 5 ependymomas, 6 low grade pilocytic and 4 high grade astrocytomas, 8 medulloblastomas and 2 neuroblastomas respectively. Clearly, the different tumour types had similar sensitivities to CCNU and their dose response curves were of a similar pattern. Fig 29a shows the chemosensitivity profiles of the cultures to CCNU (Daoy is shown in all graphs as part of the medulloblastoma tumour group). The graph clearly illustrates that different tumour types, with the exception of neuroblastomas, had similar responses to CCNU. However, the range in ID$_{50}$ values did differ within the same tumour group as well as between the different tumour histologies. For example, MBs as a group of tumours showed a 4-fold difference in ID$_{50}$ values between the individual cultures (with a range of between 2.8-13.1 µg/ml). Ependymoma cultures had similar ID$_{50}$ values to medulloblastomas (3.17-10.08 µg/ml), although the difference in sensitivity between the most sensitive and the most resistant was about 3-fold. Pilocytic and high grade astrocytomas had slightly different chemosensitivities where for the pilocytic tumours, the range was between 5-11 µg/ml with a 2-fold difference between the the most sensitive and resistant cultures. Whilst for the malignant astrocytomas the range was between 8.1-10.4, with a 1.3-fold difference. The neuroblastomas also showed a 4-fold difference between cultures and were the most sensitive to CCNU with ID$_{50}$'s of 0.75 and 3.4 µg/ml respectively.

The median ID$_{50}$'s for medulloblastomas was 6.4 µg/ml, for ependymomas, 8.1 µg/ml, for pilocytic and high grade astrocytomas 6.5 µg/ml and 8.1 µg/ml respectively. Of interest is the difference of the median values of the short-term MB cultures and that of Daoy, where the median values for both groups were 6.4 and 8.2 µg/ml. These short-term cultures have shown a different response to CCNU to that of the established cell line, in that the former group are more sensitive. The median ID$_{50}$ value of Daoy was comparable to that of high grade astrocytomas and the ependymomas (Fig 30 a, 8.1 µg/ml). The pilocytic astrocytomas were slightly more sensitive to CCNU (6.5 µg/ml) than higher grade astrocytomas. Of the short-term cultures, high grade astrocytomas and ependymomas were the most resistant to CCNU compared with the other tumour groups with a median value close to that of Daoy. In contrast, the neuroblastomas were the most sensitive with a median value of only 2.1 µg/ml. TE671 was markedly resistant to CCNU with an ID$_{50}$ value of 14.5 µg/ml (Fig. 30a and Table 15).

A pair-wise comparison of the differences in sensitivity between different groups of tumours was performed using the Mann-Whitney test. Neuroblastomas were significantly more sensitive than both pilocytic astrocytomas (p=0.0334) and medulloblastomas (p=0.045), but the difference between neuroblastomas and ependymomas did nearly reach significance (p=0.054). There was also no significant difference between the neuroblastomas and the high grade astrocytomas (p=0.056).
These graphs show the dose response curves of a number of different cultures derived from different tumour types to CCNU (Fig 28a), PCB (Fig 28b) and VCR (Fig 28c) respectively.

Fig 28a shows the meaned dose response curves of 5 ependymoma, 6 pilocytic and 4 high grade astrocytoma, 8 medulloblastoma and 2 neuroblastoma cultures respectively to CCNU. The graph illustrates that most of the cultures studied responded in a similar manner, with the exception of the neuroblastoma derived cultures which were slightly more sensitive.

Fig 28b shows the meaned dose response curves of 7 ependymoma, 6 pilocytic and 5 high grade astrocytoma, 12 medulloblastoma and 2 neuroblastoma cultures respectively to PCB. Clearly the graph shows that there was hardly any difference in the response to PCB of the various cultures screened.

Fig 28c shows the meaned dose response curves of 5 ependymoma, 6 pilocytic and 5 high grade astrocytoma, 11 medulloblastoma and 2 neuroblastoma cultures respectively to VCR. In contrast to CCNU and PCB, there was a marked difference in the cultures response to VCR, where the neuroblastomas were the most sensitive, the ependymomas the most resistant and the remaining tumours behaving in a similar manner.
Fig 28a: Meaned dose response curves to CCNU for the different tumour types

- Ependymoma
- Low grade pilocytic astrocytoma
- High grade astrocytoma
- Medulloblastoma
- Neuroblastoma

Drug dose (μg/ml) vs. Percentage cell survival
Fig 28b: Meaned dose response curves to PCB for the different tumour types

- Ependymoma
- Low grade pilocytic astrocytoma
- High grade astrocytoma
- Medulloblastoma
- Neuroblastoma

Drug dose (µg/ml)

Percentage cell survival

1  4 0 1
1  3 0 -
0 0 -
0 (0 -
(0 4 -
Ü 4 0 -
I 3 0 -
3 0 -
2 0 -
1 0 -
0 0 -
0 0 -
100 1000 10000 100000
Fig 28c: Meaned dose response curves to VCR for the different tumour types
Procarbazine

Fig 28b represents the mean dose response curves of the different tumour types studied against PCB. The number of cultures comprised of 7 ependymoma, 6 pilocytic and 5 high grade astrocytomas, 12 medulloblastoma and 2 neuroblastomas. All tumour types responded in virtually the same manner. Fig 29b shows the chemosensitivity profiles of the cultures to PCB. All tumour types appeared to have rather similar sensitivities to this drug with the exception of Daoy and TE671 (Table 15). The medulloblastomas showed the widest range of sensitivities with ID$_{50}$ values ranging from 905-17070 µg/ml, about a 19-fold difference. Daoy was one of the most resistant cell lines to this drug, with an ID$_{50}$ value of 5083 µg/ml. One short-term cell line from the MB group of tumours had the highest value of 17070 µg/ml, and a number of cultures had values greater than 3000 µg/ml. The ependymomas showed less variation in sensitivity with ID$_{50}$ values between 1347 and 2842 µg/ml. The most sensitive cell line had a value of 1347 µg/ml, whilst the remainder of cultures were found to fall into a range between 2400-3022 µg/ml. The heterogeneity of values within this group was not as pronounced as for medulloblastomas. Pilocytic astrocytomas, had a range of 1987-3202 µg/ml. The values fell into three distinct groups, one less than 2000 µg/ml, another with values greater than 2400 µg/ml and finally those with greater than 3100 µg/ml. In contrast, the high grade astrocytomas did display a degree of heterogeneity with a range of values between 468-2952 µg/ml, a 6-fold difference. Finally, the two neuroblastoma cultures, had ID$_{50}$ values of 2374 and 3847 µg/ml.

 Comparisons of median ID$_{50}$ values between different tumour histologies, showed that high grade astrocytomas were more sensitive (median 1641 µg/ml) than the pilocytic astrocytomas (median 2573 µg/ml). Ependymomas and pilocytic astrocytomas had similar median values (>2500 µg/ml) and they were both slightly more resistant than medulloblastomas (median 2468 µg/ml). Neuroblastomas were the most resistant of all the short-term cultures (median 3110µg/ml, see Table 15). In contrast to the short-term cell lines, Daoy and TE671 were extremely resistant to PCB (median 5083 and 4672 µg/ml respectively, Fig. 30b). Ependymomas were more resistant than high grade astrocytomas (p=0.017) and Daoy (p=0.05) to PCB, but the difference between the other tumour groups did not reach significance. TE671 was consistently more resistant to PCB than high (p=0.04) and low grade pilocytic astrocytomas (p=0.03) and medulloblastomas (p=0.03). Also TE671 was more resistant to PCB than ependymomas (p=0.008).

Vincristine

Fig 28c shows the mean dose response curves to VCR for the different tumour types. The data was comprised of 5 ependymoma, 6 pilocytic and 5 high grade astrocytomas, 11 medulloblastoma and 2 neuroblastoma cultures. Greater heterogeneity between this drug compared to CCNU or PCB was observed in the cultures response to VCR. Fig 29c shows the response of cultures to VCR. The greatest differences between individual tumours within the
same tumour group were seen with VCR. Extreme sensitivity to VCR was shown by neuroblastomas with ID\(_{50}\) values between 1.1x10\(^{-4}\) - 1.7x10\(^{-3}\) µg/ml. In contrast, ependymomas were extremely resistant with ID\(_{50}\)s between 1.9x10\(^{-3}\) - 1.8x10\(^{-2}\) µg/ml. The high grade astrocytomas (5.5x10\(^{-4}\) - 4.1x10\(^{-3}\) µg/ml), medulloblastomas (3.2x10\(^{-4}\) - 4.8x10\(^{-3}\) µg/ml) and low grade pilocytic astrocytomas (1.4x10\(^{-3}\) - 1.2x10\(^{-2}\) µg/ml) were of similar and intermediate sensitivity.

Medulloblastomas displayed a 15-fold difference from the most sensitive to the most resistant. High grade and pilocytic astrocytomas showed a 7-fold and 21-fold difference respectively, and ependymomas displayed a 9-fold difference between the most and least resistant. In addition, the neuroblastomas showed a 16-fold difference between the most sensitive and the most resistant. However, there were only two such samples.

The median ID\(_{50}\)'s for medulloblastomas, pilocytic and high grade astrocytomas was very similar (≥ 0.0015 µg/ml, see Table 13). The high grade astrocytomas had a median ID\(_{50}\) of 0.0022 µg/ml and the pilocytic astrocytomas had a median ID\(_{50}\) of 0.0015 µg/ml. The ependymomas, the most resistant of all the short-term cell lines had a median of 0.0042 µg/ml. Ependymoma cultures were significantly more resistant than MBs (p=0.026), low grade pilocytic astrocytomas (p=0.041), and neuroblastomas (p=0.041). However, difference between the high grade astrocytomas and ependymomas did not reach significance (p=0.088).

Daoy had a median 2-fold greater than that of short-term cell lines derived from medulloblastomas (0.0046 µg/ml). In contrast, the cell line, TE671 was found to be the most sensitive, with a median of only 0.0007 µg/ml (Fig. 30c). Again TE671 was significantly more sensitive to VCR than the other tumour groups; ependymomas (p=0.0045), high grade astrocytomas (p=0.01), but only slightly more sensitive than the only primitive neuroepithelial tumour studied (p=0.05). The difference in response to VCR between TE671 and the MB cultures approached significance (p=0.08).
Fig 29a

This graph shows the distribution of response of cultures derived from 5 ependymomas, 6 pilocytic and 4 high grade astrocytomases, 8 medulloblastomas and 2 neuroblastomas to CCNU.
Fig 29a
Distribution of \( ID_{50} \) values for cultures derived from five groups of childhood brain tumour to CCNU

![Graph showing the distribution of \( ID_{50} \) values for cultures derived from five groups of childhood brain tumours to CCNU.](image)

- **Ependymoma**
- **Low grade pilocytic astrocytoma**
- **High grade astrocytoma**
- **Medulloblastoma**
- **Neuroblastoma**
This graph shows the distribution of response of cultures derived from 7 ependymomas, 6 pilocytic and 5 high grade astrocytomas, 12 medulloblastomas and 2 neuroblastomas to PCB.
Fig 29b

Distribution of ID$_{50}$ values for cultures derived from five groups of childhood brain tumour to PCB

Cumulative proportion

Drug dose (μg/ml)

- Ependymoma
- Low grade pilocytic astrocytoma
- High grade astrocytoma
- Medulloblastoma
- Neuroblastoma
This graph shows the distribution of response of cultures derived from 5 ependymomas, 6 pilocytic and 5 high grade astrocytomas, 11 medulloblastomas and 2 neuroblastomas to VCR.
Fig 29c
Distribution of ID_{50} values for cultures derived from five groups of childhood brain tumour to VCR.

- Drug dose (µg/ml)
- Cumulative proportion

- Ependymoma
- Low grade pilocytic astrocytoma
- High grade astrocytoma
- Medulloblastoma
- Neuroblastoma
These graphs show the dose response curves of TE671 and Daoy to the three drugs screened, CCNU (Fig 30a), PCB (Fig 30b) and VCR (Fig 30c) respectively.
30a Sensitivity of TE671 and DAOY to CCNU

30b Sensitivity of TE671 and DAOY to Procarbazine

30c Sensitivity of TE671 and DAOY to Vincristine
Patterns of cross resistance

Cross resistance was not evident between any of the drugs used in this study within any one of the tumour groups (Table 16). The regression coefficient was determined by plotting the ID$_{50}$ values in a pair-wise fashion within each group of tumours. Neuroblastomas were excluded from this analysis as there were only two cultures used in this study.

