INDUCTION OF TUMOUR DIFFERENTIATION AS A TOOL IN CHEMOTHERAPY OF GLIOMAS

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ABSTRACT

During in-vitro cultivation murine astrocytoma cells derived from a spontaneous murine astrocytoma undergo de-differentiation as evidenced by a fall in the markers of astrocytic differentiation glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS). Growth in culture was accompanied by a rise in the polyamine marker enzyme, ornithine decarboxylase (ODC), cell proliferation and increase in protein. Exposure of VMDk P497 cells to dexamethasone-containing growth medium after plating on day 0 produced a decrease in proliferation with a concomitant induction in GS activity by sixfold. This functional differentiation was independent of the age of the cultures. The effect of dexamethasone was mediated at the transcriptional level through cytosolic glucocorticoid receptors, the presence of which was confirmed during all phases of culture in vitro. Inhibition of ODC activity with difluoromethylornithine (DFMO) resulted in a decrease in cell proliferation and elevation of GS activity. A time-course experiment showed that inhibition of ODC resulted in a rise in GS activity within 24 hours of treatment and this biochemical differentiation lasted for more than 48 hours. When VMDk P497 cells were subcutaneously implanted into syngeneic VM mice, tumours of short latency with a Gompertzian growth pattern were obtained. DFMO administered in drinking water alone did not arrest

growth of these tumours. BCNU alone, administered as a single intraperitoneal dose of 30 mg/kg, produced a short but statistically significant growth delay. However, when DFMO pretreatment was combined with single dose BCNU, it potentiated the cytotoxic effect of BCNU. Analysis of changes taking place at the molecular level showed a greater inhibition of ODC in tumours treated with combined therapy than in those which were treated with either BCNU or DFMO alone. Tumour samples from mice treated with combined therapy had less deoxyribonucleic acid (DNA) than samples of tumours from mice treated with either BCNU or DFMO alone.

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TABLES AND ILLUSTRATIONS.

ABBREVIATIONS

ABTS	Azino diethyl benzthiazoline sulfonic acid
ADP	Adenosine diphosphate
ANOVA(R)	Analysis of variance
ATP	Adenosine triphosphate
BCNU	1,3, bis(2-chloroethyl-1)-nitrosourea
BSA	Bovine serum albumin
CNS	Central Nervous System
CSF	Cerebrospinal fluid
ci	Curie
dBcAMP	dibutyryl cyclic adenosine monophosphate
DFMO	Difluoromethylornithine
°C	degrees centigrade
DMSO	Dimethyl sulphoxide
DNA	De-oxy-ribo-nucleic-acid
EGF	Epidermal growth factor
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
f	femmto
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocynate
GABA	Gamma aminobutyric acid
GFA	Glial fibrillary acidic protein
GGF	Glial growth factor
g	gram(s)
GM	growth medium

GS	glutamine synthetase
HBSS	Hank's balanced salt solution
h	hours
HCl	hydrogen chloride
Ig G	Immunoglobulin G
KCl	potassium chloride
l	Litre
m	milli
M	molar
ND	not detected
n	nano
NGF	Nerve growth factor
NS	not significant
ODC	Ornithine decarboxylase
p	Phosphate
PAGE	Polyacrimide gel electrophoresis
PAP	Peroxidase-anti-peroxidase
PBS	Phosphate buffered saline
PCA	Perchloric acid
PDGF	Platelet derived growth factor
PEP	Phospho-enol-pyruvate
RNA	Ribonucleic acid
SAMD	S-adenosylmethionine decarboxylase
SEM	standard error of the mean
SMA	spontaneous murine astrocytoma
v/v	volume for volume
w/v	weight for volume
WHO	World Health Organisation

μ	micro
%	percent

Advances in the treatment of gliomas have been slow. The most major advance has been in the combination of surgery, radiotherapy and chemotherapy with 1,3 bis (2-chloroethyl-1)-nitrosourea (BCNU) which has produced an increase in median survival and in the proportion of patients surviving 18 months from surgery (Walker et al, 1980; Green et al, 1983). However the outlook is still bad and 90% of patients with anaplastic gliomas will be dead within two years of diagnosis (Walker, 1973; Walker, et al, 1978). More biological information on gliomas is needed to improve the outlook. Some of this information may be obtained through the use of animal models.

Anaplastic astrocytomas have been described by Fraser in mice from the inbred VM strain (Fraser, 1971). The cell line VMDk P497 was established from one of these tumours by Serano et al (1980). These tumours express the astrocytic markers glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS) (Pilkington et al, 1983). There is an inverse relationship between GFAP expression and the degree of malignancy of astrocytomas (Duffy, 1982; Duffy et al, 1980). GS has been associated with differentiation of normal astrocytes during development (Patel et al, 1983a; Weir et al, 1984).

Ornithine decarboxylase (ODC) is an enzyme which

catalyses the conversion of ornithine to putrescine, the rate limiting step in polyamine synthesis (Janne and Raina, 1968; Pegg and Williams-Ashman, 1968; Russell and Snyder, 1968). Polyamines are associated with cell proliferation and differentiation (Mattson et al, 1984; Slotkin and Bartolome, 1986). Each tissue has its own characteristic ODC/polyamine developmental pattern (Slotkin and Bartolome, 1986). Intracellular polyamine deficiency may be created by use of α-difluoromethyl ornithine (DFMO), an irreversible inhibitor of ODC (Metcalf et al, 1978).

Long-term survival of patients with glioma correlates with the degree of tumour differentiation (Thomas, 1983). The primary aim of this programme of work is to examine the hypothesis that induction of differentiation in tumour cells may be of therapeutic value in the management of patients with glioma (Patel et al, 1986). Initially, changes in the astrocytic markers GFAP and GS and in ODC activity in VMDk P497 cells in vitro were examined. The potential for differentiation in vitro using drugs known to induce tumour differentiation like glucocorticoids (Pilkington et al, 1983; Patel et al, 1986) was examined. These findings were compared with changes in cytosolic glucocorticoid receptor levels in the same system during growth in vitro. Intracellular polyamine deficiency was created in VMDk P497 cells by treatment with DFMO and the effect of

this on cell proliferation and differentiation was studied. A time-course experiment was carried out to determine the effect of exposure on degree of inhibition of ODC and GS activity. VMDk P497-(P1) cells growing as subcutaneous implants in syngeneic mice were used to test the hypothesis that greater therapeutic response can be obtained in the management of gliomas by combining treatment with an established cytotoxic drug, BCNU, with a drug which can slow cell proliferation and induce cell differentiation, DFMO. Tumour response was monitored by assessing changes in tumour volume as well as ODC enzyme and DNA levels in tumour samples.

1.1. BRAIN TUMOURS: GLIOMAS

1.1.1 AETIOLOGY

1.1.1.1. Genetic factors

Some tumours of the nervous system are associated with well defined genetic disorders, for example, von Recklinghausen's disease, tuberous sclerosis, von Hippel-Lindau's disease and Sturge Weber's disease (Butler et al, 1982). Von Recklinghausen's disease is inherited in an autosomal dominant manner and its frequency in the general population ranges from 1:2000 to 1:3000. This disease is commonly associated with the development of Schwannomas, gliomas and meningiomas (Butler et al, 1982). The frequency of tuberous sclerosis is however far lower, with an incidence in the general population of between 1:30000 and 1:50000. The cerebral lesions are firm, hyperplastic nodules made up of large, abnormal glial cells. The incidence of malignant change in these cells is between 1 and 3%; producing benign giant cell astrocytomas (Butler et al, 1982).

Tumours of the nervous system with a clear hereditary background are extremely rare and in most sporadic cases of brain tumour evidence for a genetic factor is usually lacking. Both environmental and constitutional factors are involved in the origin of cancer. Environmental factors such as ionising

radiation, ultra-violet light, chemical carcinogens and diet are thought to account for about 80% of human cancers (Tijssen et al, 1982). Malignant transformation is a multi-step process. The first lesion is probably located in DNA and this change in genetic information is transmitted to daughter cells. Proliferation of initiated cells may be maintained by exogenous substances (promoters) such as phorbol esters, hormones or by physical effects like virus infection or repeated mechanical trauma. The whole process may take many years (Tijssen et al, 1982).

Some cancers exist in a dominantly heritable form as well as in a non-hereditary form. For example, retinoblastoma, in which 40% of the cases are hereditary. In this form of cancer, the first mutation occurs in the germinal cell and somatic mutations later occur at the same site (Tijssen et al, 1982). Rarely, cases of brain tumours occurring in twins have been reported in the literature (Leavitt, 1928). It is likely that molecular genetic studies using material from twins and families with intracranial tumours may yield more information on genetic mechanisms.

There are specific genes in susceptible cells called proto-oncogenes which are responsible for malignant transformation of cells. When the normal controls which regulate their expression are removed they are capable

of influencing cell growth and differentiation (Land et al, 1983; Stehelin et al, 1976; Willecke and Schaefer, 1984).

1.1.1.2. Physical factors

The scientific evidence for a correlation between trauma and tumour growth may be provided by examination of individual human cases, experimental investigation and analysis of statistical data on brain tumours in series of brain injured patients. Such analyses have, however, produced conflicting results. Cushing and Eisenherdt (1938) considered head injury to be of importance in the development of some meningiomas; they obtained a history of head injury in 33% of 313 cases. However, Parker and Kernohan (1931) found that the association of brain tumour with trauma was not significantly higher compared with the association of head injury with other diseases, or the history of trauma in healthy individuals of the same age. It is possible that trauma could play a role in initiating or aggravating the clinical symptoms of a tumour already present within the cranial cavity.

Traumatic alteration of neuroepithelial tissue can trigger autonomous growth during the regenerative process (the "misregeneration" theory of the classical pathologists) (Zulch, 1986). Cases have been reported in the literature where traumatic alteration of brain tissue and its coverings has led to a regenerative

process which eventually becomes neoplastic (for review see Zulch, 1986).

To experimentally assess this possibility trauma has been used as a co-carcinogen with N-ethyl-N-nitrosourea (ENU) in experimental animals. Morantz and Shain (1978) made a stab wound into the left cerebral hemisphere in Fisher rats treated concurrently with ENU. In the traumatised group gliomas were induced in 73% of animals, while in the non-traumatised control group, gliomas were induced in only 47% of animals.

Anderson and Treip (1984) reported three cases of radiation-induced intracranial neoplasms following therapeutic irradiation. The patients developed astrocytoma, meningioma and ependymoma. The second intracranial neoplasms were histologically different from the initial tumours, had long latent intervals and they all developed within the irradiated fields. Fibrosarcoma has been reported following post-operative radiation for ependymoma in a 15 year old girl (Zulch, 1965). Whether ionising radiation acts as a direct carcinogen or whether it causes damage to normal tissue which renders it more susceptible to subsequent neoplastic change is unclear.

1.1.1.3. Chemical factors

Several classes of chemical carcinogen have been shown to cause nervous system tumours in experimental

animals. Some of these require topical intracranial application while others require systemic administration (Kleihues and Rajewsky, 1984). Epidemiological studies have suggested a link between occupational exposure to chemicals and an increased incidence of intracranial tumours (Selikoff et al, 1979) although no environmental carcinogen has been unequivocally identified as a causative agent in human brain cancer. Oberling et al (1936) were able to produce hypophyseal adenomas in rats by applying crystals of benzpyrene to the cortex. Weil (1938) injected methylcholanthrene into the brain of six rats and produced an epidermoid carcinoma in one rat and sarcomas in the others. Methylnitrosourea has been shown to induce tumours of the peripheral and CNS in rats (Druckrey et al, 1972; Kleihues et al, 1968).

1.1.1.4. Biological factors

Some viruses can induce brain tumours when inoculated intracerebrally into experimental animals. Three groups of viruses have been used to produce brain tumours, adenovirus, papova-virus and oncorna virus. Oncorna viruses can produce differentiated astrocytomas and glioblastomas in rats. Cultured human glial cells can be transformed by infection with both DNA and ribonucleic acid (RNA) viruses in vitro (Bullard and Bigner, 1980).

Virus-like particles have been reported in human

brain tumours by Cuatico et al (1976). They examined 31 cases using molecular hybridization techniques and found particles in 77% of cases. These particles could not be detected in any of the non-neoplastic brain tissues examined. Scherneck et al (1979) tested a human glioblastoma multiforme in short-term culture by indirect immunofluorescence and showed SV40-related tumour antigen and capsid antigen in 95% of cells. Zulch (1986) examined by electron microscopy more than 120 brain tumours and found virus-like particles in only a single case of intracerebral fibroma. There is little evidence to support the view that brain tumours in man are virus-induced (Zulch, 1986).

1.1.2. CLASSIFICATION

Virchow was the first to recognise the supporting elements of the nervous system and called them neuroglia (Virchow, 1863). He also created the term glioma and classified these tumours according to the cell type. Tooth (1912) studied 500 cases of brain tumours collected between 1902 and 1911 at The National Hospital, Queen Square, London, of which 258 were gliomas. This was the first report of an extensive histological study of neurosurgical material. Tooth emphasised the correlation between morphological structure and clinical course. Ribbert (1918) emphasised the comparison between tumour cells with the developmental stages of normal glia and so laid down the foundations for the classifications used

today.

Bailey and Cushing (1926) used the metallic impregnation techniques of Cajal and Hortega, combined with other staining methods, to study more than 400 verified gliomas. From this study Cushing developed a new approach correlating biological features such as macroscopic appearance, point of origin, and method of growth and spread, with the cellular architecture of the tumour. His subsequent classification was based on the resemblance of tumour cells to embryonic cells in various stages of differentiation. Tumours were therefore classified according to the morphological stages through which each cell was thought to pass in embryogenesis. This classification was later modified by Bailey (1932) to include choroid plexus papilloma, medulloblastoma, glioblastoma multiforme, spongioblastoma, astroblastoma, astrocytoma, neuroepithelioma, ependymoma, pinealoma, ganglioneuroma and oligodendroglioma. This classification was useful because of its clinical value in correlating the types of tumour with survival times.

Broder (1920) suggested that tumours of the same cellular type could be divided into four grades of malignancy depending on the degree of cellular anaplasia. This was based on the assumption that tumours were derived from de-differentiation of mature cells. Grade I and II tumours were composed almost entirely of well

differentiated cells while grade IV tumours were composed of less than 25% of differentiated cells (Broder, 1925).

Kernohan and Sayre applied Broder's ideas to the grading of gliomas (Kernohan and Sayre, 1952; Kernohan et al, 1949). They proposed four grades of malignancy and applied these to astrocytomas, ependymomas and oligodendrogliomas. They found a direct correlation between the degree of anaplasia and post-operative survival.

The World Health Organisation (WHO) has attempted to provide an internationally acceptable classification for brain tumours (Zulch, 1986). This classification closely resembles that of Russell and Rubinstein which is widely used in the United States (Russell and Rubinstein, 1963). The WHO scheme uses technically simple histological procedures and gives a gross estimation of the biological behaviour of the different tumour groups. Tumours have been classified into tumours of neuroepithelial tissues, tumours of the nerve sheath, tumours of meningeal and related tissues, primary malignant lymphomas, tumours of blood vessel origin, germ cell tumours, other malformative tumours (e.g. craniopharyngiomas), vascular malformations, tumours of the anterior pituitary, local extensions from regional tumours, metastatic tumours and unclassified tumours. This classification does not include some conditions that

are still of clinical importance in Africa, Asia and Central America such as parasitic cysts, granulomas and arachnoiditis.

1.1.3. INCIDENCE

The incidence of brain tumour for all ages is estimated to be between 4.2 and 5.4 per 100,000 population (Kurland et al, 1962; Zulch, 1965). Statistical data on brain tumours can be very misleading as these figures depend on whether they were compiled from operative or autopsy series or from death certificates. Operative series will favour operable cases which may lead to a bias in favour of those lesions where expertise exists within a neurosurgical unit. Other factors which may affect these figures are racial and ethnic distribution within the referral area. A study done of the incidence of brain tumours in 27 countries (Kurland et al, 1962) revealed apparently low rates in Chile, Japan and Mexico. The annual age-adjusted rate for most of the countries ranged from 4 to 5 per 100,000. In Israel, the age adjusted mortality rates for CNS tumours was 12.8/100,000 (Leibowitz et al, 1971). Goldberg and Kurland (1962) suggested that the low mortality rate observed in Mexico (1/100,000) was due to "artefacts of reporting and diagnosis." The low rates of occurrence of brain tumours reported from the "developing" world, are probably related to under-reporting due to lack of specialised medical facilities.