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>ID$_{50}$ CCNU/PCB</th>
<th>ID$_{50}$ CCNU/VCR</th>
<th>ID$_{50}$ PCB/VCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>p value</td>
<td>$r^2$</td>
</tr>
<tr>
<td>Medulloblastomas</td>
<td>0.12</td>
<td>0.39</td>
<td>0.17</td>
</tr>
<tr>
<td>Ependymomas</td>
<td>0.22</td>
<td>0.28</td>
<td>0.42</td>
</tr>
<tr>
<td>Pilocytic astrocytomas</td>
<td>0.07</td>
<td>0.63</td>
<td>0.44</td>
</tr>
<tr>
<td>High grade astrocytomas</td>
<td>0.02</td>
<td>0.87</td>
<td>0.11</td>
</tr>
<tr>
<td>All tumours</td>
<td>0.04</td>
<td>0.27</td>
<td>0.04</td>
</tr>
<tr>
<td>(inc. NB, MBT &amp; TE671)</td>
<td></td>
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</tr>
</tbody>
</table>

Cross resistance between any of the three drugs was not evident, although there does appear to be a degree of cross resistance between PCB and VCR in the high grade astrocytomas, this does not reach a statistical significance ($p=0.076$).

In summary, these data indicate that short-term cell lines derived from ependymomas were more resistant to VCR than other types of childhood brain tumours. Neuroblastomas were sensitive to both CCNU and VCR, and the high grade astrocytomas were the most sensitive to PCB (Table 15). The two established cell lines which were included in this study displayed rather different patterns of chemosensitivity. The chemosensitivity profile of TE671 to CCNU, PCB and VCR is clearly different from Daoy and the short-term MB cell lines included in the present study. It is markedly resistant to CCNU and PCB in vitro, but displays sensitivity to VCR. Daoy, although too was more resistant to PCB, it had similar sensitivities to VCR as the short-term MB cell lines and twice that for CCNU.
Chapter 7

Discussion

Cell culture

The aim of this research was to try to enhance the \textit{in vitro} growth of paediatric childhood brain tumours, particularly from medulloblastomas, and, ultimately, to establish panels of continuous cell lines suitable for biological studies. A further major aim was to determine whether there were any significant differences in the chemosensitivity between different types of childhood brain tumours \textit{in vitro}. Of the four main tumour types received, 31% were low grade astrocytomas (20\% pilocytic and 12\% fibrillary), 25\% MBs, 9.8\% high grade astrocytomas, 8.4\% ependymomas and 3.6\% of PNETs. This was in agreement with clinical surveys (Farwell \textit{et al}, 1977; Duffner \textit{et al}, 1986; Stiller & Bunch, 1992; Finlay \textit{et al}, 1995), indicating that there was nothing unusual about the population of patients from which these samples were drawn. The age distribution of patients who comprised the present study appeared to be similar to those reported in other clinical surveys (Nazar \textit{et al}, 1990; Bailey, 1992). The peak age for patients with ependymomas was between 0 and 2 years, while patients with MB were older with a peak incidence between 6 and 8 years of age. The present study also supports the observation that MB were more common in male patients.

Of all the solid tumour types that have been grown \textit{in vitro}, cultures derived from adult malignant gliomas have an unusual propensity to give rise to established cell lines (Ponten & Macintyre, 1968; Ponten & Westermark, 1978). This characteristic has been capitalised on by many researchers and, to date, a large number of cell lines have been produced from these tumours for experimental study (Westermark \textit{et al}, 1973; Maunoury, 1977; Bigner \textit{et al}, 1981a, b; Rutka \textit{et al}, 1987c; Westphal \textit{et al}, 1990). However, the growth characteristics of paediatric brain tumours have not been so well studied and there are only a small number of medulloblastoma (Westermark \textit{et al}, 1973; Tamura \textit{et al}, 1989a; Bigner \textit{et al}, 1990a; Pietsch \textit{et al}, 1994), ependymoma (Nakagawa \textit{et al} 1983, Mihara, 1986) and paediatric glioblastoma (Rutka \textit{et al} 1987c) cell lines and there have been no reports of established cell lines from low grade astrocytomas described in the literature.

One major factor which will have hindered systematic studies on the \textit{in vitro} growth of paediatric brain tumours is the relative rarity of these tumours. The present study has been possible only because of access to material from a large paediatric neuro-oncology practice over a number of years.

The amount of material available from any one tumour may also be an important factor in determining the success rate in establishing cell lines from these tumours. In most
cases the MB biopsy sizes were very small compared to large specimens typically processed from adult gliomas and this might have reduced the number of tumour cells available for plating. However, other studies have shown that as few as 5x10^5 cells (0.5mg) are capable of producing vigorous short-term cell lines from childhood brain tumours (Mackillop et al, 1985).

In the present study, the range in cell yield per mg of wet weight of biopsies from MBs was 2.6x10^3 - 4.4x10^4 cells/mg wet weight, with a mean value of 2x10^4 and a median value of 1.8x10^4 respectively. Therefore the samples received in the present study provided fewer numbers of cells compared to other studies (Mackillop et al, 1985). Perhaps the MBs were less efficiently digested by collagenase treatment resulting in poorer cell yields or that there were fewer cells per unit weight (i.e., were less cellular). Of the other tumour types received, the high grade astrocytomas had the greatest range in cell yield/mg wet weight, e.g., 5.3x10^2 - 3.6x10^5, together with the greatest mean value (5.8x10^4). The high grade astrocytomas were sufficiently digested by collagenase treatment resulting in higher cell yields. This is similar to adult high grade astrocytomas where high cell yields are obtained. Enzyme treatment with collagenase does seem to produce larger numbers of cells from tumour biopsies than mechanical mincing and passing through needles with decreasing diameter (Mackillop et al, 1985). There was a marked difference in the cell yield per mg wet weight of the pilocytic and fibrillar astrocytomas received. The range of values for pilocytic astrocytomas was 1.7x10^3 - 5.9x10^4 (mean 1.4x10^4), whilst for fibrillar astrocytomas it was 1.8 - 6.8x10^3 (mean 3.6x10^3) respectively. The mean cell yield per mg wet weight of biopsies derived from the pilocytic astrocytomas was nearly 4-fold greater than that for the fibrillar astrocytomas (but the medians were of similar value, = 3x10^3). This could be a reflection of the location of the fibrillar astrocytomas. This could also be an explanation as to why the pilocytic astrocytomas were better at producing short-term cultures compared to the fibrillar variant. In addition, the cell counts following collagenase digestion were markedly different between the pilocytic and the fibrillar astrocytomas. One might have be expected a similar pattern between the high grade and the fibrillar astrocytomas, since the fibrillar variant is thought to be the more malignant type, compared with the pilocytic, being the less aggressive type. Cultures derived from ependymomas and neuroblastomas fell into the groups of tumours that had the higher ranges. For example, for ependymomas, the range of values was 4.6x10^3 - 5.6x10^4, with mean and median values of 3.1x10^4 and 3.6x10^4 respectively. For neuroblastomas, there was a smaller overall range and included 2.7 - 4.3x10^4 cell/mg wet weight. These cultures had the highest values for the mean and median, 3.7x10^4 and 4.1x10^4 respectively.

The vast majority of tumour biopsies processed by collagenase digestion in the present study produced vigorous short-term cultures, suggesting that mechanical treatment is not sufficient to release all the tumour cells from these tumour types, or that mechanical digestion may have reduced cell viability. However, established cell lines, Daoy, ONS-
series and MHH-MED-series were also mechanically prepared for in vitro growth without enzymatic digestion and went on to produce long-term cell lines (Jacobsen et al., 1985; Oakes et al., 1990; Pietsch et al., 1994).

With regard to the longer term success of these cultures, there may be a number of factors which are important in determining this. All tumour samples were grown in Ham's F-10 and supplemented with 10% FCS with added antibiotics. It is possible that the medium conditions were not appropriate for sustained growth of neoplastic cells in vitro. Researchers over the years have endeavoured to improve and perfect tissue culture media in order to provide the optimum nutrients and requirements for cell growth. Newborn or foetal bovine sera have been used universally as a supplement to cell culture media as these support growth of a wide range of cell types. In one study (Pietsch et al., 1994) DMEM with high glucose concentration (4500mg/l) supplemented with 10% human umbilical cord serum and with 4mM L-glutamine was effective in increasing proliferation of newly established cell lines of the MHH-MED series compared to the same medium supplemented with foetal calf serum. Using this human serum also enhanced proliferation of other established MB cell lines, including D283 and D341 and Daoy. These findings suggest that this human serum contains an important growth-promoting factor which enhanced MB cell proliferation in vitro, which is not present in foetal calf serum. It is possible that this is a peptide growth factor, although the addition of various human growth factors including EGF, PDGF (of all types), and both acidic and basic fibroblast growth factor (FGF) did not produce the same effects as the cord serum alone (Pietsch et al., 1994). Possibly the combination of two or more growth factors are important in producing this enhanced proliferation in MB cells in vitro. It could also be due to the presence of some other factor which is important in ensuring proper development of the foetus.

A further reason why MB cell lines which have established in culture are of neuronal rather than of glial lineage, because the glial lineage lacks an autocrine loop for self-renewal. Murphy et al (1990) showed that at least 50% neuroepithelial cells derived from embryonic mice continued to proliferate in the presence of FGF, in the absence of which they rapidly died in culture.

Unlike gliomas which tend to produce adherent cell lines, medulloblastomas in some instances produce cell lines which grow in suspension (Friedman et al., 1985; Bigner et al., 1990a; Pietsch et al., 1994). Initially, nearly all the MB cultures in the present study grew as floating aggregates and did not attach well to the culture vessel and this is in agreement with other studies (Westermark et al., 1973; Bigner et al., 1990a; Fults et al., 1992b; Pietsch et al., 1994). Pietsch et al (1994) found it to be an advantage if the floating aggregates were systematically removed from vessels where adherent cells were present, thus ensuring the continued growth of cells in suspension. In contrast, in the present study, even though these floating aggregates were transferred to agar-coated flasks, the suspension cultures failed to
produce a permanent cell line. Usually, the floating aggregates would attach to the base of the flask and cells would then grow from it but the floating aggregates progressively disappeared from the culture. Even after long periods of culture as floating cells in agar-coated flasks, these floating aggregates when subsequently transferred to flasks without agar, just stuck down and did not remain in suspension. This appears to be a very important difference between the cultures in the present study and those cited in the literature, where floating aggregates of medulloblastoma cultures did persist in long-term culture. Clearly, the present series shows that not all medulloblastoma-derived cultures sustain growth as suspension cell lines. Other tumour histologies also initially grew in suspension including astrocytomas and ependymomas, but, again, sustained growth on passaging into agar-coated flasks was not successful.

It is possible that growth in suspension may be a characteristic of certain medulloblastoma cell lines, although other cell lines from MBs have grown as adherent cells, for example, Daoy, and ONS-series of cell lines (Jacobsen et al, 1985; Tamura et al, 1989a) and one derived from a spinal PNET in the MHH-MED series (Pietsch et al, 1994). This may be related to the type of tumour, whether from a primary tumour or recurrent, or whether derived from metastasis. In this present study, 20/21 medulloblastoma samples were primaries and only one was a recurrent tumour, but none of these produced long-term cell lines and the recurrent tumour grew less well in culture compared to those derived from primary tumours. Of the Duke series of MB cell lines, only two were primary tumours, one was a metastasis and the other was derived from a CSF sample from a recurrent tumour. This was also similar to those derived from the MHH-MED series. In addition, a spinal PNET of the MHH-MED series (Pietsch et al, 1994) and the PF5K cell lines were derived from a PNET in the cerebral hemispheres (Fults et al, 1992b). Clearly, the site and type of tumour (whether primary or metastasis or recurrent) does not determine the success in producing established cell lines. The improved success rate seen using material from tumours which have either spread locally or metastasized, suggests that a subset of cells with the ability to grow in suspension may have been sampled for in culture. Another interesting finding from a study was that if the FGF concentration was increased, the cells altered from being non-adherent round cells to more flattened cell types which adhered to the substratum (Murphy et al, 1990). Possibly further studies with this growth factor may result in more MB cell lines being established which are adherent as opposed to growing in suspension.

A possible reason for the lack of continuous suspension cell lines from the MB tumour group in the present study could be that the cells were not initiated as xenografts in contrast to the cell lines from the Duke series and this method of culture initiation may predispose towards growth in suspension when they are subsequently grown in vitro as well as establishment in long-term culture.
It has been suggested that amplification of c-myc is an important prerequisite for establishment of MB cell lines in vitro (Bigner et al., 1990a) and such amplification has been documented in the established cell lines, D341, D384, D425 and xenografts of D382 (Bigner et al., 1990a). Cell lines D341, D384 and D425 and their xenografts showed 10-30 copies of the c-myc gene. In these cell lines amplification of other oncogenes such as N-myc, EGFR or gli was not found. However, Pietsch et al. (1994) detected amplification of c-myc in only 1/4 of established cell lines. The amplification of c-myc has been observed in other cell lines derived from childhood tumours, including neuroepithelioma cell lines where these lines were found to have high expression of c-myc which is thought to be related to an increase in copy number of chromosome 8 seen in these cell lines (Sacchi et al., 1991).

It might be argued that there are two types of established cell lines derived from MBs, those which are adherent cell lines, have no c-myc amplification and are of glial origin (i.e., Daoy), and those which include non-adherent cells which have c-myc amplification and are of neuronal origin (i.e., those of the Duke series). However, the results from this present study do not support this hypothesis, since the MB short-term adherent cell lines very often displayed neuronal characteristics in vitro since they expressed SYN and NF. However, about 50% of the MB cultures were also to some degree GFAP positive. Although, c-myc amplification of the ONS-series of established MB cell lines has not been reported, these are adherent cell lines which express NF. The adherent PSFK cell lines do not have c-myc amplification but do express nestin but not neurofilament proteins. This may suggest that establishment of cell lines from cerebral PNETs, involves another mechanism compared to MB where gene amplification and neuronal differentiation is a prerequisite.

Adult malignant astrocytomas grow well in culture, and about 50% will produce continuous cell lines (Darling, 1990). In the present study, however, it was not possible to establish a single cell line from the eight samples of childhood malignant astrocytomas available for study. Similar results have been reported by others (Westermark et al., 1973; Camins et al., 1980; Palmer et al., 1981) and there is only one glioma cell line (SF-188) derived from a child which has been adequately described in the literature (Rutka et al., 1987c).

In cultures derived from adult high grade astrocytomas, it has been suggested that there is a relationship between the morphology of the cells present in the initial primary culture and the subsequent establishment of a long-term cell line. If the dominant cell type was spindle-shaped, then the culture was likely to establish in culture, whilst those composed of astrocyte-like cells with cytoplasmic processes did not usually establish in culture (Bigner et al., 1981b). The high grade astrocytoma cultures in the present study did in some cases include cultures with spindle-shaped cells (in less than 50% of cultures) but these did not go on to produce established cell lines. In contrast to adult glioma-derived cell lines, in
the present study, morphology alone was not an important criterion in predicting the establishment of a cell line derived from paediatric high grade astrocytomas.

Some studies have shown that adult glioma cells with a homogeneous near diploid karyotype were stable in short-term culture but failed to establish long-term (Mark, 1971) whilst those with more complex karyotypes did produce permanent cell lines (Bigner et al, 1987). Possibly, the genetic profiles of paediatric high grade astrocytomas are less complex, with fewer abnormalities than their adult counterparts. C-myc amplification does not appear to be important in the establishment of cultures from high grade gliomas in adults, although the single GB cell line (SF-188) derived from an 8-year old boy with a GB in the right frontal lobe did show c-myc amplification (Trent et al, 1986). The content of c-myc RNA of this cell line was 2.5 fold and at least 20-fold higher than that found in RNA from fibroblasts and placenta respectively. This finding suggests that c-myc may be important for the establishment of cell lines from not just MBs but also astrocytomas and perhaps, paediatric brain tumours in general. Wasson et al (1990) also found a lower erbB1 amplification in glial brain tumours in children compared to adults, although this may be a reflection of the preponderance of lower grade glial tumours seen in the paediatric population. However, 1/2 GB displayed amplification of erbB1 gene which suggests that paediatric GB may have a similar frequency of erbB1 oncogene amplification to that seen in adults (Wasson et al, 1990).

There appears to be distinct genetic differences between high grade astrocytomas of adults and children despite their neuropathological similarities. Loss of sequences on chromosome 10 is restricted to glioblastoma multiforme in adults and seems to be a prerequisite for the development of this anaplastic tumour. Presumably, the genes lost on chromosome 10 are not involved in the development of the neuropathological appearance of this tumour as childhood malignant gliomas resemble their adult counterparts closely but do not have losses on chromosome 10 (Louis et al, 1993b; Rasheed et al, 1994). However, many biopsies of grade III astrocytomas in adults give rise to established cell lines and these tumours do not exhibit losses on chromosome 10, indicating that any genes important in determining cell immortalization in vitro are not located on this chromosome.

The CDKN2 gene, located on chromosome 9 which codes for cyclic dependent kinase inhibitor which is important in controlling the cell cycle, has been found to be deleted in a number of anaplastic astrocytomas and glioblastomas in adults but not in lower grade tumours (Giani & Finocchiaro, 1994; Jen et al, 1994; Schmidt et al, 1994) or in MBs and ependymomas (Jen et al, 1994). This deletion has also been reported very frequently in glioma cell lines (Kamb et al, 1994; Nobori et al, 1994). This suggests that deletions in the CDKN2 gene, may not only be important in the progression of low grade astrocytomas to the higher grades, but that this may confer a growth advantage in these cells in vitro. As this gene does not appear
to be deleted in most paediatric brain tumours which do not establish readily in vitro, it is tempting to suggest that this is one of the genes important in cell immortalization in vitro.

Another possible reason for being unable to establish paediatric astrocytoma cell lines in vitro could be due to the absence of self-stimulation by factors such as PDGF (Huang et al, 1986). Many adult malignant glioma cell lines express both PDGF and PDGF receptors. These findings indicate that an autocrine loop of growth stimulation involving PDGF may be present in glioma cell lines (Nister et al, 1988). Co-expression of PDGF genes and of PDGF-type B receptor in vivo and in human astrocytic biopsies has also been reported (Hermansson et al, 1988; Maxwell et al, 1990). Such results indicate that the presence of this autocrine growth loop may be important in tumour progression and perhaps important for in vitro growth as well. PDGF stimulates the proliferation of O2-A progenitor cells and has been shown to prevent them from differentiating prematurely into oligodendrocytes in cultures (Noble et al, 1988; Richardson et al, 1988). It was also observed that exposure to PDGF of O-2A progenitor cells in the presence of basic fibroblast growth factor (bFGF) resulted in cells undergoing continuous renewal without differentiating into oligodendrocytes (Bogler et al, 1990). There is no information available on the growth factor expression, or responsiveness on the single GB cell line derived from a paediatric tumour, and it remains a possibility that these cells do not possess an autocrine loop for self-renewal.

Ependymomas produced vigorous short-term cell lines but none went on to establish in culture. That there are only two established cell lines reported in the literature from this tumour group indicates that these tumours do not show a propensity for in vitro growth. A number of reasons could account for this, and may indeed include some of the above explanations for medulloblastomas and all astrocytomas from the present series of paediatric brain tumours. The in vitro characteristics of the two cell lines in the literature have not been comprehensively documented, e.g., whether these lines have c-myc or any other amplification to explain their immortalization, or whether alterations in other genes are involved.

Effects of substrata on the long-term growth of cells derived from childhood brain tumours

Over the years, researchers have investigated substratum requirements and defined media for many CNS-derived cell types (Bottenstein & Sato, 1980; Rutka, 1986; Westphal et al, 1987; Mori et al, 1991; Wikstrand et al, 1991). Pre-treatment of vessel surfaces with various components of the natural ECM, including COL, FN, LMN and a synthetic basement membrane, Matrigel and various synthetic polymeric amines have been described (Rutka, 1986; Westphal et al, 1987; Mori et al, 1991; Wikstrand et al, 1991).
The primary cultures in this present study displayed a spectrum of responses to the various components employed. In some cases, there would be vigorous growth on all surfaces, where cells attached to the substrates resulting in good yields especially between initial setup and first passage level (i.e., P₀-P₁). In other cases, most cells did not attach and just floated in suspension, whilst those cells that did attach ceased to proliferate and died. Some cultures did better on one particular substrate compared with another and within one tumour histology heterogeneity in response to the substrates was also observed. Growth was said to be successful if the culture reached passage level 3 without signs of deterioration and could be frozen in liquid nitrogen for future studies. The shortest period of time over which this occurred was 3 weeks and the longest was 6 weeks. All cultures grew on cell culture treated plastic-ware, and most grew on surfaces coated with FN, LMN and PLL. For example, at least 50% MBs grew to passage levels beyond 3 on PLL, LMN and FN, whilst for low grade and high grade astrocytomas, 75% or more of the cultures grew well on these substrates. However, only a small proportion of cultures grew successfully on Cell-tak (6/24) and vitrogen (8/13). Cells did not readily attach to glass and no cultures grew on Matrigel. All cultures grew on at least three surfaces, indicating that viable cells were present in the cell suspension prepared from these tumours.

Cells derived high grade astrocytomas did not grow well on Cell-tak but did grow well on vitrogen. In contrast cells derived from low grade pilocytic and fibrillary astrocytomas were observed to respond in a heterogeneous manner. For example, two cultures derived pilocytic astrocytoma grew well on vitrogen, whilst one derived from a fibrillary astrocytoma grew well on Cell-tak. Cells derived from MBs tended not to grow on vitrogen, but were not specifically inhibited from doing so on Cell-tak. The ependymomas were not specifically inhibited on either surface. Only three cultures (out of 13) grew on glass, one was derived from a MB, one from a neuroblastoma and one from a low grade pilocytic astrocytoma. None of the higher grade or fibrillary astrocytoma derived cultures grew on glass. With regard to histology, there was no consistent pattern observed in the small number of cultures which failed to grow on PLL, FN and LMN.

Westphal et al (1987) showed that 80% of primary adult glioma cultures showed a marked improvement in initial plating efficiency, colony formation and speed of attachment when plated on ECM derived from bovine corneal endothelium. Cells derived from high grade astrocytomas failed to attach to plastic while on the ECM-coated flasks they did. This study also showed that these cultures derived from samples following radiotherapy grew better on ECM than those which had not received this treatment. In addition, for the higher grade tumours, gradual lysis of the matrix was observed due to the release of proteolytic enzymes by these cultures (Westphal et al, 1987). These researchers also investigated the effect of ECM on cellular differentiation in vitro. They found that growth on ECM did not
change the expression of GFAP where it was present and this also applied to FN. However, in the present series of high grade gliomas of childhood tumours, there were individual cultures where growth on a particular substrate did change the expression of GFAP and FN. For example, one HGA (IN1419), when screened for GFAP on five substrates, showed differences in expression. When grown on plastic and Cell-tak, GFAP expression was seen in more than 50% of cells, but on LMN and PLL the expression was seen in less than 10% of cells, and on FN substrate, the cells were all negative. Another HGA (IN1495), when grown on plastic, FN, PLL, Cell-tak, Matrigel and vitrogen, was negative for GFAP but positive for FN in all cells respectively, except when grown on Matrigel, where the cells were found to be weakly positive for GFAP in more than 50% of cells. Clearly these results do not agree with those found for adult gliomas in which the ECM does not appear to influence cellular differentiation, while, in the present study, for HGAs, it definitely did. In contrast, cultures derived from LGAs, the expression of GFAP or FN was not influenced by growth on particular substrates (Westphal et al, 1987).

The results from the present study indicate that cells derived from medulloblastomas, both low (pilocytic and fibrillary) and high grade astrocytomas, ependymomas as well as neuroblastomas, attached and grew on surfaces coated with LMN and FN in preference to other substrates used and this is in agreement with data produced by others (Bogenmann et al, 1983). However, in the present study, there was a difference between pilocytic and fibrillary astrocytomas, where 7/10 (70%) pilocytic cultures grew better on plastic and LMN, in contrast to only 2/10 (20%) fibrillary cultures (the remainder having favoured FN with regard to enhanced and sustained growth). In addition, there was one culture derived from a fibrillary astrocytoma (IN1751) which grew consistently well on three surfaces, reaching passage level 21 on plastic, 22 on FN and 29 on LMN respectively. This pattern of growth was never observed with the pilocytic tumours.

Rutka et al (1987a) showed that MB cells attached to and grew on monolayers of leptomeningeal cells which produced an ECM composed of FN, LMN and COL types, I, II and IV. Fibronectin is a dimeric glycoprotein and is important in cell aggregation and attachment of cells to the substratum because of its affinity for COL. It has been reported that FN expression is lost to some degree in transformed glial cells (Bigner et al, 1981a; Sherbet et al, 1982) in both short and long-term cultures. Sherbet (1987) suggested that the absence of surface FN may explain the inability of human glioma cells to interact and adhere to the BM of capillaries, hence explaining the lack of systemic metastases from these tumours. The results from the astrocytoma-derived cultures indicate that FN is not lost from the surfaces of childhood astrocytoma cells and these cultures did well on vitrogen compared to the other tumour groups. This has been confirmed since the majority of cultures derived from both low (pilocytic and fibrillary) and high astrocytomas expressed FN (Tables 11c, 11d and 11e). Conversely, as virtually all the MB derived cultures were positive for FN, lack of
attachment to vitrogen is unlikely to be a consequence of the absence of surface FN on these cells. Obviously, other factors are important for these cells to enable attachment to a collagen derived substratum.