In children younger than 15 years of age, tumours of the nervous system are the second most frequent type of cancer; exceeded only by leukaemia. Two-thirds of tumours occurring in childhood are in the posterior fossa. Perhaps three quarters of these are gliomas; ependymomas are more common above the tentorium and cerebellar astrocytomas below the tentorium. The frequency of intracranial tumours decreases in adolescence and then increases to reach a second peak in later adult life. In adults, brain tumours are slightly more common in males than females by a ratio of 5:4 (Zulch, 1965). Most commonly these tumours are high grade supra-tentorial malignant gliomas.

Brain tumours are said to be less common in the black race compared with the white. In the United States, the ethnic distribution of 16,311 cases of primary CNS tumour seen at the Armed Forces Institute of Pathology showed a case ratio of caucasians to negro of 13:1 compared to a population ratio of 8:1 (Fan et al, 1977). Gliomas were more frequent in caucasians than in negroes, but pituitary adenomas, meningiomas and nerve sheath tumours were more frequent in the negroes. Most series from the developing world show a high frequency of "tumours" due to infections e.g tuberculomas (Dastur et al 1968; Dastur and Lalitha, 1969).

1.1.4. DIAGNOSIS

Symptoms due to brain tumours can be divided into four groups; headache, seizures, focal neurological deficits and generalised cerebral dysfunction. The headaches are usually not severe, worse in the morning, mild and throbbing, usually unilateral and on the same side as the mass. Supratentorial masses produce frontal or unlocalised headaches and posterior fossa masses produce occipital, sub-occipital or generalised headaches. Seizures are more common in slow growing tumours (60 to 90% of cases) than in rapidly growing ones (30% of cases) (Penfield and Feindel, 1947). Focal and temporal lobe seizures are more common than grand-mal seizures (Rasmussen, 1975). Neoplasms interfere with brain function through replacement of brain parenchyma, oedema in surrounding tissue, compression of the micro-circulation, production of herniation syndromes and occlusion of major cerebral arteries. This usually leads to a localised dysfunction and a focal deficit. Increased intracranial pressure and shift will lead to generalised cerebral dysfunction. This is usually due to the bulk of the neoplasm or surrounding peripheral oedema and hydrocephalus. This may manifest itself through impairment of intellectual function and coma. A good history and complete general and neurological examination are important in making a diagnosis of intracranial neoplasm. The history should include onset of disease and its progression and the time of appearance of focal

neurological deficits, as well as any associated diseases in the patient or his family.

Plain skull x-rays may help in localising the tumour if calcification is present in the tumour or if there is bony erosion. Increased intracranial pressure is usually shown by displacement of a calcified pineal gland or erosion of the sella. The electroencephalogram may be abnormal with an anaplastic lesions and may also indicate its location (Frankel and German, 1958). The electroencephalogram may be of particular importance in patients with temporal lobe epilepsy especially in establishing the side of the neoplasm (Rasmussen, 1975). Computed tomography is the most important investigation in the evaluation of a patient with an intracranial tumour. It is the most informative study as well as being safe and painless. Cerebral angiography is useful in planning the operation and it may also help in identifying the tumour.

1.1.5. MANAGEMENT

1.1.5.1. Surgery

The first attempt at removal of a supra-tentorial glioma was reported by Bennett and Godlee (1884). Advances in anaesthesia and methods of achieving haemostasis and the introduction of Bailey and Cushing's classification of gliomas (Bailey and Cushing, 1926) led to much improved results although the mortality rates

were still of the order of 70%. Other factors which helped make surgery of the nervous system safer were the introduction of ventriculography by Dandy (1918; 1919) and cerebral angiography by Moniz (1927).

Dandy advocated a radical surgical approach to the management of brain tumours and believed that the results of surgery could only be improved by radical excision of the tumour (Dandy, 1921). Bailey (1932) on the other hand adopted a more restrained approach and believed that the extent of attempted tumour removal should depend on the pathological type and position, relying more on lobectomy (frontal, occipital or temporal). Horsley (1893) and MacEwen (1893) introduced external decompression for relief of symptoms due to raised intracranial pressure and Cushing (1905) modified this method by introducing the more cosmetically acceptable sub-temporal decompression. Here the external expansion was hidden beneath the temporal muscles and beneath the floor of the middle cranial fossa. In spite of all these efforts the overall results were poor and this led some neurosurgeons to advocate only burr-hole biopsy to establish a pathological diagnosis (Cairns, 1935).

Surgery within the posterior fossa became much safer with the introduction of the sitting position by De Martell (1931). The role of accurate histological diagnosis in the planning of surgical resections of

posterior fossa tumours was realised by Cushing (1930; 1931) who found that the prognosis of cerebellar astrocytomas was improved by radical surgery whilst the prognosis of medulloblastoma was not.

Apart from the perfection of neurosurgical techniques and anaesthesia, another factor which helped improve mortality was the introduction of shunting of cerebrospinal fluid (CSF) from lateral ventricles to the jugular vein by Nulsen and Spitz (1952) and later to the heart by Pudenz et al (1957).

It is generally accepted that surgery is always necessary to establish a histological diagnosis and formulate a plan of management. Tumours located in hazardous sites, for example the thalamus and brain stem, may be approached by CT-directed biopsy. The role of major surgical procedures in the management of gliomas is debatable. Total removal of tumour appears to improve the short term survival rates when compared with partial removal (Frankel and German, 1958); after 6 months 45% of 50 patients after total removal were alive whereas only 20% of 105 patients who had partial removal were living. However, long-term benefits are less well documented. After 3 years only 20% of patients in both groups were still alive. Similar results were obtained by Jelsma and Bucy (1967).

1.1.5.2. Radiotherapy

Analysis of survival figures following postoperative radiation therapy collected from a number of studies carried out between 1940 and 1973 (Salazar et al, 1976) shows a small improvement in results; for astrocytoma grades I and II 58-86% of patients survived one year, 31-64% survived three years and 26-50% survived five years; for grade III and IV 20-44% survived one year, 0-16% three years, but only 0-9% five years.

Radiotherapy is not without risks. Non-dividing cells such as those found in the brain do not usually show early radiation damage but delayed necrosis can occur (Brismar et al, 1976). Local effects of radiotherapy may occur in the scalp such as skin erythema and epilation. When irradiation is used in combination with cytotoxic drugs the hair-loss may be permanent. Radiation may also cause systemic reactions such as lethargy, nausea and vomiting but these effects are usually transient.

There is no doubt that radiation has, to a small extent, improved survival times over surgery alone. In malignant glioma one and five year survival rates are higher in patients receiving radiation therapy than in those who do not. Survival rates in patients receiving radiotherapy ranged from 3-9% at five years, and 0-6% in those patients who did not receive radiotherapy. For

patients with differentiated cerebral astrocytomas, reported 5 year survival rates are much better, ranging between 32 and 63% (Capra, 1980). Further improvement in these figures using currently available methods of radiotherapy after surgical treatment is unlikely and therefore new approaches will have to found.

1.1.5.3. Chemotherapy

Surgery and radiation therapy does occasionally cure low-grade astrocytomas, oligodendrogliomas and ependymomas. However, the same cannot be said for the more malignant tumours like glioblastoma multiforme. Fifty percent of patients with glioblastoma multiforme survive for an average of 37 weeks (Renaudin et al, 1973; Walker, 1977). One avenue to improve these figures lies in the discovery of new cytotoxic drugs effective against gliomas.

Cytotoxic drugs for use against tumours within the central nervous system must be able to penetrate the blood brain barrier (BBB). This was first demonstrated in 1962 in experiments using the murine L1210 leukaemia cell line (Chirigos et al, 1962). It was found that cyclophosphamide was effective against subcutaneous implants but not intracerebral implants because the drug failed to reach the brain in sufficient concentrations. Normal brain capillaries exclude nonionised hydrophilic molecules with a molecular weight greater than 180. The

transport of lipophilic drugs through the BBB is quite rapid (Paoletti et al, 1986). Consequently, drugs that cross the BBB and are effective throughout the cell cycle such as BCNU, will be of greatest value in treating malignant brain tumours.

The major cellular constituents of tumour mass consist of non-proliferating anoxic cells in G_0 phase and dead cells (Hoshino and Wilson, 1975). Studies using tritiated thymidine indicate the percentage of cells engaged in the synthesis of DNA at any one time is between 5 and 30% in glioblastomas and 1% or less in astrocytomas (Hoshino et al, 1972; Hoshino and Wilson, 1975). A characteristic feature of glial tumours is that as they become more malignant, cellular proliferation increases and they outgrow their blood supply and thus become hypoxic and eventually undergo necrosis. Although in comparison with many other types of tumour, malignant gliomas have a low growth fraction, smaller tumours are more likely to have a higher growth fraction than larger ones (Levin and Wilson, 1978). This means that therapy effective against smaller tumours might not be effective against larger tumours and vice-versa (Levin and Wilson, 1978). If maximum therapeutic effect is to be obtained a drug should reach its site of action in adequate concentrations for an adequate period of time (Levin, 1975) regardless of whether the drug crosses a normal capillary or a leaky tumour capillary. Sub-optimal drug

delivery can induce drug resistance in neoplastic cells and this can be manifested as impaired intracellular transport of the drug, enhanced intracellular detoxification of the drug or repair of sub-lethal drug-induced damage. Drugs usually diffuse from capillary to cells down a concentration gradient and this depends on the concentration of the drug in the capillary lumen and on the rate of chemical and bio-transformation of the drug as it moves through the tumour's extracellular and intracellular spaces (Levin et al, 1976). Drugs with a short plasma half-life and those that bind protein rapidly and extensively are unlikely to achieve uniformly adequate concentration within tumours. Malignant gliomas are extremely infiltrative and their infiltration usually precedes the development of leaky capillaries. This may explain why anticancer drugs that cross the blood brain barrier are more effective in the treatment of malignant gliomas.

1.1.6. BRAIN TUMOUR MODELS

1.1.6.1. The ideal animal model

More biological information is needed to improve the outlook for the management of gliomas. Because of the limitations involved in clinical research much of this information will be obtained through the use of animal models and ultimately this information will be utilised in the planning of new clinical trials. Ideally, the experimental model would be a spontaneous, uniformly

fatal, astrocytic murine tumour (Wilson, 1978). The requirements of an ideal animal model are: that it should be spontaneous in origin, glial in composition, uniformly fatal within a reasonable time period and have the therapeutic profile of human glioma (Wilson, 1978). The model should also be capable of in vitro growth and be transplantable intracranially or subcutaneously in syngeneic animals. The ability to grow tumour cells in vitro enables single cell cloning to be carried out, making biochemical and molecular biological studies easier to perform.

1.1.6.2. Transplantable animal models

These are animal models of human tumours which are capable of being serially transplanted. These tumours may be transplanted into syngeneic animals of the same genetic background or, in heterotransplant models, tumours of different genetic background may be transplanted into immunosuppressed or congenitally immunodeficient animals (Rygaard and Povlsen, 1969) or into an "immunologically privileged site" in a normal animal such as the brain, anterior chamber of the eye or the cheek pouch. The advantages of the transplantable system are uniformity of growth and short latency times; factors which combine to make it a less expensive model with a high morphological correlation between the tumours. Some of these models, such as the VMDk model, have the further added advantage of being capable of

subcutaneous growth and also of being grown *in vitro* (Pilkington *et al*, 1983). A major disadvantage of transplantable models is that when introduced intracranially this results in the breakdown of the BBB and this may affect therapeutic sensitivities.

1.1.6.3. The VM murine brain tumour model

Fraser (1971) described spontaneous development of anaplastic astrocytomas in 1.6% of inbred VM mice and was able to transplant these tumours serially into the brains of syngeneic mice (Fraser, 1980). This tumour model, however, could not be transplanted extracranially and the cell population of the tumours was pleomorphic including cells which morphologically resembled macrophages (Pilkington *et al*, 1982). Serano *et al* (1980) established five cell lines from the transplanted spontaneous murine astrocytoma (SMA) of the VM strain and three of these: VMDk P497, VMDk P560 and VMDk P540 have been extensively characterised (Pilkington *et al*, 1983), confirming the astrocytic nature of the cells.

Cell line VMDk P497 was established by mincing whole mouse brain containing the transplanted SMA, followed by trypsinization for 5-10 minutes, the subsequent cell suspension was cultured in monolayer (Serano *et al*, 1980). Cell lines VMDk P540 and VMDk P560 were established by intraperitoneal implantation of syngeneic animals with suspensions of the SMA. Some of the animals

developed intraperitoneal tumour nodules. Nodules were minced and explanted into tissue culture. The established line which resulted was designated VMDk P540. The remaining nodules were injected subcutaneously into animals. The mice developed tumours. Minced subcutaneous tumour was explanted and cell line VMDk P560 was derived from a successful explant.

1.1.7. THE CELL CYCLE

The cell cycle is a term used to describe the period between one mitosis and the next. The cycle has been divided into four phases: G_1, the period between mitosis and the onset of DNA synthesis; S, the period of DNA synthesis; G_2, the period between completion of DNA synthesis and mitosis and M the period of mitosis (Howard and Pelc, 1953).

Animal cell populations may be divided into three groups in relation to DNA synthesis and mitosis: in group 1 are the continously dividing or cycling cells, in group 2 are those that leave the cycle but can be induced to synthesise DNA and divide by an appropriate stimulus i.e., resting or non-cycling cells, and in group 3 are those that have permanently left the cycle and are destined to die (non-dividing cells) (Baserga, 1981).

Lajtha introduced the concept that normal cells, as an alternative to proliferation, can move out of the

cycle or become arrested for extended periods of time but still retain the capacity of reactivation (Lajtha, 1963). He designated this state as G_0 and proposed that cells entered it by leaving the cycle from the G_1 period. Cycling cells are more amenable to killing by proliferation-dependent agents such as vincristine while resting cells are less susceptible to the cytotoxic effects of these type of drugs. Resting cells can, however, be drawn into cycle to replace any proliferating cells destroyed by cytotoxic treatment.

1.1.8. TUMOUR GROWTH KINETICS

Skipper et al (1964) suggested that the ability to eradicate malignant cells with a drug was dependent not only on the dose of the drug but also on the number of tumour cells present. Drug-induced cytotoxicity is said to follow "first order kinetics" which means that a given treatment destroys a constant fraction of cells rather than a fixed number. That is, a treatment which reduces a tumour cell population of one million to ten cells should reduce a population of 100,000 to one cell. Therefore, drug treatment is most likely to be curative only when a small number of malignant cells are present, which necessitates the integration of systemic treatment with surgery and radiotherapy which removes or destroys the bulk of the tumour mass.

In general, experimental animal tumours and human

tumours in situ have a volume doubling time that increases with tumour size. This progressive retardation is associated with vascular insufficiency, accumulating necrosis and an increasing drain on the host's metabolism. This is termed Gompertzian growth (Laird, 1969). In this type of growth the growth rate is least for both very small and very large tumours and reaches a maximum at a point called the "inflection point" when the tumour is about 37% of the maximum size (Norton and Simon, 1977).

1.2. ASTROCYTIC MARKERS

1.2.1. Glutamine synthetase

1.2.1.1. Role in cell metabolism

The synthesis of glutamine provides cells with a mechanism for removal of ammonia and for its storage. Glutamine plays a central role in the metabolism of nitrogen, amino acids and protein. It is the most prevalent amino acid in blood plasma and it is required for growth of cells in tissue culture. Glutamine is essential for brain function and its hydrolysis by renal glutaminase plays a key role in acid-base balance. Glutamine and the enzyme that catalyses its synthesis, glutamine synthetase (GS), are of considerable importance in cellular metabolism and function (Meister, 1980).