Both polylysine and FN have been reported to be important for the attachment and growth of cells derived from gliomas (e.g., U251MG) and neuroblastomas (B104 rat neuroblastoma) in culture (Bottenstein & Sato, 1980). On poly-D-lysine (PDL), cell lines U251MG and Daoy attached to and showed cell body extensions on this surface (Wikstrand et al, 1991), whilst in the present series, poly-L-lysine (PLL) inhibited cell spread and did not sustain growth of MB and ependymoma cell lines. In contrast, pilocytic astrocytoma derived cultures did well on this substrate reaching passage levels 10 and 11 in two cases and 7 in another two, whilst for the fibrillary lower passage levels were reached (passage level 4-5), suggesting that polylysine (both L and D isomers) is a good substrate for cell lines derived from astrocytomas but not all glial derived tumours. The present study indicated that cells derived from LGAs (both pilocytic and fibrillary) grew well on PLL (despite the fibrillary variant having grown less well), whilst those derived from HGAs did not. These differences may be related to the degree of differentiation of the cells. For example, PLL is an electrostatic substrate and the degree of adhesion to this substrate is determined by the electrostatic charge of the cells. It is possible that there is an association between the phenotypic differentiation of cells derived from different grades of gliomas and the surface electrostatic charge of the cells.

Long-term growth of some cultures was also affected by the substratum. For example, three MB, three low grade astrocytoma and three out of six ependymomas also grew to higher passage levels on LMN compared to plastic and FN. These cell lines showed enhanced sustained growth on LMN (reaching in some cases, nearly double that on either FN or plastic). The growth enhancing effects of LMN has also been reported in other human glioma cell lines derived from adults (SX763 and SX767) where the most rapid attachment in vitro occurred on LMN (Giese et al, 1993). This has also been observed for Daoy (Wikstrand et al, 1991), where growth was enhanced on LMN compared with other substrates. This finding is interesting, since it appears that LMN enhances the attachment and growth in vitro of cell lines of glial origin. LMN has been shown to be produced by early astrocytes in primary culture (Liesi et al, 1983) and is also synthesised by astrocytes after injury to adult CNS (Liesi et al, 1984) suggesting that this is a normal physiological response in some glial-derived cells, including MBs.

Cell adhesion to components of the ECM has been shown to be mediated by various receptors called integrins (Hynes, 1987). A number of integrins (receptors for LMN) have been reported for established cell lines and gliomas in situ (Gehlsen et al, 1988; Gladson & Cheresh, 1991; Bednarczyk & McIntyre, 1992; Malek-Hedayat & Rome, 1992).
The function of these receptors is to aid cells to attach to ECM but have also been shown to be involved in cell movement with the help of the cytoskeleton (Luna & Hitt, 1992). Cell lines derived from astrocytomas can be prevented from attaching to ECM components such as FN and COL with the appropriate integrin antibody (e.g., anti-β1) (Giese et al, 1994). Since the astrocytoma-derived cultures in the present study grew well on vitrogen, this suggests that these cultures possess integrins for this substrate, while the other brain tumour-derived cultures which did not grow on this substrate do not. Giese et al (1994) has shown that adult astrocytoma-derived cell lines could not be prevented from attaching to LMN, and in the present study, the paediatric brain tumours grew well on this substrate, suggesting that they possess at least one ligand for this substratum. This would be in agreement where numerous ligands for integrins have been discovered in LMN (Timpl et al, 1990). However, the specific integrins which mediate cell adhesion to LMN have not been identified and it maybe that another mechanism is involved for cells to attach to this substrate. Possibly a non-integrin molecule is involved in attachment to LMN and recently a receptor with a Mr 67,000 has been isolated (Sobel, 1993). Further studies are needed to determine if this molecule is important for attachment of astrocytes and glioma cells to LMN. This receptor molecule may also explain why the other tumour-derived cell lines from the paediatric brain tumours generally grew well on this substrate since a specific complex integrin is not required but that a simpler molecule, which may be more characteristic of childhood tumours rather than adult equivalents is expressed by these cells.

COLs are confined to connective tissue in gliomas in situ and these tumours are GFAP positive (Paulus et al, 1988). Primary cultures and cell lines derived from gliomas display marked deposition and synthesis of ECM components (Mc Keever et al, 1989; Paulus et al, 1993) but are GFAP negative. The development of mesenchymal features in vitro with simultaneous loss of glial differentiation has been termed "mesenchymal drift" and this change in differentiation in culture has been termed "mesenchymal differentiation" (Mc Keever et al, 1991).

Paulus et al (1994) showed that in situ glioma cells expressed β1 (common β chain of most ECM integrins) receptors but the ECM was restricted to vascular components. Early passage cells showed an increase in ECM components (COL rather than BMs), co-expression of the ECM components and GFAP by most cells and integrins β2 and β3 were upregulated in primary cultures. In 5th passage, GFAP decreased but COL expressing cells increased. Using spheroids, similar results were obtained as seen in situ but not in vitro. Therefore the different culture systems resulted in varied expression of the different ECM components and receptors and mesenchymal features of glioma cell lines, which was due to trans-differentiation of glioma cells.
Despite the fact that MB cultures attached to and grew on monolayers of leptomeningeal cells which had produced an ECM composed of FN, LMN and COL IV (Rutka et al, 1987a), in contrast, established MB cell lines did not attach to FN, LMN and COL, even though they expressed adhesion molecules, neural cell adhesion molecule (NCAM) and L1 (Wikstrand et al, 1991; 1993). However, Daoy attaches to COL IV, FN and LMN and an increase in cell body extensions was observed (Wikstrand et al, 1991). Also the lack of 5/6 established MB cell lines to attach and grow on components of normal leptomeningeal ECM (D-series) is thought to confirm their neuronal origin, whilst Daoy and an established glioma cell line (U251MG) indicate they are of glial origin (Wikstrand et al, 1991). The division into two distinct groups Daoy and D-series has been further confirmed by the antigenic profiles of these cell lines; Daoy is glial and the D-series are neuronal (He et al, 1989; 1991).

In the present study of the MB cell lines that grew better on LMN, one culture was not screened for any IFs, but the other two were found to be negative for GFAP and NF-M. However one of these cultures was found to be positive for GFAP when grown on FN substrate and more than 50% of cells were positive. Although, GFAP was not detected in all these cell lines, it could have been that the GFAP+ cells were unable to adapt to in vitro conditions, or that this expression was lost on passaging, even early on. Of the three low grade astrocytomas only one culture was found to express GFAP in a number of cells when grown on a number of substrates, including LMN and PLL and this is in agreement with other studies in which LMN and PLL appeared to maintain the in vitro proliferation of cells of glial origin (Wikstrand et al, 1991; Giese et al, 1993). Again further evidence was provided by the three ependymoma cultures in the present study, where all three expressed GFAP positivity, albeit only in less than 10% of cells, but all were positive on LMN and another additionally on PLL. However positive expression was also observed on Matrigel and Cell-tak. Although the majority of MB short-term cell lines in this study are more likely to be of neuronal origin (since the majority of established cell lines are so), the above three cultures show that there is a possibility for a MB to maintain dual lineage in vitro. Alternatively, a greater panel of markers is needed to determine accurately the origin of these cultures.

Cell kinetics

The cell kinetics of cell lines derived from brain tumours, including low and high grade astrocytomas, ependymomas, medulloblastomas and a small number of miscellaneous tumours have been studied by other researchers, and established glioma cell lines have been shown to have population doubling time ranges of between 24 and 96 hours (Manuelidis, 1965; Westermark, 1973; Maunoury, 1977; Bigner et al, 1981a). However, it should be noted that although the passage levels of some of these cell lines were as low as 25, in the majority of cases the passage levels ranged between 100-600. The single reported childhood glioblastoma cell line, SF-188 (Rutka et al, 1987c) had one of the shortest doubling times (28 hrs) and the
highest saturation density \((7 \times 10^6 \text{ cells/cm}^2)\) compared to cell lines derived from adult gliomas.

The range of cell doubling times in early passage (between passage levels 3 and 13) glioma cultures are considerably longer than that of established cell lines, in the region of 40-100 hours (Gerwick et al., 1977) and 60-264 hours (Shapiro et al., 1981). Pertuiset et al. (1985) showed that in a number of early passage cultures (between passage levels 1 and 14), the mean doubling times for five GBs was 53 hours (range 34-66 hrs), for two ependymomas was 46 hours (39 hrs and 53 hrs) and for two MBs was 83 hours (74 hrs and 92 hrs). The population doubling times of both low (pilocytic and fibrillary) and high grade astrocytomas in the present study had an overall range of 36-67 and 36-48 hours respectively, which is similar to that reported in other series. The mean population doubling time of HGAs in this present series, at 44 hours, was slightly shorter than the 53 hours reported by Pertuiset et al. (1985). In addition, the present study confirmed that low grade pilocytic and fibrillary astrocytomas are slower growing than their higher grade counterparts, where the mean population doubling times were 51, 67 and 44 hours respectively. Despite the fact that only a small number of cultures were studied (4 derived from pilocytic and 1 from a fibrillary astrocytoma) it was clear that fibrillary astrocytomas grew more slowly than cultures derived from the pilocytic astrocytomas (mean PDTs of 64 versus 50 hours respectively).

With regard to MBs, in the MHH-MED series (Pietsch et al., 1994) all cell lines were investigated at passage levels 25 or more, and the population doubling times for cell lines MHH-MED-1, 2, 4 and 5 were found to be 72, 119, 61 and 96 hours respectively (mean 87 hrs). PFSK 1 and 2C cell lines (Fults et al., 1992b) had doubling times of 30 and 48 hours respectively (mean 39 hrs) at passage level 85 or more. The population doubling times of established MB cell lines, including the D-series and others (range 19-83 hrs) were rather long and comparable to the population doubling times observed in the present series of short-term MB cell lines, where the range was between 24 and 120 hours. The overall mean of established MB cell lines is 63 hours and in the present study 59 hours, clearly showing a marked similarity of doubling times between cultures in the present series and those of established cell lines. Of the MB cultures, 4/5 had population doubling times between 50 and 82 hours and only one had a population doubling time of 24 hours (IN1527). In another series of short-term MB cell lines, the population doubling times were 74 and 92 hours, with a mean of 83 hours (Pertuiset et al., 1985), which is considerably longer than the present series.

The population doubling times of the ependymoma cultures in the present series were relatively long, with a mean of 68 hours and range of 36-96 hours compared to the limited number of previous reports, where the established cell lines KMS-II and NU-226 had doubling times of approximately 24 hours (Nakagawa et al., 1983; Mihara, 1986).
Population doubling times are important when considering in vitro chemosensitivity profiles of early passage cultures since many cultures have very long doubling times, and so assays should be performed for at least between 48-60 hours to allow for drug exposures and recoveries of at least one cell cycle (Pertuiset et al, 1985).

The saturation densities in this series of MBs had a mean value of $9.4 \times 10^4$ cells/cm$^2$, which is much lower when compared to established cell lines, like Daoy ($1.15 \times 10^6$ cells/cm$^2$) and lower than PFSK-2C ($8.7 \times 10^5$ cells/cm$^2$). Established glioma cell lines have saturation densities in the region of $9.7 \times 10^4$ - $1.1 \times 10^5$ cells/cm$^2$ (Izumu et al, 1993) and those of the SF series, were between $5-7 \times 10^6$ cells/cm$^2$ (Rutka et al, 1987c). In the present series of short-term cell lines derived from both low pilocytic and fibrillary and high grade astrocytomas the values were not within this range. However, the mean was similar for both pilocytic and high grade astrocytomas ($1 \times 10^5$ cells/cm$^2$). The saturation density of the culture derived from a low grade fibrillary astrocytoma ($1.9 \times 10^4$ cells/cm$^2$) were lower than those derived from pilocytic and the higher grade tumours. Also, the pilocytic tumours had a mean saturation cell density which was 5.2-fold greater than that found for the fibrillary tumour. In fact, the fibrillary astrocytoma derived cultures had the lowest saturation cell density of all the different tumour types studied. One cell line that did reach saturation density greater than $1 \times 10^5$ cells/cm$^2$ included an ependymoma. The established ependymoma cell line, KMS-II was found to have a saturation density of $2.2 \times 10^5$ cells/cm$^2$.

The short-term cultures in this series reached either similar or much lower saturation densities than those reported in the literature, particularly those established from adult gliomas and this may indicate that differences between adult and paediatric brain tumours extends to their kinetic properties in vitro, although, it must be borne in mind that all the cultures in the present series were at considerably lower passage levels than the established cell lines used in the other series.

In the present study overall, substrate did not appear to have a significant effect on the cell kinetics of some cultures derived from childhood brain tumours, indicating that those cell populations that grew on any one of the three substrates included the same population of cells that adapted well to the in vitro conditions, thus resulting in similar population doubling times. However, there was a marked difference between the pilocytic and fibrillary astrocytomas. For example, for the former type, there appeared to be a distinct difference in the PDTs on all three surfaces, values of 34, 85 and 44 hrs on plastic, FN and LMN respectively. In contrast, the fibrillary derived cultures showed no such variation, having values which were similar, i.e., 79, 72 and 65 hrs on plastic, FN and LMN respectively.
Markers for cell types of the CNS

Employing cell-specific markers with immunocytochemical techniques has proven to be of great importance in being able to distinguish the antigenic profiles of different types of normal cells in culture derived from the CNS (Kennedy, 1982). Such markers have been used to distinguish between the major lineages of cells present in cultures of the normal CNS. However, caution must be exercised when working with tissues derived from tumour samples since explanted CNS tissue may contain normal cells derived from the endothelium or entrapped normal cells (Collins, 1983). Another problem is that transformed glial cells derived from tumours may not express the same antigens as the normal cells from which the tumour cells were presumably derived. An illustration of this is the comparative rarity of GFAP positive cell lines derived from malignant astrocytomas. Whether this is because GFAP positive cells were unable to establish in vitro because GFAP negative tumour cells have a growth advantage, or GFAP expression is down-regulated for some reason in vitro. However, there are other glial associated markers which can be used in addition to GFAP, such as glutamine synthetase. In addition, many cell lines derived from brain tumours in culture may have changes in ploidy and structural chromosome changes, which in turn can result in the abnormal expression of CNS markers.