1.2.1.2. Historical background

Glutamine was first isolated from beet juice in 1883

by Schulze and Bosshard (as cited by Meister, 1980), and from a protein hydrolysate by Bergman in 1932 (as cited by Meister, 1980). It is found in all species in a protein-bound and free form (Meister, 1980). It is not an essential dietary amino acid for animals. GS was first isolated in a pure form from sheep brain by Palmijans, Meister and others (see Meister, 1980).

1.2.1.3. Role in the CNS

GS catalyses the following reaction in the presence of divalent cations:

L-Glutamate + ATP + Ammonia = L-Glutamine + ADP + P

GS activity is measured at its optimal pH range of 7-7.4, in the presence of L-glutamate, ATP, magnesium and ammonium ions (Meister, 1980). Glutamate and ATP bind to the enzyme and ATP is cleaved to ADP and glutamate is activated (Meister, 1980).

Glutamine is a key metabolite for the elimination of ammonia in nervous tissue and is an important precursor of both glutamate and gamma-aminobutyric acid (GABA) pools. Glutamate and GABA play an important role as synaptic transmitters in the CNS. Berl et al (1961) established that shortly after isotopically labelled glutamate was injected into the brain, the specific radioactivity of glutamine was higher than that of

glutamate, a behaviour that deviated from the usual precursor-product relationship. The total amounts of glutamine plus glutamate were constant. Since the only known route of glutamine synthesis is from glutamate, the explanation for these findings is that at least two metabolic pools of glutamate exist in nervous tissue; a "large" pool and a "small" pool in which glutamate is rapidly converted into glutamine before mixing with the "large" pool. The compartmentation of glutamate metabolism is a property peculiar to the nervous system. The two main cell types in the nervous system, the neurons and the glial cells are responsible for the "large" and the "small" pool respectively (Balazs et al, 1970). Glutamine is synthesized predominantly in glial cells and later transported into nerve endings to serve as a precursor of the neuro-transmitter pools of glutamate and GABA. Norenberg and Martinez-Hernandez (1979) have shown using ultrastructural immuno-cytochemistry, that glutamine synthetase is located in astrocytes. The enzyme was not present in other elements of the nervous system except for small amounts in a rare indeterminate glial cell. None was found in neurons or synaptic endings. Glutamine synthetase is also found in neoplastic glial cells (Norenberg, 1979).

1.2.1.4. Developmental changes in glutamine synthetase

The highest levels of GS in the nervous system have been reported in the neural retina (Moscona et al, 1980).

The retina is developmentally and functionally an extension of the brain. It arises from the embryo as a vesicle on the wall of the mid-brain and develops from a simple neuroepithelium into a system specialised for reception, integration and transmission of light signals. In the retina of the early chick embryo the level of GS is very low and increases only slowly. However, on the sixteenth day of incubation, GS levels in the retina begin to rise sharply, increasing 100-fold in five to six days, then plateaus (Moscona et al, 1980). This rise takes place after growth and cell replication in the retina has ceased and is triggered by elevation of adrenal corticosteroids in the embryo which occurs one or two days earlier (Moscona et al, 1980). GS in the retina can be induced precociously in vivo by injecting cortisol into eggs, and in vitro, in organ cultures of retinal tissue isolated from nine to fifteen day embryos, by adding cortisol to the culture medium (Moscona et al, 1980).

The induction of GS involves differential gene expression elicited by the steroid hormone. The steroid enters the cell and binds to cytoplasmic receptors forming a steroid-receptor complex which is translocated into the nucleus where it associates with chromatin and elicits transcription of GS mRNA resulting in enzyme synthesis and accumulation.

Patel et al (1983a) measured GS levels in different regions of the rat brain during development and found that there was a linear increase in GS activity during development although the major increase occurred after total cell acquisition had ceased. This increase was therefore associated with astrocyte differentiation. Glucocorticoids have been shown to regulate the activity of GS in the developing rat brain in vivo (Patel et al, 1983b).

1.2.2. Glial fibrillary acidic protein (GFAP)

1.2.2.1. Historical background

Glial fibrillary acidic protein (GFAP) was first isolated from multiple sclerosis plaques (Eng et al, 1971). Old multiple sclerosis plaques are composed of fibrous astrocytes filled with glial filaments and demyelinated axons. Fibrous gliosis is a principal event in CNS trauma and a common feature of many neurological disorders. The most prominent feature of fibrous gliosis is glial filament synthesis. The proteins of multiple sclerosis plaques when separated by polyacrylamide gel electrophoresis (PAGE) showed a major protein band with a molecular weight of 47,000-51,000 (Eng et al, 1971).

1.2.2.2. Isolation

GFAP has a tendency to polymerise and co-aggregate with other acidic proteins during extraction making isolation by chemical or physical methods difficult.

However, recent advances in the isolation of intermediate filaments, in particular the resolution of neurofilaments and glial filaments by hydroxyapatite column chromatography, now allow the isolation of large quantities of GFAP for chemical, immunological and metabolic studies (Chiu, 1981).

1.2.2.3. Properties

Most samples purified by PAGE have similar compositions consisting of high proportions of arginine, aspartic acid, glutamic acid, alanine and leucine. The terminal amino acids are either alanine, leucine or methionine (Dahl, 1976).

1.2.2.4. Antibody preparation

Various sources have been used to produce specific antibodies to GFAP; Uyeda et al (1972) used GFAP from multiple sclerosis plaques and Palfreyman et al (1979) prepared antibody against GFAP derived from cerebellar astrocytomas. Specific antibodies have been prepared against soluble (i.e. 50 mM phosphate buffer, pH 8.0 extractable) and buffer-insoluble GFAP (Eng and Uyeda, 1973), and complete immunological identity was found between the antiserum prepared from soluble and insoluble GFAP.

1.2.2.5. Assay of GFAP

Several methods have been developed for the

quantitative assay of GFAP; these include rocket immunoelectrophoresis (Jacque et al, 1976), competitive binding radioimmunoassay (Liem et al, 1978; Lowenthal et al, 1978), two-site immunoradiometric assay (Lowenthal et al, 1978) and immunodiffusion (Dahl and Bignami, 1976). Palfreyman et al (1979) have developed a radioimmunoassay using antiserum raised against GFAP purified from human cerebellar astrocytomas. Patel and Seaton (see Mwang'ombe et al, 1986) have developed a method for measurement of both soluble and insoluble GFAP using an enzyme linked immunosorbent assay (ELISA). Lee et al (1976) found that in normal tissue the major portion of GFAP antibody binding activity resided in the insoluble fraction and less than 10% in the soluble fraction.

1.2.2.6. Immunocytochemistry

Antibody to GFAP and the peroxidase-antiperoxidase method (PAP) have been used to immunocytochemically localise GFAP in formalin-fixed paraffin embedded tissue sections (Deck, 1978; Eng, 1978; Antanitus et al, 1975). These methods have been modified for use in tissue culture.

1.2.2.7. Developmental changes in GFAP

GFAP is present in the astrocytes of many species with immunocytochemical interspecies cross reactivity. In the mouse, it appears with the differentiation of

astrocytes, between 10 to 14 days postnatally (Jacque et al, 1976). In humans, GFAP has been demonstrated at gestational ages of 12 to 20 weeks in tissue culture explants from human fetal forebrain (Antanitus et al, 1975).

1.2.2.8. GFAP and differentiation of astrocytes

GFAP has been shown to be a specific marker for astrocytes (Bignami et al, 1972; Eng et al, 1971) and a major component of the intermediate 9 nm filaments of astrocytes (Eng and Kosek, 1974). There is an inverse relationship between GFAP and the degree of histological malignancy. Benign astrocytomas with well differentiated stellate astrocytes have abundant GFAP in their cytoplasm and processes but in more malignant astrocytomas, cells expressing GFAP are sparse (Duffy et al, 1980; 1982). According to a hypothesis proposed by Wickremesinghe (1971), a decrease of glial specific antigens is part of a general tendency for a progressive reduction of organ specific antigens as tumours progress from benign to malignant (Wikstrand and Bigner, 1979).

There is a strong relationship between GFAP and cell shape. Protoplasmic astrocytes express little GFAP, have large cell bodies, a large number of richly arborised processes but few filaments. Fibrillary astrocytes in white matter have smaller cell bodies and longer, thicker, highly developed processes containing many

filaments and dense GFAP (Duffy et al, 1982). During reaction to injury, protoplasmic astrocytes appear to be converted into fibrillary astrocytes with development of more abundant GFAP.

The amount of GFAP expressed by astrocytoma cells in tissue culture tends to vary from cell to cell and from culture to culture (Duffy, 1982). "Serum starvation" can induce apparent differentiation of astrocytes in culture which is characterised morphologically by a permanent increase in the number and length of processes, and immunocytochemically by a re-distribution of GFAP in the cells (Duffy, 1982). Raju (1980) reported increased GFAP in cultures of C_6 glioma after dibutyryl cyclic adenosine monophosphate (dbcAMP). There was also an increase of GFAP with increasing age of the cultures. This increase in GFAP with time in cultured astrocytes was usually observed when short-term growth without passage was employed (Bock et al, 1980). However, Spence (1981) showed that rat astrocytoma cells exposed to dbcAMP developed arborisation of processes and increased microvillous development but had no concomitant increase in intermediate filaments or GFAP as shown by scanning and transmission electron microscopy.

1.3. POLYAMINES

1.3.1. Definition and function

These are a group of structurally simple cationic

compounds, putrescine, cadaverine, spermidine and spermine. Putrescine is derived from arginine and cadaverine from lysine. They are widely distributed in living organisms including plants, bacteriophages, bacteria and animal tissues.

Polyamines have been associated with DNA structure and synthesis (Cohen et al, 1969) and with cell differentiation (Heby, 1981). Increased levels of polyamines have been observed during chick embryo development (Raina, 1963) and in rat liver after partial hepatectomy (Dykstra and Herbst, 1965). Levels of polyamines have been found to be high in tumour tissue (Russell et al, 1975) particularly CNS tumours (Kremzner, 1973).

Several attempts have been made to find a role for polyamines in clinical medicine. High levels of polyamines have been found in the CSF of patients with CNS tumours (Marton et al, 1976), especially medulloblastoma (Marton et al, 1981a). These levels rise during chemotherapy and become lower in successfully treated patients. A rise in urine and serum spermidine with chemotherapy is said to be a useful predictor of good clinical response (Russell et al, 1975).

1.3.2. Ornithine decarboxylase

The occurrence of L-ornithine decarboxylase (ODC)

in animal tissues was reported independently by Janne and Raina (1968), Pegg and Williams-Ashman (1968) and Russell and Snyder (1968). ODC levels rise dramatically in chick embryos during development and in liver after partial hepatectomy or growth hormone treatment. ODC catalyses the conversion of ornithine to putrescine, the rate-limiting step of polyamine synthesis. The transition of cells from quiescence to proliferation is associated with an increase in activity of the two decarboxylases involved in polyamine biosynthesis, ODC and S-adenosyl methionine decarboxylase (SAMD). This is followed by intracellular accumulation of putrescine, spermidine and sometimes of spermine (Tabor and Tabor, 1976; Janne et al, 1978). It has been suggested that cells at various stages of development have membrane receptors that are differentially responsive to the extracellular polyamine concentration. The more sensitive these receptors are, the lower the intracellular ODC activity and consequently the level of intracellular polyamines. Some tumours or other rapidly growing cells may have membrane receptors that are poorly responsive to polyamines. Such cells will maintain high intracellular ODC and polyamine levels and become excretors of polyamines (Canellakis et al, 1978).

1.3.3. Properties of ODC

The half-life of ODC, as determined by Russell and Snyder (1969), is between 15 and 45 minutes. The half-life of an enzyme is related to the cellular requirements

for rapid change in intracellular concentration and to the enzyme's role in intermediary metabolism (Goldberg and Dice, 1975). The short half-life of ODC has been attributed to its cellular function as a rate-limiting step of polyamine synthesis (Russell and Snyder, 1969). Ono et al (1972) estimated its molecular weight to be approximately 100,000. It has been suggested that the active enzyme has a molecular weight of approximately 55,000, but forms an inactive dimer in the absence of dithiothreitol. Co-factors which are necessary for maximal enzyme activity are thiol groups, pyridoxal phosphate and calcium ions.

1.3.4. ODC anti-enzyme

An anti-enzyme to ODC was first demonstrated by Canellakis et al (1978). This reacted specifically with ODC, neutralised its activity and had a relatively short half-life. It has been postulated that ODC anti-enzyme is normally attached to subcellular components (mainly the nucleus and ribosomes) and that polyamines at millimolar concentrations cause its release (Heller et al, 1977).

1.3.5. Regulation of ODC activity

The synthesis and activity of ODC is controlled through decay of enzyme activity and ODC anti-enzyme, which is activated when cellular polyamine levels reach a threshold level. The anti-enzyme neutralises cellular

ODC. The synthesis of ODC is also subject to regulation by polyamines (Russell and Snyder, 1969; Canellakis et al, 1978; Heller et al, 1977). The product of the reaction catalysed by ODC (putrescine) is an obligatory activator of SAMD which is the enzyme that regulates synthesis of spermidine and spermine (Tabor and Tabor, 1976; Janne et al, 1978; Duffy and Kremzner, 1977; Bachrach, 1980). Regulation of ODC activity involves membrane receptors and it has been postulated that tumour cells which are rich in polyamines have lost some of these receptor sites (Canellakis et al, 1978).

1.3.6. Ornithine decarboxylase inhibitors

Specific inhibitors of the polyamine biosynthetic enzymes have been employed in mammalian cells to create polyamine deficiency. In mammalian cells polyamine biosynthesis involves the sequential action of two decarboxylases and two transferases. The inhibitors of polyamine biosynthesis that have been developed so far are directed towards the carboxylases. The reasons for this are that firstly decarboxylation of ornithine is the rate limiting step of the polyamine biosynthetic pathway (Williams-Ashman et al, 1968), and secondly the complexity of the catalytic mechanisms of the transferases is incompletely understood. The mechanism of action of pyridoxal phosphate dependent and to a lesser extent, pyruvate dependent α-amino acid decarboxylases is well established (Boecker and Snell,

1972).

The first irreversible synthetic inhibitors of ODC described were α-difluoromethyl ornithine (DFMO) and α-acetylenic putrescine (Metcalf et al, 1978). These compounds belong to the class of enzyme activated irreversible inhibitors known as kCat inhibitors (Rando, 1974) or suicide enzyme activators. These compounds are usually substrate or product analogues of the target enzyme and are chemically inert. Their action requires an enzyme catalysed activation which generates an electrophilic form of the substrate analogue inside the active site of the enzyme. A subsequent reaction of this activated intermediate with a nucleophilic residue of the active site leads by covalent linkage to irreversible inactivation of the enzyme.

Experimental approaches used to create polyamine deficiency in mammalian cells have some short-comings. Normally, control mechanisms operating intracellularly and at the cell membrane, maintain a critical intracellular polyamine concentration essential for optimal rates of the metabolic processes (Russell and Snyder, 1969; Canellakis et al, 1978; Heller et al, 1977). As an immediate consequence of polyamine deficiency, ODC and SAMD activities increase and oppose the action of the inhibitors, hence the advantage of using irreversible inhibitors.

1.3.7. Polyamines and the cell cycle

The levels of ODC and SAMD have been shown to increase when quiescent, G_1-arrested cells are stimulated to proliferate. In mouse kidney cells, the activities of both these enzymes were elevated biphasically during lytic infection with polyoma virus (Goldstein et al, 1976). The first rise in activity occurred almost immediately after infection and the second peak followed the induction of DNA synthesis. The mechanism of polyamine action in DNA replication is not understood. Polyamines probably serve as co-factors in DNA synthesis perhaps by stimulating DNA polymerase (Stalker et al, 1976).