Desmin
Desmin is the IF that is expressed by cells of muscle origin. Therefore one would not expect to see this expressed in brain tumours. However, desmin was apparently expressed in isolated cells in cultures derived from MBs in the present study, but not in other tumour types and such expression has been reported elsewhere (Bonnin & Rubinstein, 1984; Molenaar et al, 1989; Gould et al, 1990). Desmin expression has been documented in frozen sections and in primary cultures of newborn rat brains (Dahl & Bignami, 1982) and was found to be co-localised with GFAP. Unexpected expression of IF proteins, particularly desmin has been reported in a number of glial-derived tumours, including five astrocytomas, three oligodendrogliomas and six GBs (Franke et al, 1991). It was observed that the majority of tumour cells expressed desmin in astrocytomas and oligodendrogliomas, although expression was more heterogeneous in GBs (Franke et al, 1991). However, in one study only 1/23 gliomas (including low grade, anaplastic astrocytomas, GB, ependymomas among others) was found to express desmin and that was a GB (Hirato et al, 1994). It seems likely that the apparent expression of desmin in some MB cultures is because desmin antibodies recognise cross-reacting epitopes in GFAP, thus mimicking desmin expression in gliomas (Bilzer et al, 1989; Dahl et al, 1989).

Cytokeratin
Cytokeratin expression is thought to be specific for epithelial cells and recognises tumours derived from such lineages, and should not be expected to be expressed in the brain tumour cultures studied. However, this antigen has been found to be expressed in non-epithelial
tumours (Sime et al, 1989) and in differentiated neuroectodermal tumours (Mork et al, 1988; Ng & Lo, 1989).

In the present study this antigen was expressed in all ependymomas, but only in a small proportion of cells (= 10%). However, expression of this antigen in ependymomas in situ is uncommon with only 1/17 cases of ependymomas positive for this antigen (Mannoji & Becker, 1988), although cytokeratins (acidic and basic) are expressed in normal ependyma (Sarnat, 1992).

The incidence of cytokeratin expression in the MB cultures in the present series appears to be quite high (8/16 were positive) compared to some series, where only a small proportion of tumours expressed this antigen (Grant et al, 1988; Molenaar et al, 1989; Patt & Zimmer, 1992), and in other series no expression has been observed (Cruz-Sanchez, 1989, Sime et al, 1989). In the present series it was found that substrate determined the level of cytokeratin expression, for example, 8/16 MBs expressed this antigen in less than 50% of cells when grown on plastic and 1/2 cultures on glass. A third of MB cultures were positive again to the same degree on LMN. In contrast the same level of expression was found to be in 1/4 on Cell-tak and PLL and only in 1/5 on FN.

Extensive cytokeratin positivity of different molecular weights has been documented in childhood astrocytomas of all grades in situ (Bodey et al, 1991) and, in the present study, expression was observed to varying degrees in 4/6 high grade astrocytomas and 5/11 low grade astrocytomas. On glass (50%), on FN and PLL (=30%) and on Cell-tak, LMN and plastic (25% or less) were positive in less than 50% of cells stained. Vitrogen and Matrigel appeared not to support the growth of cytokeratin positive cells. However, there was a difference between the two variants, where 4/8 (50%) pilocytic derived cultures expressed this antigen to a certain degree, whilst for the fibrillary, only 1/3 (33%) of cultures expressed cytokeratin and this on only one surface (FN). The HGAs expressed cytokeratin in more than 50% of cells stained in half or more cultures screened on plastic, LMN and FN. Also 1/4 on LMN and 1/2 on glass positive expression was seen in more than 50% of cells stained respectively, in this tumour group. This would be in agreement with other studies where cytokeratin expression has also been documented in adult gliomas (Cosgrove et al, 1989; Ng & Lo, 1989).

Unexpected staining in other tumour types was seen in 2/3 NB cultures. Since all the paediatric cultures expressed this antigen to various degrees, it could be that the culture conditions encouraged the cells to differentiate along an epithelial lineage. It has been reported that diverse transformed cell lines of non-epithelial origin have rare cells that emerge spontaneously which, in addition to VIM, synthesize certain cytokeratins (Knapp & Franke, 1989). Although IFs are useful markers in general for the tissue of origin of a highly differentiated tumour cell population, they are not universal indicators of tissue
differentiation and so may account for the unexpected expression of cytokeratin in the present study of childhood brain tumours. This further emphasises that depending on the tumour type and tissue of origin a large panel of markers is needed to determine correctly the origin of these neoplastic cells in vitro.

Cytokeratin expression changes when cells are transferred to in vitro conditions, where they may express a broader spectrum of keratin polypeptides (Breitkreutz et al., 1981). Usually some of the tissue-specific keratins are reduced under in vitro conditions, while more keratins of the hyperproliferative and simplified epithelial type, i.e., those unusual for the tissue of origin, are expressed (Fusenig et al., 1991).

**Fibronectin**

FN is a non-collagenous component of ECM (Vaheri & Mosher, 1978). It appears to be present in higher levels on transformed, neoplastic fibroblasts, compared to their normal counterparts (Vaheri et al., 1976; Vaheri & Mosher, 1978). FN has not been detected in adult brain or in gliomas in situ (Schachner et al., 1978; Chronwall et al., 1983). Using immunocytochemical techniques, detection of surface FN has been unsuccessful (Vaheri et al., 1976; Franks & Burrow, 1986). Additionally, an inverse reciprocity exists between FN and GFAP expression by glial cells in vitro (Paetau et al., 1980; Westphal et al., 1990).

Cultures derived from MBs, PNETs, ependymomas and neuroblastomas expressed FN in all cells irrespective of the growth substrates. However, the cultures derived from low (pilocytic and fibrillary) and high grade astrocytomas showed marked variation in expression of this antigen. Although double staining was not employed, there were a number of cultures derived from low and high grade astrocytomas cultures which expressed GFAP and FN. On plastic, 7/11 LGA-derived cultures (5 pilocytic and 2 fibrillary), all cells were positive for FN and also expressed GFAP to varying degrees, while the remaining 4/11 cultures (3 pilocytic and 1 fibrillary) were negative for GFAP, but positive for FN. However, in some cases it appeared that mutually exclusive expression of FN and GFAP could occur. For example, a HGA culture, IN1566 was found to be negative for FN and GFAP on vitrogen and Cell-tak, but positive for FN and negative for GFAP on PLL, LMN and FN. On plastic the culture was GFAP positive and negative for FN. The LGA culture, IN1567 (fibrillary), was negative for both GFAP and FN on FN, LMN, PLL, Cell-tak, vitrogen and glass but was positive for FN on plastic and GFAP negative, indicating that depending on the substrate used, different cell populations were encouraged, or that the interaction between surface coating and the cells' surface, modulated GFAP expression.

Ependymomas, grown on LMN, PLL, Cell-tak, Matrigel, vitrogen and glass, expressed FN strongly in all cells and GFAP to varying degrees, while on plastic and FN, there was clearly a different pattern of expression, in which all cells were positive for FN but only one
or two cultures expressed GFAP in a small number of cells (= 10% or less). This sort of pattern was also seen with neuroblastoma cultures, where on plastic and FN substrates, all cells expressed FN but only 1/2 cultures expressed GFAP and that was very weak and in very few cells. In contrast to ependymomas, the neuroblastomas, were positive for FN and negative for GFAP on LMN, Cell-tak and glass substrates.

However, one should be aware when using immunocytochemical techniques for assessing FN production by cultured glial cells, that one should be able to distinguish between those cells which produce FN and those which bind it to their cell surface or internalise it. Primary cultures may also contain cells other than neoplastic glial cells (e.g., leptomeningeal cells) which may express FN.

**GFAP**

GFAP is the most widely used reliable marker of astrocytes. *In situ*, gliomas express GFAP in a manner which is inversely proportional to cellular anaplasia, i.e., the better differentiated cells expressing more GFAP (Lolait *et al.*, 1983). Cell lines of astrocytic origin such as high grade astrocytomas *in vitro*, express this antigen in only a limited number of cell lines. For example, of 13 high grade glioma-derived cell lines screened only one cell line, U251MG was found to express GFAP at a high passage level (Bigner *et al.*, 1981a) and this has been mirrored by other studies (Maunoury, 1977; Rutka *et al.*, 1987c; Westphal *et al.*, 1990).

Heterogeneity of established glioma cell lines with respect to GFAP expression has been documented, where one cell line (U251MG) was composed of 80% GFAP positive cells compared to a subline U251MG sp (with spindle cells) where all cells were GFAP positive (Bigner *et al.*, 1981a). In addition to variations between different tumour samples, Yung *et al.* (1982) showed that clonal cells derived from the same tumour biopsy, not only had different morphologies but also differences in the proportion of GFAP positive cells.

Primary cultures from malignant astrocytomas can have a large proportion of cells expressing GFAP, but with repeated passages, the number of GFAP cells decreases and may be absent altogether by passages 5 or 6 (Lolait *et al.*, 1983; Kennedy *et al.*, 1987; Mc Keever *et al.*, 1987). In short-term cultures from malignant astrocytomas, the dominance of a GFAP-/FN+ aneuploid cell population has been documented (Kennedy *et al.*, 1987). It appears that there is a gradual decrease in the number of either normal glial cells or GFAP positive neoplastic cells, being replaced either by less well differentiated neoplastic cells or a gradual de-differentiation of cells. In some cases it may be that cultures prepared as explants retain GFAP positivity and this is present in a greater number of cells and for longer *in vitro* compared to those prepared using a digestion technique (Darling, 1991). This may account for the lack of GFAP cell lines from the paediatric tumours cultured, particularly from the astrocytomas.
One might expect to see GFAP expression in all tumours derived from both low and high grade astrocytoma cell lines, since they are of glial origin. However, GFAP expression is inversely proportional to malignancy, where the lower grade astrocytomas express this antigen to a higher degree than their higher grade counterparts. It is interesting, that cells derived from both high and low grade astrocytomas in the present series of paediatric astrocytomas expressed GFAP. All cell lines derived from the HGAs expressed GFAP to varying degrees on at least one substrate as compared to only 7/11 cultures from the low grade group. For a number of HGA cultures the substrate was not a significant factor in regard to GFAP positivity, where for example, cell lines, IN1419, IN1523 and IN2080 expressed GFAP on 4/5, 3/5 and 2/2 surfaces respectively, whilst for other cultures such as IN1495, IN1566 and IN2087, positivity was observed when grown on a particular substrate (1/6, 1/7 and 1/3 coated surfaces respectively). The substratum on which the culture was positive was different in all three cell lines, i.e., Matrigel for IN1495, plastic for IN1566 and laminin for culture IN2087. One reason for such high GFAP expression could be the low passage levels at which these lines were screened, since the majority of cell lines were screened at passage levels no greater than 5. This indicates that there are some GFAP neoplastic cells in these HGAs which are capable of sustained growth in vitro. These results are in agreement with one study where GFAP positivity was observed in cultures at passage level 11 derived from paediatric astrocytomas (Mapstone & Galloway, 1991).

In contrast, cultures derived from LGAs (pilocytic & fibrillary) expressed less GFAP than the higher grade tumours. 4/11 cultures were negative for GFAP when grown on either 7, 6, 4 surfaces or just one substrate. However, consistency in expression in this group was more evident than that seen in the high grade group, where 4/11 cultures were positive for GFAP to varying degrees on the surfaces on which the cultures were grown and included, IN1524 (pilocytic, 6 surfaces), IN1751 (fibrillary, 5 surfaces), IN1930 (fibrillary) and IN2110 (pilocytic, each 3 surfaces). The effect of substratum on GFAP expression appeared to be less prominent than that for HGAs. One reason for the lack of GFAP positive cells in cultures derived from paediatric low grade astrocytomas could be that this population of cells cannot adapt to growth in culture conditions or that GFAP-negative cells dominated the GFAP-positive cells in culture. The major difference between the pilocytic and fibrillary tumour types, was that in the fibrillary cultures a greater number of cells were GFAP positive (i.e., >10%), compared to the pilocytic tumours (<10% GFAP+). In addition, in three fibrillary derived cultures, >50% of cells were positive for GFAP when grown on plastic and FN compared to the pilocytic tumours where no such expression was seen. This is in contrast to a recent finding where in low grade astrocytomas (including both pilocytic and non-pilocytic types), pilocytic tumours had fewer GFAP and A2B5 positive cells and grew slower than fibrillary astrocytomas, indicating that these two types of tumours arise from a different astrocytic lineage (Pollack et al, 1995).
Because of the bipotential nature of MBs one might have expected to see GFAP expression in some cells within cultures derived from these tumours. GFAP was not always found in MB cell lines, although such expression has been documented in biopsies (Palmer et al., 1981; Molenaar et al., 1989). Half of the MB cultures screened in this present series were found to have a number of GFAP positive cells present in vitro and is in agreement with results observed in sections of tumours (Zeltzer et al., 1990; Goldberg-Stern et al., 1991) and in vitro (Mapstone & Galloway, 1991). Of the 8/16 MB cultures investigated, three cultures were positive to varying degrees for GFAP when grown on surfaces (IN1562 (1/1), IN2062 (3/3) and IN1405 (3/3) respectively). Of the remaining cultures, IN1545 (1/4), IN1583 (4/5), IN1782 (2/3) and IN2085 (1/3) positivity was observed on at least one or more surfaces. The intensity of GFAP expression ranged from being weakly positive in more than 50% of cells or strongly positive in less than 10% of cells. No particular substrate appeared to dominate in terms of supporting GFAP positive cell growth, but the range of substrates included plastic, FN, LMN PLL and Cell-tak.

GFAP positive cells were present in the initial brain biopsy specimen of MB from which the cell line D283 was derived. However, GFAP expression was not present in the cell line or in subcutaneous xenografts of the cell line. Such observations may be an indication that GFAP positive cells in MB biopsy specimens and in vitro are reactive astrocytes or remains of degenerated astrocytes which have been phagocytosed by macrophages (Schindler & Gullotta, 1983). However, there is evidence that GFAP positive cells are in fact neoplastic cells and are present in at least some MBs in situ.

In the ependymoma cultures, positivity was seen on at least one surface but never on all surfaces. GFAP expression was seen to varying degrees in less than 10% to more than 50% of cells, but such positivity was only weak, unlike astrocytomas where it was consistently stronger. Positive expression of GFAP has been reported in ependymomas in situ but not in cell lines derived from them in vitro (Duffy et al., 1979; Cruz-Sanchez et al., 1988; Mannoji & Becker, 1988). Cell lines IN1497, IN1563, IN1759 and IN1932 contained positive cells to varying degrees on 6/8, 4/6, 2/3 and 2/3 surfaces respectively. But cultures IN1638, IN2030 and IN1594 were found to be positive for GFAP on 1/2, 1/2 and 2/5 surfaces respectively. In the case of these tumours, the substratum appeared to have an influence on the expression of this antigen. Culture IN2030, although grouped with the ependymomas, is an ependymoblastoma and is regarded as part of the PNET groups of tumours (i.e., a PNET with ependymal differentiation), and so expression of GFAP is therefore not unusual. One out of three NBs was negative for GFAP when screened on 5/5 surfaces, but cultures IN1921 and IN2059 were found to be positive for GFAP on 1/1 and 1/3 surfaces. This is an unusual finding since such tumours are of neuronal origin and such expression is not expected. GFAP expression in these tumours has been reported in situ (Kemshead et al., 1983).
Vimentin

VIM is usually not or only weakly expressed by mature CNS astrocytes, although, VIM expression usually precedes GFAP expression during development (Dahl et al., 1981). In vitro, VIM has been reported to be expressed in human glioma cell lines (Paetau et al., 1979) and in nearly all astrocytomas screened (Herpers et al., 1986; Schiffer et al., 1986). VIM is also expressed in MBs (Cruz-Sanchez, 1989; Sime et al., 1989; Pietsch et al., 1994) and in both low and high grade astrocytomas (Roessmann et al., 1983; Bodey et al., 1991; Kashima et al., 1993). It has been suggested that commencement of VIM synthesis in astrocytes coincides with the initial events leading to malignant transformation (Herpers et al., 1986).

Co-expression of VIM and GFAP has been reported (Sotelo et al., 1980; Osborn et al., 1981). Quinlan & Franke (1983) showed that cultured glioma cells synthesised GFAP and VIM, suggesting that a close relationship exists between the two IFs. This has been confirmed where co-expression of these two IFs was observed (Paetau et al., 1979). Although double staining was not performed in the present study, cultures where there was VIM positivity in the majority of cells also had a proportion of cells that were GFAP positive, indicating that co-expression in some of the cultures screened may have been present and this included cultures derived from low and high grade astrocytomas, MBs and ependymomas. Also, Bilzer et al. (1991) reported also that as cultures are passaged in culture and lose their GFAP expression, the levels of FN and VIM expression increase. In the present study, the astrocytoma-derived cultures may have supported this hypothesis, since nearly all these cultures expressed VIM and FN. In addition, there was a marked difference between the pilocytic and fibrillary derived astrocytoma cultures with regard to VIM expression. For example, all cultures from the pilocytic type were found to be positive for this antigen on all surfaces, whilst those derived from fibrillary astrocytomas heterogeneity in expression was observed (i.e., cultures were either positive in >50% of cells or negative). Substrate also appeared to influence the phenotypic profile of one fibrillary derived culture (IN1567), where positive expression was found when cells were grown on FN, LMN and VT, but not on plastic, PLL, CT and glass. This pattern of expression was found in a single fibrillary derived culture, which was different from the remaining fibrillary cultures. It is interesting that this aberrant culture was derived from a tumour located in the cerebellum rather than the cerebral hemispheres.

Expression of VIM in epithelial tumour cells in vivo is a feature thought to be specific for cells of mesodermal origin and characteristic of mesenchymal cells such as fibroblasts and endothelial cells (Banks-Schlegel et al., 1985). This expression may be associated with regulatory defects in differentiation (re-expression of embryonic phenotype) or it may represent a random event as a consequence of genetic abnormalities in tumour cells which affect the expression of differentiation-related genes.
Neurofilaments & Synaptophysin

Neurofilament proteins are neuron-specific gene products that are expressed by nearly all neurons with long projecting axons (Trojanowski et al., 1986; Schmidt et al., 1987). Therefore in the present study only tumours derived from this lineage would be expected to express this antigen, namely neuroblastomas and some or all of the MBs. Indeed, all three NB cultures expressed this IF to varying degrees. Also, as expected, twelve out of sixteen (75%) MB cultures were positive for NF-M, although this expression ranged from less than 10% to more than 50% of cells. Such expression has been reported in established MB cell lines, including the Duke series (He et al., 1989) and ONS (Tamura et al., 1989a) series, although not by the MHH-MED series (Pietsch et al., 1994). In the Duke MB series, D283 and D341 appear to be composed of cells with an advanced stage of neuronal differentiation as they express higher levels of NF-H and NF-M compared to NF-L. In vitro the two subunits, NF-H and NF-M were present in much higher percentage of D283 cells than NF-L whilst in vivo, all three subunits were present. These cells therefore, expressed the IF proteins of committed embryonic and mature non-dividing neurons. Cell line D341 (Friedman et al., 1988a) was found to be negative for NF-L and only a small population of cells expressed NF-M and NF-H. Daoy on the other hand did not express either NF or GFAP (He et al., 1989), but did express other neuroectodermal and glial associated antigens, indicating that Daoy cells were of a glial rather than neuronal lineage.

Immunocytochemical studies have shown that when MBs screened for NF and GFAP, 50% showed glial, 25% showed neuronal and 13% showed both glial and neuronal differentiation (Roessmann et al., 1983). It is assumed that MBs are derived from a pluripotential cell which has the ability to differentiate down either a glial or neuronal lineage (Rorke, 1985). In the present study, a small number of MB cell lines are composed of cells some of which express GFAP and some NF. Although double staining was not carried out, this data provides further evidence for the bi-modal differentiation potential of MB (Cruz-Sanchez et al., 1989; He et al., 1989; Gould et al., 1990).

The presence of synaptophysin and NFs can distinguish MBs from other brain tumours. Expression of synaptophysin (a panneuroendocrine marker) in MBs (Gould et al., 1987; Schwechheimer et al., 1987; Molenaar et al., 1989) suggests a close association between neuroectodermal and neuroendocrine tumours. There is evidence to presume that PNETs and peripheral neuroectodermal tumours are "neural types of neuroendocrine tumours" (Tischler et al., 1977; De Lellis & Wolfe, 1983; Gould et al., 1987) and this is further supported by demonstrations of a number of neuropeptides in the same MBs (Gould et al., 1990). Synaptophysin was detected to varying degrees in a number of cell lines screened derived from MBs and is a marker frequently observed in biopsies of primary MB tumours (Schwechheimer et al., 1987). Since MB cultures were positive for synaptophysin this indicates that these cultures represented early stages of neuronal differentiation.
A number of MB cell lines have shown to be of neuronal lineage (D283, D341, D384 and D458). These cell lines grew in suspension and formed 3-D aggregates spontaneously and all have been shown to express the neuronal marker, nestin (Tohyama et al, 1992). In addition these cell lines also expressed synaptophysin and nearly all expressed the three forms of the neurofilament proteins but have been shown to be negative for GFAP or Class I or II MHC antigens. In contrast to gliomas, these cells also displayed ganglioside expression which is similar to other neuroectodermally-derived tumours. On the other-hand, Daoy has been shown not to express any neuronal markers but has been shown to express glial markers. Daoy differs from the aforementioned cell lines in that it is an adherent cell line and such a difference may account for the differences in phenotypic expression. Thus Daoy may represent a MB which has differentiated along a glial lineage but that of a very immature stem cell prior to nestin expression and so this cell line may not be a true representative of a MB (Tohyama et al, 1992).

Cell lines ONS-76 and ONS-81 (Tamura et al, 1989a) have been shown to express neurofilament but not GFAP or S-100 antigens. They grew as adherent lines and when exposed to IFN-γ, expressed Class II MHC antigens, possibly indicating glial rather than neuronal differentiation. Such findings therefore confirmed that MBs originate from a bipotential cell that has the ability to differentiate along either a glial or neuronal lineage (Tamura et al, 1989a). Most authors have concluded that MB cells in vitro exhibit predominantly a neuronal phenotype (Trojanowski et al, 1987; He et al, 1989; He et al, 1991). Cell lines derived from MBs in the present study confirm this. As a group of tumours, MBs appear to be less mature than NBs in the PNS (Molenaar et al, 1990). Except for Daoy, the D series and the ONS cell lines showed the molecular phenotype of immature neurons (Trojanowski et al, 1992, 1994).

Recently, metabolic studies of human PNET cell lines by proton nuclear magnetic resonance spectroscopy, have indicated that there is a difference between cell lines derived from cerebellar MBs and PNETs in their expression of N-acetyl aspartate (NAA) and aspartate (Florian et al, 1997). Earlier reports have indicated that NAA is a neuronal marker (Gill et al, 1990; Urenjak et al, 1992). Cell lines MHH-MED4 and Daoy, although lacking NF expression, expressed the highest amount of NAA (Florian et al, 1997). It was also found that expression of NAA by MB cell lines, was inversely related to the extent of neuronal differentiation observed by immunocytochemistry (Pietsch et al, 1994; Florian et al, 1997). These findings suggest that their cell of origin may be one of a progenitor cell with an oligodendrocyte type 2 astrocyte cell phenotype (Florian et al, 1997).

Surprisingly, some expression of NF-M was found among the astrocytoma cultures, where in 3/8 (38%) and 2/6 (33%) of pilocytic and high grade astrocytomas were weakly positive in less than 10% of cells respectively. In contrast, the three cultures derived from
fibrillary astrocytomas were negative for this antigen. Expression of the three triplet subunits has been reported (though not simultaneously) in childhood astrocytomas of various grades (Bodey et al, 1990). Cultures where there was GFAP staining, also showed NF-M expression, although this may not occur in the same cells and this was observed in astrocytomas in situ (Bodey et al, 1990). However, it is not known whether such expression is due to normal, neoplastic derived or trapped intratumoral neuronal elements. A single ependymoma culture (IN1563) showed very weak expression of NF-M.

**Chemosensitivity**

A major aim of this project was to develop cell lines from paediatric brain tumours which can be used for therapeutic investigations. Cultures were screened against three drugs which are known to be clinically effective against adult brain tumours (Edwards et al, 1980; Friedman et al, 1988b) and which are often components in regimens used in large scale clinical trials against childhood brain tumours. The three drugs used in this study have different modes of action. CCNU is a small, lipid soluble alkylating agent which has the ability to cross the blood-brain-barrier (BBB). PCB is also an alkylating agent but has methylating activity, and like CCNU, can also cross the BBB. VCR on the other-hand has a limited capacity to cross the BBB and acts by blocking mitosis. The activity of PCB in vitro has lead to some controversy, as this drug in vivo requires activation by liver microsomes. However, a number of studies have shown in vitro activity of PCB against cultures derived from a number of tumour types including, brain tumours (Morgan et al, 1983; Thomas et al, 1985), suggesting that activation of this drug can occur either spontaneously in solution or that certain cell types can activate this drug.