1.3.8. Polyamines and cell differentiation

The cellular polyamine concentration is related to the rate of cell proliferation and to cell differentiation, although the mechanisms through which this occurs are unclear (Jetten and Shirley, 1985; Pegg and McCann, 1982). Polyamines are also substrates for transglutaminase enzymes whose activity is regulated by calcium. Polyamines and calcium are associated with the polymerisation and depolymerisation of cytoskeletal elements (Gibbs, 1980). Calcium also plays a role in the formation of putrescine, spermidine and spermine (Jensen et al, 1987). Gibbs (1980) suggested that a microtubule-microfilament structure may convey growth regulatory

information within the cell, a process which could be dependent on ODC activity.

1.4. HORMONE RECEPTORS

1.4.1. Definition

Hormone receptors are not a single chemically defined group of macromolecules. Steroid hormone receptors are found within the cytoplasm and cell nucleus. The term receptor is reserved for a cellular component which is capable of recognising and selectively interacting with the hormone and which is then capable of generating a signal that initiates the chain of events leading to a biological response (Clarke and Peck, 1977).

1.4.2. Criteria

The criteria used to establish whether a hormone binding site falls within the above definition (Clarke and Peck, 1977) are: (i) Since there are a limited number of receptors per cell, binding should be saturable; (ii) binding should be reversible and of high affinity, since most hormone effects are transient and occur in response to relatively low free hormone concentrations; (iii) binding should be specific for the hormone and related hormone analogues, with a strong positive correlation between the binding affinities of different analogues for the putative receptor and their observed agonist or antagonist activities in vivo; (iv) binding should be tissue-specific and confined to those organs that show

a response to the hormone; (v) it should be possible to demonstrate that hormone binding is in some way functionally linked to the initiation of a hormonal response.

The first three of the above criteria are generally accepted for the definition of a CNS hormone receptor site (Clarke and Peck, 1977).

1.4.3. Mode of action

Steroid hormone action involves the initial attachment of the hormone to a cytoplasmic receptor site and subsequent translocation of all or part of the hormone receptor complex to the cell nucleus. Within the nucleus the receptor complex binds to the chromatin and initiates changes in gene expression, which ultimately are translated into an overall cellular response (King, 1974; O'Malley, 1974).

1.4.4. Properties

Steroid hormone receptors are thermolabile acidic proteins which are released into the soluble (cytosol) fraction of the cytoplasm following tissue disruption, unless the tissue has been exposed to steroids, in which case, a proportion of the receptors will also be found bound within the cell nucleus. Complexes between the receptors and hormonal steroids are precipitable with cations such as protamine (Clarke and Peck, 1977; King,

1974; Lieberburg, 1978).

Free sulphydryl groups appear to be essential for the integrity of the receptor hormone binding site. Binding activity is destroyed by treatment with sulphydryl group blocking agents and is rapidly lost under cell free conditions by contact with air, unless reducing agents such as ß-mercaptoethanol, thioglycerol or dithiothreitol are incorporated into the medium. At low temperature (0-4°C) and in low ionic strength buffers (<50mM), the receptor steroid complexes sediment at around 6-10S.

1.4.5. Specific and non-specific binding

The binding of ligands by a receptor is selective and therefore defined as "specific" or "non-specific". "Non-specific" binding results from interaction of ligands with sites that are of low affinity and high capacity relative to the receptor. The total bound ligand in a system composed of specific and non-specific sites is the sum of that bound to receptor plus that bound to non-specific sites. Total binding and non-specific binding are non-saturable (Peck and Kelner, 1982).

Direct measurement of non-specific binding can be made by inhibiting labelled ligand binding using a non-labelled competitive ligand (Peck and Kelner, 1982). The receptor sites are exposed to multiple concentrations of

radioactive ligand in the presence and absence of an excess of non-radioactive competitive ligand. The total binding represents radiolabelled ligand that is bound to both receptor sites and non-specific sites. Non-specific binding is the radioactive ligand bound in the presence of excess unlabelled competitive ligand. Receptor sites are estimated by subtracting non-specific binding from total binding. The use of inhibition to determine receptor binding parameters is based on the assumption that the non-labelled ligand is a competitive inhibitor and that the non-specific binding sites are of low affinity and high capacity relative to the receptor system (Peck and Kelner, 1982).

1.5. ENZYME INDUCTION

De novo biosynthesis of certain enzymes are subject to a switch-on mechanism elicited by components in the surrounding medium. The induction processes may effect a change in phenotype, allowing further production of energy required for metabolism and growth.

Usually major inducible enzymes are formed by de novo synthesis from amino acids (Jacob and Monod, 1961) rather than activation of inactive peptide or enzyme precursor. The overall rate of enzyme biosynthesis is controlled by the rates of transcription and translation of mRNA.

Jacob and Monod (1961) formulated their concept of mRNA as the intermediary through which nuclear genes exert their control of the types and quantities of proteins formed by a cell. They suggested mechanisms by which genetic information encoded within a gene could be translated to synthesis of that protein within the cytoplasm. They also suggested how the rate of formation of that protein could be controlled by regulatory genes. According to their hypothesis, structural genes are controlled by inducer molecules which modulate the binding of repressor proteins which in turn repress the expression of structural genes (Jacob and Monod, 1961).

Basal (uninduced) enzyme and induced enzyme are biochemically similar and made by transcription of the structurally identical genes. A slow or occasional transcription of these genes does occur which is accelerated by the switch-on effect of the inducer. The induced activity divided by the basal activity of a particular enzyme is known as the induction ratio. Certain substances related structurally to the natural inducer of a particular system may be much better inducers, whether they serve as productive substrate of the inducible enzyme or not. When induction is obtained without hydrolysis of inducer it is termed gratuitous induction.

1.6. GROWTH FACTORS

These are substances, frequently polypeptides, that stimulate cell proliferation and may promote cell differentiation of specific target cells (Levi-Montelcini and Calissaro, 1986). These growth factors interact with specific surface receptors on a target cell. Some tumour cells manufacture their own growth factors *in situ* and *in vitro* and possess functional receptors for them. These cells therefore become self stimulating or "autocrine" (Schmidek, 1987). Growth factors or their receptors may be related to products of oncogenes for example, the c-erb B oncogene encodes the epidermal growth factor (EGF) receptor and c-sis encodes one of the polypeptides of platelet derived growth factor (PDGF) (Schmidek, 1987).

EGF was discovered during studies on nerve growth factor (NGF) activity in submaxillary gland extracts. EGF receptors are present on astrocytes and, to a lesser extent, oligodendrocytes (Schmidek, 1987). Overexpression of EGF receptors has been reported in various primary brain tumours (Hunter, 1984; Leutz and Schachner, 1981; Libermann *et al*, 1984, 1985).

PDGF is released from platelets when blood vessels are injured as one of the initial stages of tissue repair. Some glioma cell lines secrete PDGF although this agent does not cause cellular transformation by itself (Nister *et al*, 1984; Pantazis *et al*, 1985).

Human glial cells from brain biopsies, transformed rat glioma cell lines and neuroblastoma cells have been shown to release NGF. The activity of NGF also extends to non-neuronal cells such as chromaffin and mast cells (Schmidek, 1987). The murine NGF gene has been cloned and found to code for a 307 amino-acid precursor which can be processed to form two biologically active 118 amino-acid proteins that are linked together (Schmidek, 1987).

Glial growth factor (GGF), fibroblast growth factor (FGF), EGF and PDGF cause division of astrocytes in vitro (Lemke and Brockes, 1984). Other growth factors which have also been shown to stimulate DNA synthesis and cell division of astrocytes are bombesin, vasopressin, substance P and substance K (Thoenen and Edgar, 1985).

1.7. CELL DIFFERENTIATION

1.7.1. Genetic regulation

The process of differentiation requires a heritable alteration in the patterns of genes in one of the two progeny cells arising from the same parent cell. This may occur through a number of mechanisms:

1.7.1.1. Somatic mutation

In this case, there is an alteration in certain nucleotide sequences in DNA resulting in production of different proteins. Although this may occur spontaneously, it can be induced by certain drugs, chemicals and radiation.

1.7.1.2. Genetic recombination

In this process, specific gene sequences are split and re-spliced into different parts of the genome.

1.7.1.3. Gene amplification

Here, extra copies of certain genes are generated in response to external stimuli, for example, amplification of the gene for dihydrofolate reductase in cells selected for resistance to methotrexate (Schimke, 1978).

1.7.1.4. Selective transcription

Specific transcription of genes in one cell type as opposed to another within a mixed population of cells.

1.7.1.5. Selective translation

This occurs, for example, in cells exposed to hormones that induce synthesis of specific proteins. There is translation of only certain mRNA species in cells controlled by signals from the environment.

The altered gene expression may be maintained through passage of the protein itself or its mRNA from parent to progeny at each mitosis (Temin, 1976).

1.7.2. The role of micro-environment on differentiation

There are several factors in the micro-environment that contribute to the differentiation of cells (Bunge

and Waksman, 1985). These include positional effects and polarity of a given cell in relation to the rest of the developing cellular mass; interactions of cells with surrounding stroma or supportive tissue and direct cell-to-cell contact; gradients of oxygen, nutrients and ions in the area of developing multicellular mass; and presence of specific growth and differentiation factors. Information transfer between cells could occur by direct cell-to-cell contact or via hormones or growth factors secreted by one cell type which act locally. Desmosomes and junctional complexes provide orientation to the cell and are a means of chemical and electrical communication.

Calcium ion concentration plays a key role in cellular proliferation and differentiation. Low calcium concentrations result in dedifferentiation, absence of desmosomes and reduced cell-to-cell contact. The levels of calcium are modulated by a calcium binding protein called calmodulin. The calmodulin-calcium complex is involved in the activation of several enzyme systems e.g., protein kinase, phosphodiesterase and adenylate cyclase which controls cellular levels of cAMP. cAMP plays a key role in differentiation of certain cell types.

1.8. CELL PROLIFERATION AND RESTRICTION POINT CONTROL

In normal tissue there is a well controlled balance between cell division, cell differentiation and cell

death and this balance is disrupted in cancer (Gross, 1968). Normal cells respond to a variety of suboptimal conditions by entering a quiescent phase in the cell cycle called G_0. There is a decision point in G_1 phase at which time the cell must make a commitment to continue into S phase or stop in G_1. If this waiting period is prolonged, the cells are said to be in G_0 phase. If cells make the commitment they continue through S, G_2, M and return to G_1. If the cells are blocked in S, G_2 or M phase for any length of time they die. This point in G_1 is termed the "restriction point" (Pardee, 1974). There are a number of environmental conditions which shift cells *in vitro* into G_0. These include limitations of serum and of certain essential amino acids such as isoleucine and glutamine, glucose deprivation and deprivation of certain lipids (Pardee, 1978). Cells at high cell density *in vitro* also tend to enter the quiescent phase. The biochemical mechanism of restriction point control is not known. It is probably related to the accumulation of a labile initiator protein that triggers entry into S phase. It has been postulated that serum growth factors may be responsible for inducing synthesis of this protein (Pardee, 1978).

The growth characteristics of transformed cells suggest that they have lost stringent restriction point control. They continue to grow under conditions of high cell density, low serum concentration or suboptimal

nutrient concentrations that block normal cell growth. Transformed cells may have altered control of the synthesis or degradation of initiator proteins or have a decreased requirement for them.

1.9. INDUCTION OF TUMOUR DIFFERENTIATION

Cancer can be considered as a disorder of normal cell differentiation (Pierce et al, 1978). Neoplastic cells are blocked prior to achieving their fully differentiated state and maintain the capacity to divide, a property that most normal cells lose as they mature. This blocked differentiation is not totally irreversible and some certain types of cancer cells can be induced to differentiate to less malignant states by exposure to certain factors.

Developing cells produce protein factors which act as signals for the control of proliferation and differentiation. These signals cause the cell to switch between proliferation and differentiation. Differentiation occurs in the G_1 phase of the cell cycle (Scott et al, 1982). The cell is thought to contain a proliferation factor which responds to outside signals operating at specific receptors of the cell membrane. Outside signals include both growth and differentiation factors elaborated by various cells within interrelated tissues or organs. This proliferation factor may be similar to the long advocated labile protein (Bloch,

1983) said to be required for cell progression from G_1 to S.

Oncogenes when activated are responsible for the conversion of normal cells into tumour cells and they cause a permanent halt in the differentiation process by holding the affected cells in the proliferation mode. This may be achieved when the proliferation factor levels are elevated, its response to modulation by growth factors is altered or its response to differentiation factors is altered. During normal haematopoiesis, an immature stem cell proceeds via several differentiation steps to become a mature cell that lacks the capacity to proliferate (Till and McCulloch, 1980). Arrest of differentiation, without loss of proliferative activity, may occur at any one of these steps and this may give rise to immature cells. Leukaemic cell differentiation *in vitro* may be induced by certain phorbol esters (Breitman *et al*, 1980); 12-O-tetradecanoyl phorbol-13-acetate (TPA) can inhibit binding of EGF to its receptor (Lee and Weinstein, 1978) and retinoic acid can interfere with the mitogenic response induced by EGF and by serum factors (Jetten, 1982). Phorbol esters initiate maturation events through their ability to modulate growth factor binding and activity. A decrease in growth factor dependent events enhances the cells' sensitivity to the action of differentiation factors (Lotem and Sachs, 1979).

Agents which interact specifically with DNA may cause inhibition of proliferation-associated DNA synthesis or function and cause unfreezing of the proliferation mode by curtailing the message required for synthesis of the proliferation factor. Takeda et al (1982) showed that in vitro only 2-4% of ML-1 myeloblastic leukaemia cells differentiate spontaneously to intermediate stages of maturity, while in the presence of DNA-specific inhibitors, a majority of viable cells undergo differentiation. This drug-induced differentiation involves the conversion of the myeloblastic leukaemic cells to cells with morphological characteristics of monocytes or macrophages. Many of these DNA-specific inhibitors result in loss of cell viability and an appropriate dose sufficient to cause differentiation induction but which causes minimal cell death needs to be carefully determined. This would be the dose capable of decreasing the expression of proliferation-associated information without blocking subsequent events related to differentiation. Differentiation proceeds from a specific stage in G_1 (Scott et al, 1982) and so cells present in another phase of the cell cycle in which they are optimally sensitive to the action of a drug would be destined to die rather than to differentiate. Cells that enter such a phase when the initial drug concentration is already diminished, or which are present in less sensitive phases of the cell

cycle, would be able to proceed to G_1 and to maturation.

It is now possible to introduce genes into cells and study gene regulation. It has proved possible in various cell lines to induce differentiation by gene transfer or transplantation (Alema et al, 1985). These techniques involve calcium precipitation, electroporation and microinjection of DNA directly into the cells. The calcium precipitation method is used to co-precipitate DNA with calcium phosphate. The DNA-calcium phosphate particles are then mixed with the cells to be transfected. The complex binds to the surface of cells and is ingested into the cell by endocytosis. The DNA reaches the cytoplasm and later the cell nucleus of the recipient cell. Some of the cells incorporate the foreign DNA into their genome and express the appropriate gene product. The electroporation method utilises a high voltage of short duration to render the cell membrane transiently permeable which allows the entry of DNA. It seems likely that these techniques, will, in the future be of considerable importance for studies on the genetic basis of differentiation in cells from human malignant glioma.

1.10 AIMS OF THE STUDY

The major objectives of the study are as follows:

(a) To carry out a quantitative study of the growth

characteristics and expression of astrocytic markers, GFAP and GS, in VMDk P497 cells in vitro.

(b) To carry out a quantitative study of the effect of dexamethasone on GS levels during cultivation in vitro.

(c) To study the mechanisms by which dexamethasone mediates biochemical effects on VMDk P497 cells in vitro.

(d) To study the role of ODC (and polyamines) on the proliferation of VMDk P497 cells in vitro.

(e) To study the effect of inhibition of ODC activity (and depletion of intracellular polyamine levels) on the proliferation of VMDk P497 cells in vitro and to determine whether inhibition of cell proliferation produces apparent differentiation in these cells.

(f) To determine whether pre-treatment of syngeneic VM mice with oral DFMO is capable of enhancing the modest growth-delay produced by BCNU using subcutaneous implants of VMDk P497-(P1) tumour. The biochemical changes induced by such treatment will also be investigated.

2.1. Media and reagents for cell culture

These were supplied by Flow Laboratories, Rickmansworth, Hertfordshire unless otherwise stated.

Dimethylsulphoxide (DMSO): This was supplied by Sigma, Poole, Dorset. It was sterilised by autoclaving at 121°C in tightly capped glass bottles and stored frozen at 4°C.

Ham's F-10 medium: This was supplied as a x 1 concentrate with 12.0 mg/l phenol red and 20 mM HEPES buffer and stored at 4°C.

Hanks' balanced salt solution (HBSS): Supplied as a sterile x 1 concentrate with phenol red, supplemented with 0.35 g/l sodium bicarbonate.

PBS solution: Prepared from phosphate buffered saline tablets (modified Dulbecco's formula, without calcium and magnesium). Each tablet was dissolved in 100 mls of distilled water and autoclaved at 121°C.

Penicillin and streptomycin: Supplied as a mixed sterile solution of penicillin (5000 i.u./ml) and streptomycin (5000 μg/ml) and stored frozen at -20°C until use.

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 murine and human glioma cell lines in monolayer cultures.

Growth medium (GM): Ham's F-10 medium with 50 i.u./ml penicillin and 50 μg/ml streptomycin, buffered with 20 mM HEPES and supplemented with 10% v/v FCS.

Trypsin solution: This was supplied as a 0.25 % w/v solution of trypsin in HBSS (1:300) with 0.5 g/l sodium bicarbonate, 200 i.u./ml penicillin and 100 μg/ml streptomycin but without calcium, magnesium and phenol red. This was aliquoted in 10-20 ml lots in plastic universals and stored frozen at -20°C.

2.2. Plastic and glassware for cell culture

Nunclon (Gibco, Paisley, Scotland) or Lux (Flow) tissue culture flasks were used in all these experiments. 24-well tissue-culture-treated multiple well plates were used for growth curves (Flow Laboratories or Sterilin, Hounslow, Middlesex). Multiple well plates were sealed with clear mylar plate sealers (Flow). Plastic pipettes (Sterilin) were used for measuring liquids for cell culture, enzyme assays and drug treatments. Cells were frozen in polypropylene screw-capped cryotubes (1.2 ml capacity, Gibco).

Glass coverslips, 9 x 22 mm or 18 x 18 mm, no. 1 thickness were supplied by Chance Proper, Warley, West Midlands.

2.3. VM strain mice

The inbred VM mice are bred locally at the Institute of Neurology, Queen Square.

2.4. Murine astrocytoma cell lines, VMDk P 497 and VMDk P497-P(1)

VMDk P497 is a permanent glioma cell line derived from the VM spontaneous murine astrocytoma and was a gift from Dr. D. Bigner, Duke University Medical Center, Durham, North Carolina. All the in-vitro experiments were done with this cell line. The cell line was not used beyond passage 40. VMDk P497-P(1) was established from the parent VMDk P497. It was derived from a single animal treated with procarbazine (Bradford et al, 1986). The in-vivo experiments were done with this cell line. The cell line was used at passage 15.

2.5. Passaging of glioma cell cultures

Monolayer cultures were washed twice in HBSS and 2-5 ml trypsin solution added to each flask. The flasks were incubated for 5-15 minutes to allow the cells to become detached from the surface of the flask. Growth medium (10 ml) was added to each flask and the cell suspension was pipetted to complete disaggregation. Cells

were then diluted and plated for enzyme or receptor assay, passaged or frozen. For routine passaging, split ratios of 1:5 or 1:10 were used.

2.6. Cell freezing

Cells in monolayer cultures were trypsinised, counted and the concentration adjusted to give approximately 1 x 10^6 cells/ml. Sterile DMSO was added to give a 10% v/v solution and 1 ml aliquots pipetted into cell freezing vials. These vials were placed in foam-expanded polystyrene boxes (wall thickness approximately 7.5 mm) and then placed in a -70^0 C freezer. After 18 hours the vials were transferred to the liquid phase of a liquid nitrogen freezer. The cell lines were frozen routinely at every passage level to maitain adequate stocks.

2.7. Mycoplasma screening

The cell lines were screened for mycoplasma contamination using a fluorochrome method (modified from Chen, 1977). Cells were grown for 48 hours in 6-well plates containing glass coverslips. The coverslips were removed, fixed in methanol, washed with PBS solution and treated with Hoechst 33258 (10 μg/ml, Sigma) solution for 15-20 minutes at room temperature. The coverslips were then washed in PBS solution and mounted in PBS/glycerol (10 parts: 90 parts) and examined under a Leitz Dialux EB 20 fluorescence microscope. No cells showing any

extranuclear fluorescence indicative of mycoplasma contamination were detected during this series of experiments.

2.8. Preparation of cells for biochemical assays and determination of growth rate in vitro

Cell cultures in exponential growth phase were trypsinised and diluted in growth medium to give 1-2 x 10^4 cells/ml. 0.1 ml of this diluted cell suspension (1-2 x 10^3 cells) was added to each well of a 96-well microtitration plate using a 5 ml Hamilton syringe (V.A. Howe, London) and repeating dispenser. Wide bore blunt needles (19G, Monoject, Sherwood Medical, St. Louis, USA) were used with the syringe to minimise cell damage. Plates were sealed with Mylar film and incubated at 37°C. Cell counts were carried out using these plates to confirm that the cells were in exponential or plateau phase during biochemical assays. The plates were refed with fresh growth medium at daily intervals. At each time point medium was aspirated from the wells and the wells washed with 0.2 ml of trypsin solution. The cells were then incubated at 36°C with 0.1 ml of fresh trypsin solution, resuspended in 20 mls of isotonic saline (Isoton, Coulter Electronics, Luton, Bedfordshire) and counted in a precalibrated model D Industrial Coulter Counter fitted with a 140 μm orifice tube. Growth curves were plotted on semilogarithmic graph paper and the doubling time determined. The growth was considered to be

exponential if a plot of cell numbers versus time was linear when plotted on semilog graph paper.

To study the effect of dexamethasone on the rate of cell growth *in vitro*, cells were plated on 96-well microtitration plates at a concentration of 2×10^3 cells per well. Cultures were set up in two groups. Group 1 acted as untreated controls. Group 2 cultures were grown in growth medium containing 1 μM dexamethasone. The number of cells per well was determined on days 5, 6, 7 and 8 after plating and growth curves plotted.

To study the effect of DFMO on the rate of cell growth *in vitro* cells were plated on 96-well microtitration plates at a concentration of 2×10^3 cells per well. Cultures were set up in two groups. Group 1 cultures, acted as controls and were grown in normal growth medium. Group 2 cultures were grown in growth medium containing 5 mM DFMO. The number of cells per well was determined on days 2, 3, 4, 5, 6 and 8 after plating and growth curves plotted.

For biochemical assays cultures in exponential growth were trypsinised and diluted in growth medium to give 1×10^5 cells per ml. The diluted cell suspension was used to set up the following experiments:

(a) To study changes in cell number, protein content, GS

activity and GFAP levels during growth in vitro. The diluted cell suspension was added to 75 cm^2 flasks to give 2 x 10^5 cells per flask. The cells were harvested on days 4, 5, 6, 7 and 8 after plating. Before cell homogenisation the number of cells per flask was determined.

(b) To study the effect of dexamethasone on cell number, protein content and GS activity, the diluted cell suspension was added to 75cm^2 flasks to give 2 x 10^5 cells per flask. Cultures were set up in four groups. Group 1 cultures were untreated controls for GS and protein assay. Group 2 cultures were untreated controls used to determine number of cells per flask. Group 3 cultures were treated with 1 μM dexamethasone for 24 hours prior to harvest. The samples were assayed for GS activity and protein content. Group 4 cultures were treated with 1 μM dexamethasone for 24 hours and the number of cells per flask determined. The cultures were harvested on days 5, 6 and 8 after plating.

(c) To study changes in glucocorticoid receptor levels during growth in vitro, the diluted cell suspension was plated in 75cm^2 flasks at a concentration of 2 x 10^5 cells per flask. Cultures were set up in two groups. Group 1 cultures were used for cytosolic glucocorticoid receptor assay and group 2 cultures were used to determine cell number. Group 1 cultures were harvested into ice-cold

buffer solution A (containing EDTA, dithiothreitol, sodium molybdate, TRIS and potassium chloride) at pH 7.4 (see section 2.18). The harvested samples were used for a fixed-point glucocorticoid receptor binding assay (see section 2.19).

(d) To study changes in ODC activity during growth in vitro and the effects of these changes on cell proliferation, the diluted cell suspension was added to 75cm^2 flasks to give 2 x 10^5 cells per flask. Cultures were set up in two groups. Group 1 cultures were used for ODC assay and protein estimation. Group 2 cultures were used to determine cell number. Cultures were harvested 2, 4, 6 and 8 days after plating.

(e) Time-course experiment to study the effects of treatment with DFMO on ODC and GS. The diluted cell suspension was added to 75cm^2 flasks to give 4 x 10^5 cells per flask. Cultures were set up in four groups. Group 1 cultures were untreated controls for ODC and GS assay. Group 2 cultures were untreated controls for determination of cell number. Group 3 cultures were treated with 5mM DFMO for 12, 24, 36 or 48 hours before harvest and assay for GS, ODC and protein. Group 4 cultures were treated with 5mM DFMO for 12, 24, 36 or 48 hours, and the number of cells per flask estimated. Cultures in the four groups were harvested after four days in vitro.

2.9. Subcutaneous implantation of mice with VMDk P497-P(1) cells

Monolayers were harvested using a rubber policeman and resuspended in growth medium. Mice were anaesthetised with ether, and injected with 10 μl of growth medium containing 1 x 10^6 cells subcutaneously in both flanks. Cells were used at the 15th *in vitro* passage. The mice were between 90th and 120th days of life. The mice were fed on pellets and water *ad libitum*. They were maintained five per cage in a room with a light-dark cycle (12 hours on, 12 hours off). Experiments were performed between 7 a.m. and 7 p.m.

2.10. Freezing tumour fragments

Mice were killed by cervical dislocation and the subcutaneous tumours removed by dissection. The tumours were weighed and subdivided into small pieces of approximately equal sizes. Tumours were placed in cell freezing vials in 1 ml of GM containing 10% DMSO v/v. Tumours were frozen using a method similar to that for cell freezing and then stored in the liquid phase of a liquid nitrogen freezer.

2.11. Subcutaneous implantation of mice with tumour fragments.

Mice were anaesthetised with ether and a small incision made on the skin. Tumour fragments were implanted into both flanks using a fine trocar and

cannula. The mice were allowed to recover and randomly divided into four groups of five mice, groups A-D. Tumour growth was assessed by measuring the size of the tumours in 3 perpendicular axes using a vernier caliper and the mice were weighed regularly. Treatment was started when the tumours became visible, this was termed day 1. Group A acted as an untreated control and was fed on distilled water and pellets. Group B was fed on mouse pellets and 1% DFMO. Group C was fed on distilled water and pellets from day 1-17, and given BCNU (30 mg/kg body weight, single dose, intraperitoneal) on the tenth day. Group D was fed on mouse pellets and 1% DFMO from day 1-17, and given BCNU on the tenth day (30 mg/kg body weight, single intraperitoneal dose). Tumour volumes were calculated by substituting in the equation:

$$\text{volume} = \frac{\pi}{6} (d_1 \times d_2 \times d_3)$$

d_1, d_2, and d_3 were the measured diameters of the tumour obtained in three perpendicular axes. The log of tumour volume was obtained and growth curves constructed using the methods described by Bradley et al (1983).

2.12. Preparation of tumour homogenates for biochemical assays

The animals were killed 30 days after tumour implantation and tumours removed by dissection, weighed and divided into two. The first sample was used for

biochemical studies and the second sample was used for histological study. The sample for biochemical study was homogenised in ice cold 10 mM imidazole buffer (10% solution w/v), pH 7.2, using an all glass Dounce homogeniser. The sample was divided into three aliquots. The first aliquot was used for ODC assay, the second for DNA extraction and estimation and the third for total protein estimation.

2.13. Reagents and solutions for GS assay

L-[1-^{14}C] glutamic acid (specific radioactivity 59 mCi/mmol) was obtained from Amersham International, Amersham, Buckinghamshire. Other materials and chemicals used were of the highest quality and purchased from the sources mentioned in previous publications (Patel and Hunt, 1985; Patel et al, 1982; Patel, 1982; Patel et al, 1983a, 1983b).

Buffer solution 1:

Imidazole	0.681 g.
Magnesium chloride	0.254 g.
Sodium glutamate	0.845 g.
Ouabain	0.073 g.
Triton X-100	0.160 ml.
Mercaptoethanol	0.139 ml.

The above reagents were dissolved in 40 mls of distilled water and the pH of the solution adjusted to 7.2 with 0.1 N HCl. Distilled water was added to make up to a total

volume of 50 mls.

Solution 2:

Ammonium chloride 0.107 g.

Dissolved in 50 mls of distilled water.

Imidazole buffer 0.01M:

Imidazole 10mM at pH 7.2.

PBS solution:

One tablet of PBS dissolved in 100 mls of distilled water and warmed to 37°C.

Isotonic saline:

Sodium chloride 4.5 g.

In 1000 mls of distilled water and warmed to 37°C.

2.14. Cell harvesting for biochemical assays

Cells in monolayer culture were washed three times with prewarmed PBS solution and once with saline at 37°C. The cells were scraped into 0.01 M Imidazole buffer, pH 7.2 at 0°C with a rubber policeman and homogenised using an all glass homogeniser. Whole homogenate was used for biochemical studies.

2.15. Glutamine synthetase assay

Substrate: 0.1 ml [1-^{14}C] glutamate.

Assay mixture: This was made up of 0.1 ml [1-^{14}C]

glutamate, 0.5 ml buffer solution 1, 50 μl adenosine triphosphate (ATP), 128 mg/ml in solution 2, 50 μl phosphoenolpyruvate (PEP) 64 mg/ml in solution 2 and 20 μl pyruvate kinase. The final assay mixture (0.05 ml) contained the following concentrations: 100 mM Imidazole buffer, pH 7.2, 50 mM [1-^{14}C] glutamate (specific radioactivity 0.2 mCi/mmol), 12.5 mM $MgCl_2$, 20 mM mercaptoethanol, 10 mM ATP (sodium salt), 1 mM ouabain, 4 mM ammonium chloride, 13 mM phosphoenolpyruvate, 50 units of pyruvate kinase, 0.16% Triton X-100 and about 0.04 mg of enzyme protein. Boiled homogenates were used in blanks. After incubation at 37°C for 20 minutes the reaction was stopped by adding 1 ml of ice cold water and the reaction mixture immediately passed through a column of ion exchange resin (Dowex AG-1,X8, acetate form; size 0.7 cm diameter x 3.9 cm height and volume 1.5 ml). The [^{14}C]-glutamine which had been formed was eluted in the void volume and collected with 5 ml water wash. [1-^{14}C]-glutamic acid was retained on the column and could be eluted with 6 ml 0.5 M acetic acid. For estimation of radioactivity, the samples were mixed with a scintillation fluid and counted using a Mark III Nuclear Chicago Scintillation Spectrometer (Patel et al, 1977). A unit of GS enzyme specific activity was defined as 1 μmol of glutamine formed per hour per mg of protein.

2.16. Reagents and solutions for ODC assay

Substrate: D,L-[1-^{14}C] ornithine hydrochloride, specific radioactivity 54mCi/mmol, obtained from Amersham.

Other materials and chemicals were of the highest quality and purchased from the sources mentioned in previous publications (Patel and Hunt, 1985; Patel et al, 1982; Patel, 1982; Patel et al, 1983a, 1983b).

Imidazole-EDTA buffer:

Imidazole	500 nM	3.4 g.
EDTA	60 mM	2.2 g.

The above reagents were dissolved in 80 mls of distilled water and the pH adjusted to 7.8. The solution was made up to 100 ml.

2.4 mM pyridoxal phosphate, 0.592 mg/ml.

60 mM Dithiothreitol, 9.2 mg/ml.