Komblith et al (1981) determined the cytotoxic effect of BCNU on short-term cultures derived from both low and high grade adult astrocytomas. The range, mean and median for both groups was nearly identical, where for the LGAs, the ID$_{50}$ range was 3.9-22.4µg/ml, the mean 10.14µg/ml, and the median 7.7µg/ml, while for the HGAs, it was 3.4-23.8µg/ml, with a mean of 10.5µg/ml and a median of 7.9µg/ml (Komblith et al, 1981). The studies indicate that there is very little difference in the chemosensitivity profiles between adult low and high grade astrocytomas in vitro to BCNU. A similar result was obtained, when a microtitre assay was used to screen 120 early passaged cultures derived from human gliomas against CCNU and the median ID$_{50}$ was 9.5µg/ml (Darling, 1985).

In the present study of paediatric astrocytomas, the same sort of pattern has been observed where the mean ID$_{50}$ values for CCNU for the low grade pilocytic tumours was 8.2µg/ml and the high grade tumours was 8.5µg/ml respectively. These data are remarkably similar to that observed for adult low grade and high grade astrocytomas. Using a clonogenic
assay cells from an astrocytoma grade I culture derived from a child, the ID$_{50}$ value for BCNU was 5μg/ml (Mackillop et al, 1985). The range of ID$_{50}$ values for both low pilocytic and high grade astrocytomas in the present study of paediatric tumours was similar, range being 5.5-11μg/ml and 8-11μg/ml respectively. Thus the results from both adult and the paediatric population of astrocytomas compare similarly in terms of their *in vitro* chemosensitivities to nitrosoureas.

It is interesting to note that despite differences in the method of cell preparation which must occur in different centres and the differences in the assay methodology, the ranges and mean ID$_{50}$ values for nitrosoureas of human high grade gliomas in adults are remarkably consistent between studies. Similarly, it is striking that there is seemingly little difference between the chemosensitivities of high and low grade pilocytic astrocytomas whether derived from children or adults *in vitro*.

Tomlinson et al (1991) screened short-term cell lines derived from MBs against a number of drugs for 24 hours and found that 7/12 tumours were sensitive to BCNU (mean and median ID$_{37}$ was 3.3μg/ml and 2.0μg/ml respectively). The mean and median CCNU ID$_{50}$ values for cultures derived from MBs in the present study were 8.4μg/ml and 6.4μg/ml respectively and the range was from between 2.8 to 13.1μg/ml. Clearly, the results with BCNU show a marked sensitivity to nitrosoureas compared to the present study of MBs, but this difference is due to the ID value cut-off point. In the BCNU study (Tomlinson et al, 1991) the cut-off point recorded was only for 37% cell kill compared to the present study, where the cut-off point was for a 50% cell survival compared to the untreated controls. Daoy in the present study was also found to have a similar sensitivity to CCNU as those derived from the short-term cultures derived from MBs, and the mean CCNU ID$_{50}$ was 8.2μg/ml. This illustrates that the established cell line, Daoy, responds in a similar manner to short-term cultures derived from MBs and confirms the importance of such cell lines as well as the short-term cell lines in studying the biological characteristics of tumours *in vitro*. Also, there was a range of chemosensitivities between the different cultures derived from the same tumour histology, showing that heterogeneity is not just confined to astrocytomas.

The ependymomas (including an ependymoblastoma) had a similar chemosensitivity profile to both astrocytomas and MBs with a mean and median value of 8.2μg/ml and 8.4μg/ml respectively. The range of sensitivities was also similar being between 3.2-10.1μg/ml. The most striking difference was observed with neuroblastomas, which were found to be 4-fold more sensitive to CCNU than MBs, astrocytomas and ependymomas, with a mean ID$_{50}$ value of only 2.1μg/ml. The range was also very small, between 0.75 and 3.4μg/ml.

Cell line, TE671 was found to be the most resistant to CCNU compared to all the other tumour histologies examined, where the mean ID$_{50}$ value was 14.8μg/ml.
For PCB, a median value of 1289μg/ml for short-term glioma cultures has been reported (Darling, 1985). Paediatric tumours were more resistant to PCB than gliomas in adults. In the present paediatric series, the high grade astrocytomas were the most sensitive to PCB with a median ID₅₀ value of 1641μg/ml, whilst low grade pilocytic astrocytomas, MBs, ependymomas and neuroblastomas ranged between 2390-3580μg/ml. Interestingly, the neuroblastomas were the most resistant group of tumours with a median ID₅₀ value of 3582μg/ml.

Chemotherapeutic agents effective in experimental gliomas include BCNU, PCB, diaziquone, melphalan, cyclophosphamide and fludarabine (Schold & Bigner, 1983; Schold et al, 1984; Schold et al, 1987). However, melphalan, cyclophosphamide, ifosphamide and thiotepa are active against human MB cell lines and transplantable xenografts (Friedman et al, 1988b). In the present study however, astrocytomas and MBs appeared to have comparable sensitivities to CCNU, which is in slight contrast to that observed in the above studies. O⁶-alkylguanine-DNA alkyltransferase (AGT) activity was found to correlate with the responsiveness of tumour cells to chloroethylnitrosoureas (CENUs) in 42 surgical malignant glioma samples (Hotta et al, 1994) as well as in glioma cell lines (Beith et al, 1997). In vivo results using MBs xenografts confirmed resistance to alkylating drugs, such as CCNU and BCNU (Friedman et al, 1988b) and resistance was thought to be a consequence of the presence of high levels of AGT. Other studies, both in vitro and in vivo, have shown that tumour sensitivity to nitrosoureas and PCB is inversely related to AGT activity (Brent et al, 1985; Bodell et al, 1988; Schold et al, 1989; Nutt et al, 1995). He et al (1992), has shown AGT activity by Western and Northern blot analysis of six human MB cell lines. High levels of AGT activity were seen in D283, Daoy, D341 and D384 but little activity in D425 and D458. In glioblastoma cell lines (YH and AM) AGT activity was reported (Izumu et al, 1993). YH cells showed a high AGT activity (1196 fmol/mg) and resistance to ACNU but much lower in the tumour specimen (301 fmol/mg) although this was sufficient to confer clinical resistance to ACNU chemotherapy in the patient. Much lower AGT activity was seen in AM cells (16 fmol/mg) which were sensitive to ACNU in vitro. In contrast, the AM tumour specimen exhibited a high AGT activity (628 fmol/mg). It is likely that the observed resistance to CCNU in both grades of astrocytoma cultures, together with MBs in the present study, indicates that resistance in paediatric brain tumours is modulated by AGT activity. As cultures from neuroblastomas appear to retain sensitivity to nitrosoureas, it is possible that these cultures have little or no AGT activity.

Another possible reason for the observed chemoresistance of these short-term cultures derived from astrocytomas to CCNU could be related to the karyotypic profiles of the cells. Shapiro et al (1993) screened parental cells and clones isolated from four freshly resected tumours, an astrocytoma, an anaplastic astrocytoma and two GBs, against BCNU. Near
diploid cells were more resistant to BCNU (ID<sub>50</sub> >10µg/ml) than their sensitive hyperdiploid cells (60 or more chromosomes/metaphase) from the same tumour. It is possible that a cell population derived from paediatric high grade gliomas is composed of fewer diploid cells than their adult counterparts to account for their greater sensitivity.

In one study the mean and the median ID<sub>37</sub> for 13 MBs screened against VCR were 2.18µg/ml and 0.13µg/ml respectively (Tomlinson et al, 1991), and these cultures appear to be comparatively resistant to VCR when compared to the present study. However, this is probably explained by the short drug exposure time of only one hour which is insufficient time for a cell cycle specific drug like VCR to provide a reliable ID<sub>37</sub> value. In the present series, the MBs cultures were quite sensitive to VCR with a mean ID<sub>50</sub> of 0.0023µg/ml. However the mean ID<sub>50</sub> value (0.0046µg/ml) for Daoy was twice that for the short-term cell lines. The chemosensitivity profile of Daoy resembled those of the low grade childhood astrocytomas in the present study, which had a mean ID<sub>50</sub> value of 0.0037µg/ml. TE671 which was the most resistant to CCNU compared to the other tumour groups screened, but was remarkably sensitive to VCR, with a mean ID<sub>50</sub> value of only 0.0008µg/ml and fell within the range of sensitivities to this drug seen in patients with acute lymphoblastic leukaemia (ALL) (Pieters et al, 1989). Patients treated with ALL who had bone marrow relapse had a mean ID<sub>50</sub> to VCR of 0.046µg/ml and were less sensitive than initial ALL patients with ID<sub>50</sub> between 0.0007-0.0029µg/ml (Pieters et al, 1989). Interestingly, the median ID<sub>50</sub> results from the present study for MBs, low and high grade astrocytomas and neuroblastomas fell within the same range as the initial ALL patients group, whilst ependymomas were intermediate between the initial ALL and those with bone marrow relapse (Pieters et al, 1989).

The chemosensitivity profiles of both low pilocytic and high grade astrocytomas from children had ID<sub>50</sub> values nearly half that which was observed with adult gliomas, where in the childhood series the median ID<sub>50</sub> for VCR was 0.0015µg/ml and 0.0022µg/ml respectively and for the adults this was 0.0046µg/ml (Darling, 1985). It is apparent that childhood astrocytomas were more sensitive to VCR than their adult counterparts. This further supports the hypothesis that, although these tumours are histologically indistinguishable, there are clear differences in their biological behaviour and this extends to their response to clinically used drugs. Cultures derived from ependymomas were the most resistant to VCR compared to the other tumour groups, with a mean and median ID<sub>50</sub> values of 0.0069µg/ml and 0.0042µg/ml respectively. This may explain why chemotherapeutic regimens have not been found to be of benefit clinically for this group of tumours. The most sensitive group were the neuroblastomas with a mean and median ID<sub>50</sub> value of only 0.0012µg/ml.

While there were marked differences in sensitivity between different histological types of tumour, within any one histological group there were also differences in
chemosensitivity. This has been observed by other researchers (Darling & Thomas, 1987) where using a $^{35}$S-methionine assay, screening cultures derived from adult gliomas, the VCR ID$_{50}$ range was 3-10 logs and for both CCNU and PCB range was 1-3 logs. The authors observed that cell-cycle non-specific drugs such as CCNU and PCB appeared to have smaller chemosensitivity ranges than cell cycle specific drugs, like VCR (Darling & Thomas, 1987).

Differences in sensitivity within a single histological group of tumours was also seen in the present study of paediatric brain tumours. For example, for tumours derived from low pilocytic and high astrocytomas, the ID$_{50}$ range for CCNU was virtually identical, between 5 and 11µg/ml and 8 and 10µg/ml respectively. There was a 2- and 1.3-fold difference between the most sensitive and the most resistant cultures for the low pilocytic and high grade astrocytomas respectively. However, for PCB there does appear to be a difference, in that the low grade astrocytomas (range 1987-3202µg/ml) fell within the same range difference, but the higher grade tumours in the present study showed a 6-fold range of ID$_{50}$ values (range 468-2952µg/ml). The high grade astrocytomas clearly have different biological properties compared to the lower grade pilocytic tumours. Cultures derived from ependymomas had smaller range differences for both CCNU (range 3.17-10.08µg/ml) and PCB (1347-2842µg/ml), with range fold differences of three and two respectively, suggesting that ependymomas as a group behave in a more homogeneous manner, unlike that observed with the high grade astrocytomas. The MBs cultures in contrast, showed a much larger range difference as did the neuroblastoma cultures, both having a 4-fold difference for CCNU (ranges between 2.81-13.09µg/ml and 0.75-3.4µg/ml respectively). For PCB, the neuroblastoma cultures had a 2-fold difference, with a range of between 2374 and 3847µg/ml, while for MBs, there was a striking difference, in that, for PCB, there was a 19-fold difference in ID$_{50}$ values of the cultures screened (range 905-17070µg/ml). These findings indicate that cultures derived from MBs are as heterogeneous as those derived from paediatric and adult high grade astrocytomas.

For VCR, the results from the present study were in agreement with the adult gliomas (Darling & Thomas, 1987) where the largest differences in sensitivity were observed. For example, for low pilocytic (range 5.5x10$^{-4}$ - 1.2x10$^{-2}$ µg/ml) and high (5.5x10$^{-4}$ - 4.1x10$^{-3}$ µg/ml) grade astrocytomas, and ependymomas (1.9x10$^{-3}$ - 1.8x10$^{-2}$µg/ml) had between 22- and 9-fold difference. The range difference for cultures derived from MBs (excluding Daoy) was smaller (3.2x10$^{-4}$ - 4.8x10$^{-3}$µg/ml) with a 15-fold difference. For neuroblastomas, however, the range was 1.1x10$^{-4}$ - 1.8x10$^{-3}$µg/ml, with a 17-fold difference.