1.5 M Sulphuric acid.

Hyamine hydroxide 44%.

Potassium bicarbonate 17.5 mM.

Solution A :

Imidazole/EDTA buffer, 0.6 ml.

Pyridoxal phosphate, 0.5 ml.

Dithiothreitol, 0.5 ml.

Solution B :

Solution A, 0.2 ml.

[1-^{14}C]-ornithine, 50 μl.

2.17. ODC assay

10 μl of solution B was added to 10 μl of homogenate. The assay mixture (20 μl) contained (final concentrations): 75 mM imidazole buffer, pH 7.8, 300 μM pyridoxal phosphate, 7.5 mM dithiothreitol, 6 mM EDTA, 92.6 μM D,L-[1-^{14}C] ornithine (0.1 μCi) and about 0.04 mg enzyme protein. The samples were incubated for 30 minutes at 37°C in glass vials placed in rubber stoppered glass scintillation tubes. After 30 minutes incubation the reaction was stopped by the addition of 40 μl of 1.5 M sulphuric acid and 40 μl of 17.5 mM potassium bicarbonate solution. The liberated $^{14}CO_2$ was absorbed in hyamine and the incubation was continued overnight. Radioactivity was determined in a toulene-based scintillation fluid using a Mark III Nuclear Chicago Scintillation Spectrometer. A unit of ODC specific activity was defined as 1 nmol of L-ornithine decarboxylated per hour per mg protein.

2.18. Reagents and solutions for cytosolic glucocorticoid binding assay

[^{3}H]-Dexamethasone was obtained from Amersham. Other materials and chemicals were of the highest quality and purchased from the sources mentioned in previous publications (Patel and Hunt, 1985; Patel et al, 1982; Patel, 1982; Patel et al, 1983a, 1983b).

Buffer solution 1 (x 2 concentrated, pH 7.4):

EDTA	0.074 g/100 ml.
Dithiothreitol	0.077 g/100 ml.
Sodium molybdate	0.484 g/100 ml.
Tris	1.210 g/100 ml.
KCl	0.372 g/100 ml.

Buffer A:

50 ml solution 1 + 50 ml distilled water.

Buffer B:

50 ml solution 1 + 49 ml distilled water + 1 ml ethanol.

Stock dexamethasone:

4mM (1.742 mg/ml) in ethanol, stored at -20°C.

200 μM dexamethasone:

0.25 ml of stock dexamethasone + 4.75 ml buffer A.

[^{3}H]-dexamethasone, 10 μl, diluted to 0.852 ml with buffer B, specific activity 78 Ci/mmole.

50mM TRIS/HCl pH 7.4, containing 2 g activated charcoal and 0.2 g dextran-T 70 per 100 ml.

2.19. Glucocorticoid binding assay

The samples containing whole homogenate were centrifuged for one hour at 100,000 g at 4°C to obtain cytosol fractions. The supernatant was used for binding assay. For the fixed point assay the sample was divided into two and each treated as follows:

Sample A:

10 µl ^{3}H-dexamethasone, 150 nM.

10 µl buffer B.

80 µl cytosol.

Sample B:

10 µl ^{3}H-dexamethasone, 150 nM.

10 µl 200 µM dexamethasone (cold).

80 µl cytosol.

Binding of [^{3}H]-dexamethasone (150 nM) was performed in the presence and in the absence of 1000-fold excess of unlabelled dexamethasone. Determinations on samples A and B were carried out in triplicate. The mixtures were incubated for 2 hours at 0°C. At the end of two hours 100 µl of dextran/charcoal was added and mixed. The mixture was left to stand for 15 minutes and then centrifuged for 15 minutes at 6,000 rpm, 100 µl of the supernatant was removed, 0.9 ml distilled water added and the samples were mixed with a scintillation fluid and counted for radioactivity using a Mark III Nuclear Chicago Scintillation Spectrometer (Patel et al, 1977).

2.20. Reagents and solutions for ELISA for GFAP

All the materials were of the highest quality and purchased from the sources mentioned in previous publications (Patel and Hunt, 1985; Patel et al 1982; Patel, 1982; Patel et al, 1983a, 1983b).

PBS solution:

One tablet in 100 ml distilled water.

PBS with 1% bovine serum albumin (BSA):

2.5 g BSA in 250 ml PBS solution.

Anti-GFAP serum (polyclonal rabbit):

1:1000 dilution in PBS-with-1% BSA.

Horse radish peroxidase-conjugated sheep antirabbit immunoglobulin:

1:2000, in PBS-with-1% BSA.

ABTS reagent:

trisodium citrate with citric acid, pH 5, plus 2, 2'-azino diethyl benzthiazoline sulphonic acid, 1 mg/ml and 10 µl hydrogen peroxide.

0.5% sodium azide in PBS.

0.1% Tween-20.

Pure GFAP from adult rat spinal cord was used as standard.

2.21. ELISA

Cells were disrupted using a sonicator. Pure GFAP was made up into three dilutions for preparation of the standard curve, 1:500, 1:1000 and 1:5000. The samples, including the standards, were placed in wells on a 96-well microtitration plate and the samples treated with rabbit anti-GFAP. PBS solution was used as a blank. The plates were placed in a shaker overnight at 4°C. After overnight incubation the plates were washed with PBS solution, and then immersed in 1% BSA-in-PBS for 60 minutes at room temperature. After two hours, the plates were washed with PBS solution. Horse-radish peroxidase conjugated sheep anti-rabbit immunoglobulin at 1:2000 dilution in PBS with 1% BSA was added to the wells and the plates incubated for two hours at room temperature, after which the plates were washed with PBS solution followed by distilled water. ABTS reagent was added into each well, followed by 0.5% sodium azide in PBS. The absorbance was measured in a microtitration plate reader (BioRad model 2550). The amount of GFAP in the sample was determined from the absorbance value of the samples by comparison with the absorbance value of the standards. One unit of GFAP was defined as 1 ng of GFAP per mg of protein.

2.22. Reagents and solutions for protein estimation

The materials and chemicals used were of the highest quality and purchased fom sources which have been mentioned in previous publications (Patel and Hunt, 1985; Patel et al 1982; Patel, 1982; Patel et al 1983a, 1983b).

Lowry buffer:

Folin's solution: 1 part Folin's solution plus two parts water.

Copper sulphate solution.

Standard protein:

Bovine serum albumin (BSA) 1 mg/ml.

0.1 M sodium hydroxide.

0.3 M sodium hydroxide.

2.23. Protein estimation (Lowry et al, 1951)

Standard solutions of bovine serum albumin (1 mg/ml) were prepared at concentrations of 20 μg/ml, 40 μg/ml, 60 μg/ml, 80 μg/ml and 100 μg/ml in 0.1 M sodium hydroxide. Protein concentration in the standard samples was measured using a Technicon autoanalyser and the mean optical density of a 100 μg of standard protein obtained. Samples were diluted in 0.3 M sodium hydroxide and incubated for 20 minutes at 37°C. The samples were diluted further with distilled water and the protein concentration determined using a Technicon autoanalyser.

The amount of protein in the samples was calculated from the optical density of the samples as compared to the optical density of the 100 μg of standard protein. The concentration of protein in the samples were expressed as either 1 mg of protein per ml or g wet weight, or per flask, or per cell.

2.24. Immunocytochemistry

The rabbit anti-human GFAP was the same as that described by Palfreyman et al (1979). Anti-rabbit IgG antibody raised in goats and the fluorescein isothiocynate (FITC) conjugates were purchased from Dako, Weybridge, Surrey. Other materials and chemicals used were of the highest quality and obtained from sources mentioned in previous publications (Patel and Hunt, 1985; Patel et al 1982; Patel, 1982; Patel et al, 1983a, 1983b). Ninety five percent ethanol and 5% acetic acid, stored at -20°C, were used as fixatives. Cells grown on glass coverslips were fixed in ice cold ethanol-acetic acid (95 parts by 5 parts respectively) for fifteen minutes. The coverslips were gently washed three times in PBS. Rabbit anti-human GFAP antibody was applied at a dilution of 1:400 and cells were incubated for one hour at room temperature. At the end of this period, cells were washed in PBS and the second layer of anti-rabbit IgG applied, at a dilution of 1:50. Cells were incubated for thirty minutes. At the end of this period coverslips

were washed in PBS and mounted in PBS-glycerol (50:50) and examined under a fluorescence microscope, Leitz Dialux EB 20, incorporating reflected light fluorescence and barrier filters for FITC. Normal non-immune rabbit serum was used in place of the first antibody to provide a negative control.

To study the effect of dexamethasone on the expression of GFAP, the cells were grown in growth medium containing 1 μM dexamethasone.

2.25. Reagents and solutions for DNA extraction and estimation

1M perchloric acid (PCA).

Standard DNA:

0.4 mg/ml highly polymerised calf thymus DNA in 5.0 mM sodium hydroxide. This was diluted to give a concentration between 0.02 and 0.25 μg atom DNA-P/ml.

Diphenylamine reagent:

1.5 g diphenylamine dissolved in 100 ml glacial acetic acid + 1.5 ml concentrated sulphuric acid. Just before use 0.5 ml of a 16 mg/ml solution acetaldehyde was added.

All the reagents and chemicals were of the highest quality and obtained from sources mentioned in previous publications (Patel and Hunt, 1985; Patel et al, 1982; Patel, 1982; Patel et al, 1983a, 1983b).

2.26. DNA extraction and estimation

The samples were diluted in distilled water and 1.0 M PCA to give a concentration of between 0.02 and 0.25 μg atom DNA-P/ml. Samples were homogenised in 0.4N PCA and centrifuged for 10 minutes at 2400 rpm at 4°C. The supernatant was discarded and the pellet resuspended in 0.2 M PCA. The sample was centrifuged for ten minutes at 2400 rpm at 4°C. The supernatant was again discarded and the pellet resuspended in 1.0 M PCA, and incubated at 70°C for 20 minutes. The samples were then cooled on ice and centrifuged at 2400 rpm at 4°C. The supernatant was removed and used in the colourimetric estimation of DNA by the method of Burton (1956). Freshly prepared diphenylamine reagent was added to the DNA sample and the sample was incubated for 16 to 20 hours at 30°C. The optical density of the samples was then measured at 600 nm. The amount of DNA in the sample was calculated from the absorbance of the samples as compared to the absorbance of a 100 μg DNA-P of standard DNA.

2.27. Drugs and drug formulations

Dexamethasone sodium phosphate was supplied by

Merck, Sharp and Dohme Ltd, Hoddeston, Hertfordshire, and used as a 1 μM solution in growth medium for treatment of cell cultures. DFMO was a gift from the Merrell Dow Research Institute, Strasbourg, France (Dr. Paul Schechter). It was used as a 5 mM solution in culture medium for treatment of cell cultures and as a 1% solution, w/v in distilled water for treatment of mice. BCNU (Lundbeck, Luton, Bedfordshire) was dissolved in absolute ethanol and diluted with distilled water. The drug was given at a dose of 30 mg/kg in a single intraperitoneal injection (Bradford et al, 1990).

3.1. Morphology, growth characteristics and changes in GS and GFAP *in vitro*

3.1.1. Morphology

During exponential growth, polygonal cells with processes of different lengths pre-dominated (Plate 3.1.1). There was intertwining of the processes in some areas. An element of pleomorphism was observed although the majority of the cells were of uniform size. In the original description of VMDk P497 by Serano *et al* (1980) the cell line was a mixture of polygonal, triangular and elongated spindle cells.

3.1.2. Immunocytochemistry

VMDk P497 cells expressed GFAP *in vitro*. Small quantities of this astrocytic marker were present in all the cells and there was no apparent change in intensity of staining between day 3 and day 7 in culture (Plate 3.1.2).

3.1.3. Growth pattern

Growth *in vitro* was associated with an increase in number of cells from 1.54×10^4 cells per ml at day 3 in culture to 5.46×10^4 cells per ml at day 4 in culture and from 10.6×10^4 cells per ml on day 5 in culture to 17.6×10^4 cells per ml on day 6 in culture. There was a

greater percentage increase in the number of cells per ml during the period of exponential growth i.e. upto day six in culture (Table 3.1.1). The cell population doubling time was 21 hours (Figure 3.2.3). The cultures were confluent on day 7 (Figure 3.2.3).

3.1.4. Protein levels and cell numbers

The number of cells per flask increased during growth in culture (Table 3.1.2). The number of cells per flask on day 4 in vitro was 1.72×10^6 and on day 8, 8.72×10^6. Between days 5, 6 and 7 the number of cells per flask increased from 3.01×10^6 to 8.02×10^6 and 8.5×10^6 respectively. This increase was more marked between the fourth and sixth day, the period of exponential growth (Figure 3.1.4). The increase in cell number per flask was statistically significant upto day 6 in vitro (day 4 vs 5, $p < 0.05$; day 5 vs 6, $p < 0.01$). The increase in cell number between day 6 and day 8 in vitro was not statistically significant ($p > 0.05$). The increase in cell number per flask was associated with a similar increase in protein content per flask (Table 3.1.2 and Figure 3.1.4). The amount of protein increased from 0.24 mg/flask on day 4 in vitro to 1.55 mg/flask on day 8 in vitro. Protein levels on day 5, 6 and 7 were 0.43, 1.42 and 1.45 mg/flask respectively. The increase in protein content was statistically significant upto day 6 in vitro (day 4 vs 5, $p < 0.001$; day 5 vs 6, $p < 0.01$), the period of exponential growth (Table 3.1.2 and Figure

3.1.4). The increase in protein content between day 6 and day 8 *in vitro* was not statistically significant. The ratio of protein per cell (in mg/10^6 cells) was 0.145 on day 4, 0.143 on day 5, 0.178 on day 6, 0.177 on day 7 and 0.178 on day 8. In the VMDk P497 cell line the ratio of protein/cell during growth *in vitro* remained approximately constant indicating no appreciable change in average cell size (Table 3.1.2).

3.1.5. GFAP

VMDk P497 cells did not show any increase in GFAP levels during growth in culture. The overall amount of GFAP per flask did not change (Table 3.1.3) and the amount of GFAP per mg of protein decreased (Figure 3.1.4). GFAP levels per mg of protein decreased from 2.11 on day 4 to 0.30 on day 8. The amount of GFAP on days 5, 6 and 7 was 2.0, 0.41 and 0.37 ng/mg of protein respectively (Table 3.1.3). The decrease in GFAP in VMDk P497 cells during cultivation *in vitro* from day 5 to 6, and from day 7 to 8 was statistically significant ($p < 0.001$). These results would tend to indicate that the increase in total protein observed during cultivation *in vitro* was associated with cell proliferation rather than differentiation. Indeed, decrease in levels of GFAP in this cell system indicates de-differentiation during cultivation *in vitro*.

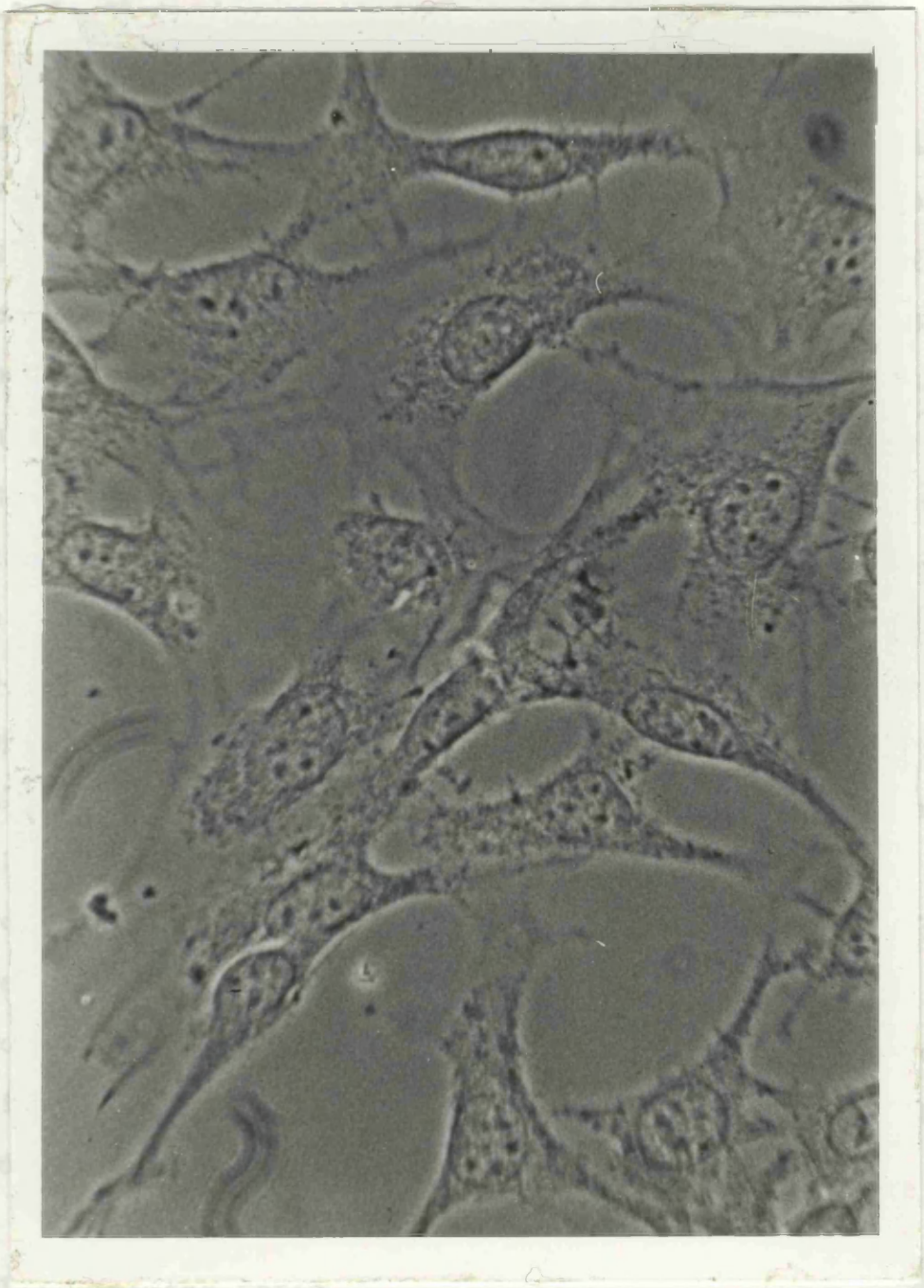

Plate 3.1.1. morphology of VMDk P497 cells (x400).

3.1.6. Glutamine synthetase

GS activity in VMDk P497 cells, in terms of nmol/h/mg protein, decreased during growth in vitro (Figure 3.1.4). This fall was also reflected in the total GS specific activity per flask (Table 3.1.3). GS specific activity in nmol decreased from 65.67 on day 5, to 5.16 on day 8. The specific activity on day 6 and 7 was 15.80 and 12.0 respectively. The decrease in GS activity in VMDk P497 cells from day 5 to day 8 in vitro was statistically significant (day 5 vs 6, $p < 0.001$; day 6 vs 7, $p < 0.05$; day 7 vs 8, $p < 0.001$). The decrease in GS activity in this cell line is further evidence for an apparent de-differentiation of the cells during cultivation in vitro. These changes are shown in Figure 3.1.4. This graph outlines the pattern of change in protein content, cell number, GFAP and GS activity during growth in vitro in this cell system. There was a progressive increase in cell number and protein content which was accompanied by a concomitant decrease in GFAP and GS activity.

3.1.7. GS/GFAP ratio

GS/GFAP ratio falls between day 5 and day 8 in culture (Table 3.1.3). This is because of reduction in GS was proportionately greater than the decrease in GFAP. The increase in total protein per flask in VMDk P497 cells during growth in culture was associated with cell

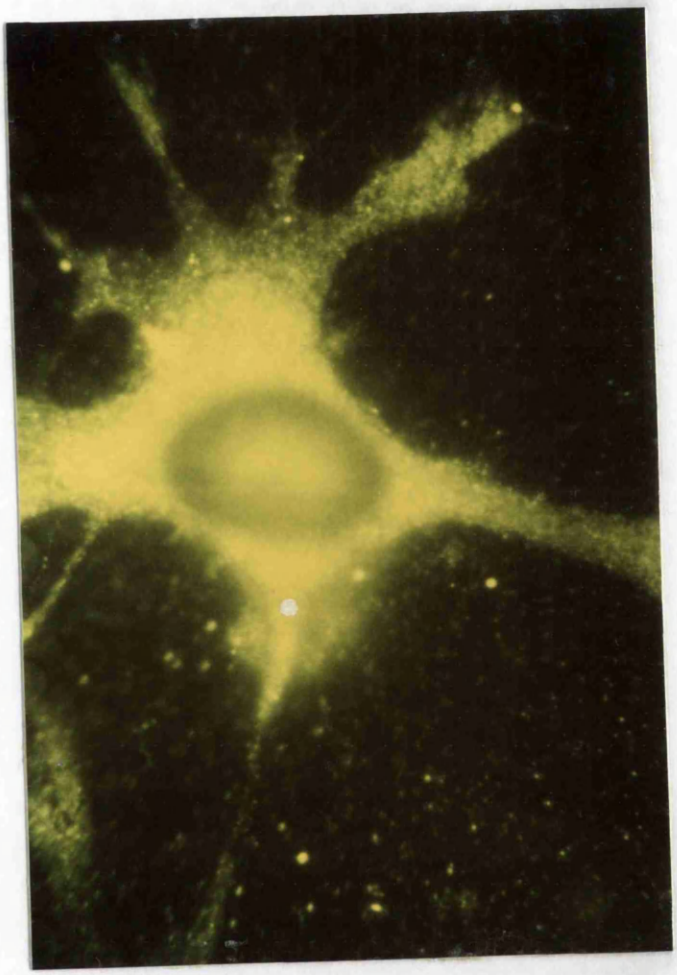

Plate 3.1.2. GFAP-positive VM cell (x1000).

TABLE 3.1.1

Changes in cell numbers per ml with development *in vitro* in the glioma cell system, VMDk P 497.

Age *in vitro* (days)	Number of cells per ml ($X\ 10^4$)	% increase between succesive age *in vitro*
3	1.54 ± 0.27	355
4	5.46 ± 0.25	194
5	10.60 ± 0.45	166
6	17.60 ± 0.43	122
7	21.87 ± 1.17	

Footnotes:

Each result is the mean of 3 independent experiments ± S.E.M.

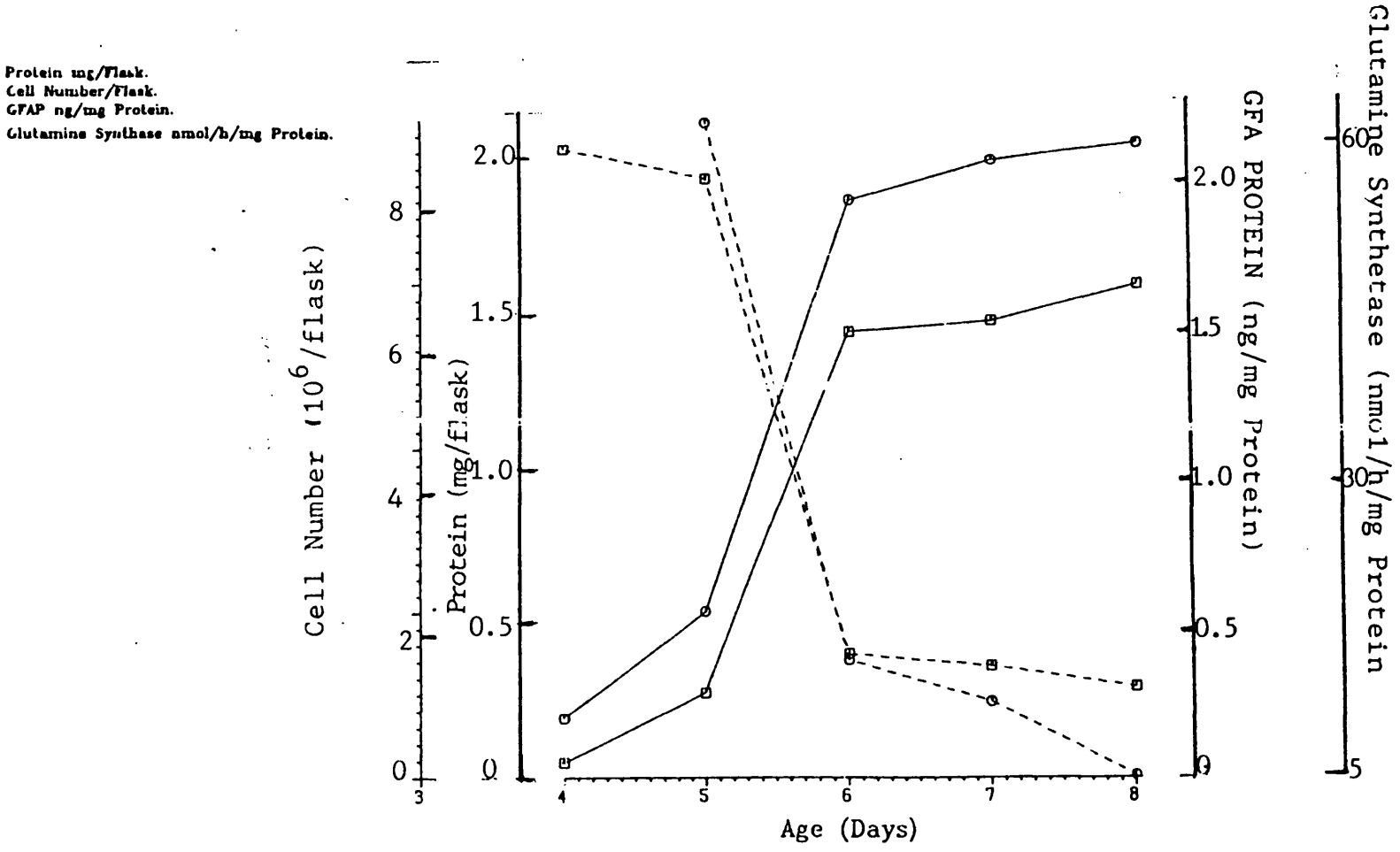

Fig 3.1.4

There is an increase in number of cells and protein levels per flask during development in culture in the VMdK P497 glioma cell system. This is accompanied by de-differentiation and fall in GFAP and GS.

TABLE 3.1.2

Changes in cell number and amount of protein per flask with development *in vitro* in the mouse glioma cell system VMDk P497.

Age *in vitro* (Days)	Number of cells (10^6/flask)	Protein mg/flask	Protein/cell ratio mg/10^6 cells
4	1.72	0.24	0.145
5	3.01	0.43	0.143
6	8.02	1.42	0.178
7	8.51	1.45	0.177
8	8.72	1.55	0.178
Residual Variance	0.332	9.63×10^{-3}	0.42×10^{-3}
Significance			
4 Vs 5	$P < 0.05$	$P < 0.001$	NS
5 Vs 6	$P < 0.01$	$P < 0.01$	NS
6 Vs 7	NS	NS	NS
7 Vs 8	NS	NS	NS

Footnotes:

Each result is the mean of 3 independent experiments. The results were analysed for statistical significance using the analysis of variance (ANOVA) one way.

(NS = No significant difference, $p > 0.05$)

TABLE 3.1.3

Developmental changes in the specific activity of Glutamine Synthetase and GFA protein concentration in cultured cells of the murine astrocytoma cell line VMDk P497.

Age in vitro (days)	GFA Protein ng/mg protein	GFA Protein ng/Flask	Glutamine Synthetase (GS) nmol/h/mg Protein; S.A.	GS S.A/Flask	GS/GFA protein ratio
4	2.11	0.57	-	-	-
5	2.00	0.86	65.67	28.12	33.73
6	0.41	0.59	15.80	22.32	38.88
7	0.37	0.54	12.00	17.38	32.49
8	0.30	0.46	5.16	7.95	17.20
Residual Variance	0.065	0.016	15.046	3.594	37.713
4 Vs 5	NS	NS	-	-	-
5 Vs 6	$P<0.001$	NS	$P<0.001$	$p<0.05$	NS
6 Vs 7	NS	NS	$P<0.05$	$P<0.05$	NS
7 Vs 8	$P<0.001$	NS	$P<0.001$	$P<0.05$	$P<0.01$

Footnotes:

Each result is the mean of 3 independent experiments. Results were analysed for statistical significance using ANOVA (one way)
(NS = No significant difference, $P>0.05$)

proliferation and not with an increase in GS and GFAP.

In summary, the VMDk P497 cell line is a good model in studies on astrocytic differentiation *in vitro*. It expresses the astrocytic markers GS and GFAP and it has well defined growth characteristics in culture. The cells have well defined exponential and plateau phases of growth. There is an increase in cell proliferation associated with an increase in protein content and a constant protein/cell ratio. Cell proliferation seems to be associated with cell de-differentiation as shown by a decrease in GFAP and GS activity.

3.2. Induction of GS by dexamethasone *in vitro*

3.2.1. Morphology

Treatment of cultures with 1 μM dexamethasone had an effect on cell morphology (Plate 3.2.1). Cells acquired longer processes, a sign of apparent morphological differentiation.

3.2.2. Immunocytochemistry

There was no increase in the intensity of GFAP staining in treated cultures (Plate 3.2.2). VMDk P497 cells were weakly positive for GFAP and any change in GFAP content as a result of treatment with dexamethasone could not be observed immunocytochemically.

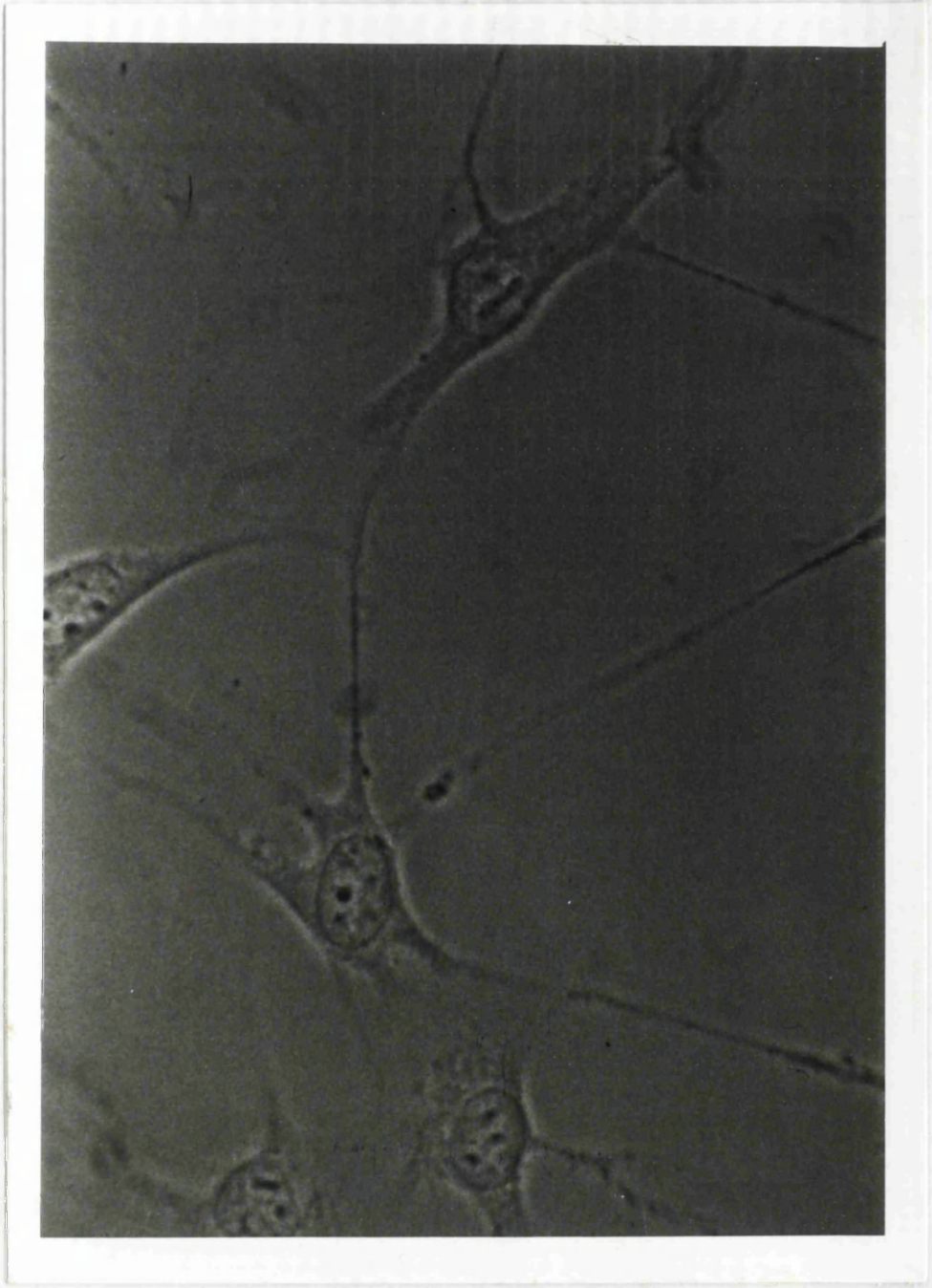

Plate 3.2.1. Dexamethasone-treated VMDk P497 cells (x 400).