TE671 was found to be the most resistant cell line to both CCNU and PCB in the present series. However, this cell line was the most sensitive to VCR and was more sensitive than the neuroblastoma cultures screened. These findings are in agreement with in vivo data which indicated that TE671 grown as subcutaneously and intracranially in athymic nude mice
(Friedman et al, 1983) was found to be more resistant to BCNU and PCB than adult malignant gliomas (Schold & Bigner, 1983), but was found to be sensitive to VCR (Friedman et al, 1983).

With regard to the clinical activity of the three drugs used in the present study against childhood brain tumours, VCR has been shown to be of benefit with minimal haematological and neurological side effects when used to treat children with low grade astrocytomas (Pons et al, 1992). Children with high grade astrocytomas have been shown to respond well to CCNU, VCR and prednisone when given after surgery and following radiotherapy in a phase II trial (Sposto et al, 1989). This has been recently confirmed in a phase III trial (CCG-945) which compared the 8-drugs-in-1-day regimen with CCNU, VCR and prednisone and the latter regimen was of greater benefit (Finlay et al, 1995). A meta analysis by Stenning et al (1987) concluded that adjuvant chemotherapy with nitrosoureas for high grade astrocytomas in adults improved survival and this was supported by a study by Sposto et al (1989) showed that 5-year event-free survival for children with high grade astrocytomas treated with adjuvant chemotherapy was 46% compared with only 18% in patients treated with radiotherapy alone.

In contrast, a clinical study (Tomita et al, 1988b) showed that cerebral PNETs in children, treated with VCR, CCNU and sometimes with PCB following radiotherapy, indicated that such tumours were chemoresistant. This was later confirmed by the first randomised trials in USA and Europe, where little benefit from chemotherapy with VCR and CCNU given after radiotherapy was observed in other brain tumours, including cerebellar MBs (Evans et al, 1990; Tait et al, 1990). Thus the in vitro results in the present study appear to confirm the clinical findings that childhood brain tumours, particularly MBs, are not very sensitive to chemotherapeutic drugs and consequently, new treatment modalities with new drugs are therefore required to improve the outcome in these patients. However, neuroblastomas are known to respond to chemotherapy, particularly to doxorubicin, VCR and teniposide (VM-26) (Goldstein et al, 1990). This present study on the in vitro chemosensitivity of NBs has confirmed these clinical findings.

In addition to the possible presence of enzyme activity such as MGMT to account for the observed resistance to alkylating agents such as CCNU, resistance to drugs may be attributed to the presence of the product of the multiple drug resistance gene (MDR1), implications of which have been discussed (Twentyman, 1995). Expression of MDR1 has been shown to confer drug resistance to tumour cells in vitro (Riordan & Ling, 1985). The gene encodes a 170kD membrane associated glycoprotein, known as P-glycoprotein (P-gp). This protein functions as an energy dependent drug efflux pump, resulting in a decrease in the intracellular concentration of cytotoxic drugs to sub-lethal levels (Thiebaut et al, 1989). P-gp has also been detected in the membrane of human glial tumours using immunohistochemical techniques (Matsumoto et al, 1991). The role of MDR1 in paediatric CNS tumours has not been
widely investigated, although a single report showed that analysis of proteins extracted from 15 childhood brain tumours (13 medulloblastomas and 2 cerebral hemisphere PNETs) using Western blot analysis, found that 2/15 patients expressed detectable levels of P-gp, while PCR detected MDR1 mRNA in 6/12 tumours (Tishler et al., 1992). MDR1 expression can be induced in vitro in the Daoy cell line, by exposing cells to sub-lethal concentrations of drugs (Tishler & Raffel, 1992). The cell line however, did not constitutively express MDR1 mRNA nor P-gp. The resultant resistant cell lines had ID$_{50}$ values which were ten times more than that of the parental cell line to actinomycin D. Even though the cell lines had not been previously exposed to etoposide, the two actinomycin-resistant cell lines had an ID$_{50}$ value which was five-fold greater than that of the parental cell line.

A report on paediatric patients with neuroblastoma disclosed that significantly more tumours expressed MDR1 mRNA when treated with chemotherapy, compared to those tumour samples that were taken from patients who had no treatment (Goldstein et al., 1990). This suggests that the high sensitivity seen in the cultures in this series may be short-lived following chemotherapy. In contrast, the observed resistance to VCR of cultures derived from ependymomas may be the result of over-expression of P-gp, since in a recent study, high levels of P-gp have been detected in ependymomas (from both children and adults) using JSB1 and C219 monoclonal antibodies in situ (Geddes et al., 1994). P-gp was detected in 29/33 ependymomas with JSB1 and 24/31 with C219 antibodies. This suggests that chemotherapy based on the drugs involved in the MDR phenotype may not be beneficial in childhood ependymomas.

**Are cells comprising short-term cell lines derived from paediatric brain tumours neoplastic?**

An important point about the cell lines used in this study is to question their malignant nature and to ensure that these cell lines are composed of tumour cells rather than normal adventitious cells present in the original tumour biopsy.

Immortalization is the ability of a cell line to divide infinitely, presumably as the result of deletion or mutation of negatively acting genes or the overexpression of dominantly acting oncogenes. This results in a loss of growth control which is manifested in growth to high cell densities in vitro, the ability to form clones in semi-solid medium and the growth on confluent monolayers of normal cells. Transformed cells also have a reduced requirement for serum. Malignancy is essentially an in vivo phenomenon and indicates that the cells have acquired the ability to produce invasive tumours when either implanted in vivo into an isologous host or transplanted as a xenograft into an immuno-deprived animal.
With regard to growth characteristics, such as anchorage independent growth, either in agar or on stirred suspension, although in the present study, cultures were not examined for growth in semi-solid medium, suspension cells were grown in small culture flasks, the base of which there was a layer of agar. Some of the cultures did indeed grow for a short time in an anchorage-independent manner. As has been discussed earlier, one reason could be that the cells were very delicate or that the growth medium employed was not optimal. Similarly, in the majority of cultures derived from the paediatric brain tumours in this study, loss of contact inhibition was observed, since cells which reached 60-80% confluency began to pile up, forming small aggregates of piled cells. The cell kinetic properties of the short-term cell lines in the present study also indicate that the cells were transformed, as the cultures had PDTs and saturation cell densities, similar to those of the continuous cell line derived from the various tumour groups studied.

Genetic properties of the short-term cell lines were not investigated at the time of the present study. However, subsequently this has become a focus of activity using some of the cell lines described in this thesis and other lines derived in the same way which have indicated that genetic alterations can be detected in most short-term lines derived from medulloblastoma (Stratton et al, 1991), ependymoma (Stratton et al, 1989a; Warr et al, 1993; Warr et al, 1995; Anderson et al, 1996) and high grade malignant glioma (Warr et al, 1993).

Structural alterations may also occur in transformed cells, and these include modification to the actin cytoskeleton, loss of cell surface-associated fibronectin, increased lectin agglutination, modification of the ECM and alterations in cell adhesion molecules. Clearly, from what has been discussed previously, some of the short-term cell lines in the present study do indeed have some of the above features.

In summary, the cell lines used in these studies display characteristics which are consistent with the presence of neoplastic cells in vitro. They display disordered growth in vitro with evidence of cell piling resulting in high saturation densities. They often display loss of cell surface fibronectin, alterations in cytoskeletal structure and display karyotypic abnormalities demonstrable using interphase cytogenetics and loss of genetic material. Further studies such as determining the serum dependency, tumourigenicity and the invasive properties of these cells are needed to further confirm the transformed phenotype of the cell lines used in the present study.

Summary of achievements and plans for the future

This is the first systematic study of the growth of short-term cell lines derived from childhood brain tumours. This included a wide range of tumour types and was not confined to medulloblastomas. In the majority of cases, cell lines were produced which in many cases
reached passage levels as high as 20. These cell lines appeared to retain the antigenic profiles consistent with those of the tumours of origin. Differences between the pilocytic and the fibrillary low grade astrocytomas were observed confirming the clinical differences seen with these two variants. These variations with respect to cell kinetics, phenotypic profiles and overall growth characteristics, although not significant, still indicate a pattern that is important. Interestingly, in some instances, the pilocytic astrocytomas behaved in a similar manner to that of the higher graded astrocytomas. Such a finding may be regarded as unusual, as one might have expected for the pilocytic astrocytomas to have behaved differently, as their prognosis is far better than that of the high grade astrocytomas. This is the first report of differential sensitivity between different types of human brain tumour. This study has shown that neuroblastomas were extremely sensitive to CCNU and VCR compared to the other tumour groups. Cultures derived from medulloblastomas and both low pilocytic and high grade astrocytomas were similar in their chemosensitivities. In contrast, ependymomas were markedly resistant to VCR. The chemosensitivity profiles of cell lines derived from low pilocytic and high grade astrocytomas, and medulloblastomas were similar to those of high grade astrocytomas in adults. This reinforces the need to identify new drugs which have efficacy against childhood brain tumours.

Further studies

The present study of childhood brain tumours in vitro has produced results which have opened up other areas of research that could be investigated and include:

i) The role of c-myc in predicting the establishment of childhood brain tumours. There seems to be an interesting connection between c-myc amplification and established cell lines produced from paediatric brain tumours. This does not seem to be restricted to medulloblastomas, and may be a feature of cell lines derived from many different types of childhood brain tumours. It would seem reasonable to examine the role of c-myc and the ability of this gene to immortalize paediatric brain tumour cells in vitro. Is it possible to detect c-myc amplification in biopsy material and does this predict establishment of cell lines derived from this biopsy?

ii) Is it possible to improve the success rate in producing established cells from paediatric brain tumours?

Is it possible to use cell fractionation techniques to isolate populations of cells from disaggregated tumour biopsies, perhaps by using density gradient centrifugation or some other technique, and grow established cell lines from these cells?
Is it possible to isolate clones of cells which will ultimately form established cell lines, by plating disaggregated tumour biopsy material on feeder layers of 3T3 cells or normal glial cells which are killed by mitomycin-c treatment or irradiation?

iii) The growth factor repertoire of childhood brain tumours is only very poorly understood and cell lines, like these described in the present series are ideal tools for investigating this. What growth factors are mitogenic for particular tumour types in vitro? Do astrocytomas of childhood have the same repertoire of growth factors as their adult counterparts?

iv) What are the karyotypic features of these cell lines? It was not possible to carry out karyotypic analysis of these cell lines as part of the present study. Limited work has been done with some of these cell lines which indicates that in many cases, the karyotypic abnormalities seen are rather subtle. For example, in some ependymomas, cultures appeared to have small translocations involving the distal part of the q arm of chromosome 22 which may harbour a tumour suppressor gene which is of importance in these tumours. However, it would certainly be reasonable to use these cell lines for a systematic karyotypic analysis using conventional metaphase techniques, or perhaps, to use DNA isolated from these cells to screen the whole genome of these groups of tumours for deletions or areas of amplification using comparative genomic hybridization (CGH).

v) What are the mechanisms of drug resistance in these cell lines? Is resistance to nitrosoureas mediated by AGT, or by other mechanisms (e.g., glutathione or glutathione-S-transferase). Do ependymoma cultures express P-gp or is resistance to natural products, like VCR produced by other mechanisms. Are there other cell membrane drug pumps present in these cells?

Gene amplification is often a mechanism by which tumour cells produce drug resistance, and karyotypically gene amplification is evidenced by double minute chromosomes or homogeneously staining regions. It is known that double minutes are often present in medulloblastoma cell lines. What are they? Do they represent amplification of genes important in the development of therapeutic resistance, or are they a consequence of oncogene over-expression? Are they c-myc or another known oncogene? Using CGH, can we detect new regions of amplification in these tumour cell lines which would provide clues about genes which are involved in drug resistance or oncogene amplification?

vi) Does surface coating influence the types of cells which grow from these tumours, or does it modulate the antigenic expression of cells? By removing cells from one surface coating and culturing them on another, does this change the antigenic phenotype of the cells?

Does surface coating influence chemosensitivity in vitro?
vii) Further biological studies using these cell lines is warranted. What is the expression of
nestin in these cultures and what other neuroectodermal markers might be important. What
integrins do these cells express? Do they provide clues to determine alternative surface
coatings which might improve cell growth?

viii) Can we use these cell lines to screen panels of existing cytotoxic drugs for activity against
particular groups of tumours? Can we identify drugs which will be effective against
ependymomas?

Can we identify novel cytotoxic drugs which will be effective against particular groups of
childhood brain tumour?

ix) Can we use very short-term cell lines (passage levels 1-4) for chemosensitivity testing in
individual patients?

x) Need to further investigate the differences between the pilocytic and fibrillary
astrocytomas, in terms of growth characteristics, and their genetic and chemosensitivity
profiles, thereby give more ammunition in the treatment and management of these tumours.
## APPENDIX I

### Patient's characteristics

<table>
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