3.2.3. Growth pattern

When VMDk P497 cells were cultured in growth medium containing 1 μM dexamethasone, the overall shape of the growth curve was similar to that of untreated controls (Figure 3.2.3). Prolonged exposure to growth medium containing dexamethasone, however, was associated with a decrease in cell number (Table 3.2.1). Cultures grown in growth medium containing dexamethasone had fewer cells per ml compared to control cultures. This effect was more marked after 5 days *in vitro*. The difference in number of cells between the treated and untreated cultures from day 3 to day 5 was not statistically significant. However the difference in number of cells between treated and untreated cultures at day 6 and day 7 *in vitro* was. Treatment of cultures with dexamethasone appeared to be associated with a lower terminal cell density.

3.2.4. Protein levels and cell numbers

Cultures at 5, 6, 7 and 8 days *in vitro* were treated with dexamethasone for 24 hours to find out whether this would have any effect on the total protein content, cell number and average cell size. Treatment of cultures with 1 μM dexamethasone for 24 hours, did not have any affect on cell proliferation (Table 3.2.2). The difference in the number of cells between treated and untreated cultures was not statistically significant. This lack of effect on cell proliferation was observed in cultures shortly after plating as well as in longer-term cultures.

TABLE 3.2.1

Effect of dexamethasone on proliferation of mouse astrocytoma cells - VMDk P497

Age in vitro (days)	Number of cells per ml (x 10^4)		
	Untreated controls		Dexamethasone
3	1.54± 0.27	NS	1.38± 0.28
4	5.46± 0.25	NS	5.43± 0.17
5	10.60± 0.45	NS	10.11± 3.33
6	17.60± 0.43	$p < 0.05$	10.25± 1.83
7	21.87± 1.17	$P < 0.05$	14.66± 0.79

Footnotes:

Each result is the mean of 3 independent experiments ± S.E.M. The results were analysed for statistical significance using the paired t-test.

(NS = No significant difference, $P > 0.05$)

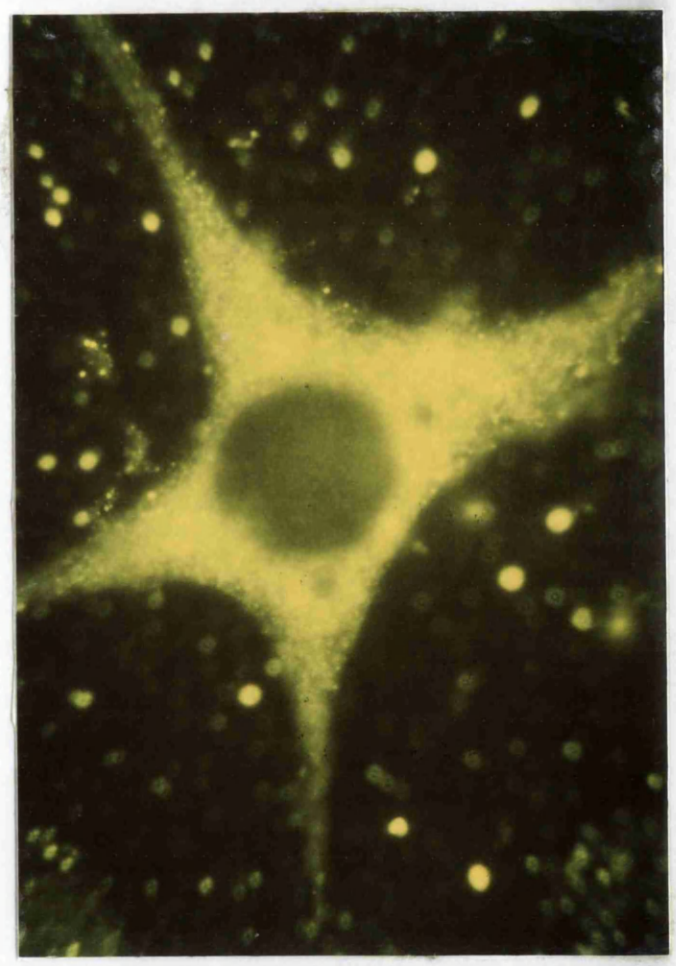

Plate 3.2.2. Immunofluorescence for GFAP in dexamethasone-treated VMDk P497 cell (x 1000).

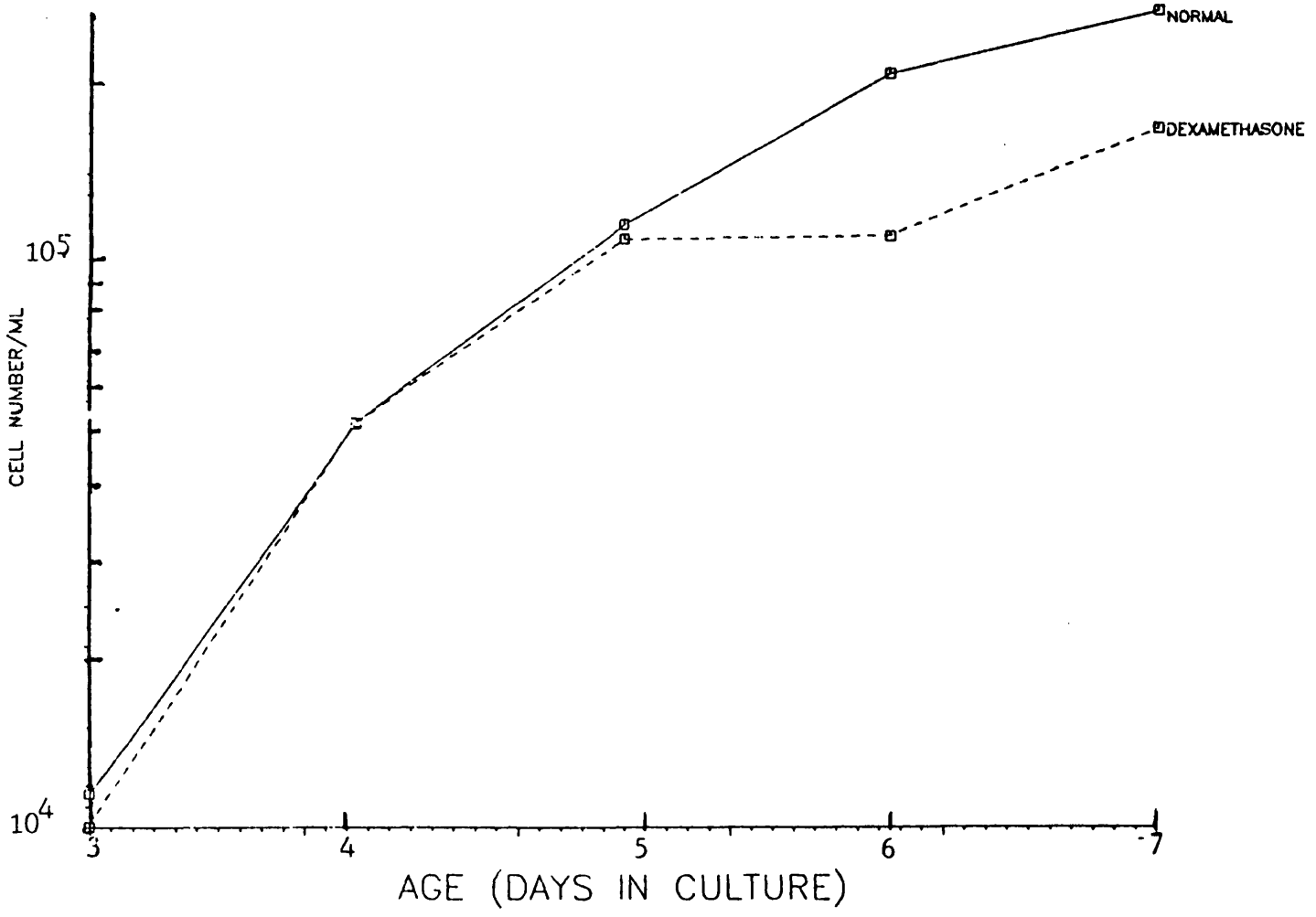

Fig 3.2.3

The glioma cell line VMdK P497 remains in exponential growth for upto 6 days in culture with a cell population doubling time of 21 hours. Dexamethasone slowed down cell proliferation.

Table 3.2.2.

Effect of dexamethasone on number of cells and protein content in developing mouse astrocytoma cells.

Age in vitro (Days)	No of cells (10^6/flask) control	dexa	Protein µg/ flask control	dexa	Protein/10^6 Cell (µg) control	dexa
5	3.30±0.15	3.45±0.12	439±3.2	532±50	133± 5	156± 19.
		NS		NS		NS
6	8.30±0.17	8.36±0.23	1420±110	1530±70	171±17.1	183± 7
		NS		NS		NS
7	9.20±0.15	9.46±0.20	1800±150	1630±430	196±19.6	171±43.
		NS		NS		NS
8	10.43±0.17	10.16±0.17	2030±110	1960±190	195±8.0	192±17
		NS		NS		NS

Footnotes:

Each result is the mean of 3 independent experiments ± S.E.M. The results were analysed for statistical significance using the paired t-test.
(NS = No significant difference, $P > 0.05$).

TABLE 3.2.3

Induction by dexamethasone of Glutamine Synthetase activity in developing mouse astrocytoma cells in culture.

Age in vitro (Days)	Glutamine Synthetase, nmol/h/mg Protein S.A. untreated Control	Dexa	Glutamine Synthetase Activity Per 10^6 cells untreated Control	Dexa	Induction of GS. As a % of Control
5	65± 4.3	260±11.5	20.03±2.12	75.83±3.81	400
		$p < 0.01$		$P < 0.01$	
6	15.8±0.65	91.33±11.4	1.89±0.49	10.98±1.60	600
		$P < 0.05$		$P < 0.05$	
8	35.6±14.8	158.33±35.3	3.38±1.36	15.56±3.51	450
		$P < 0.05$		$P < 0.05$	

Footnotes:

Each result is the mean of 3 independent experiments ± S.E.M. The results were analysed for statistical significance using the paired t-test.

(NS = No significant difference, $P > 0.05$).

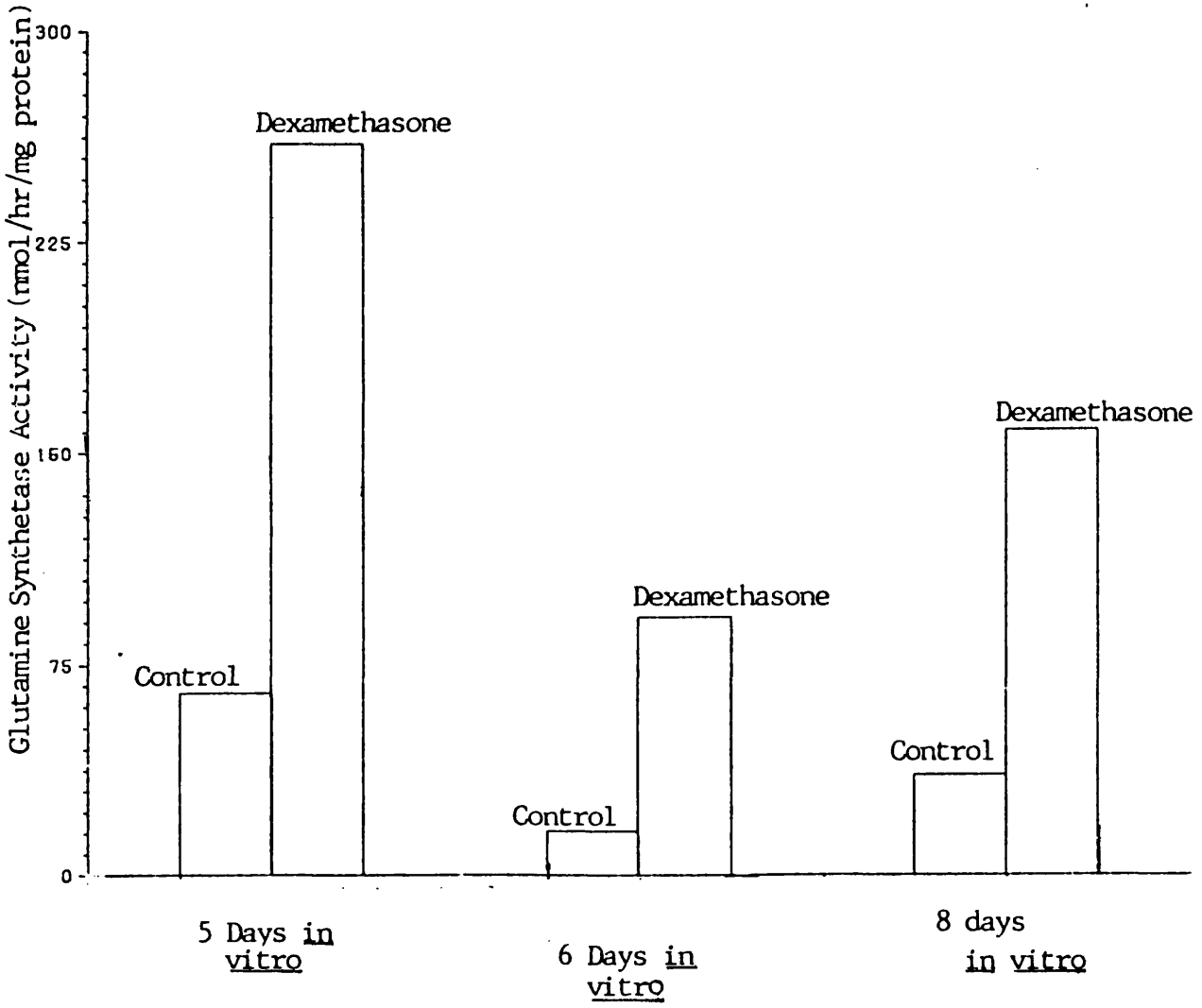

Fig. 3.2.4

Treatment of established VMdK P497 glioma cultures with dexamethasone for 24 hours caused a four to sixfold rise in GS activity. The effect was independent of the age of the cultures.

Treatment of VMDk P497 cells with 1 μM dexamethasone for a period of 24 hours had no effect on total protein level per flask (Table 3.2.2) when compared to untreated control cultures. Dexamethasone had no effect on cellular content of protein or average cell-size (Table 3.2.2).

3.2.5. Glutamine synthetase

Treatment of VMDk P497 cells with 1 μM dexamethasone for a period of 24 hours caused a four to sixfold rise in GS activity (Table 3.2.3). The level of induction of GS activity by dexamethasone was 400% in 5 day old cultures, 600% in 6 day old cultures and 450% in 8 day old cultures. These results are shown in Figure 3.2.4. The increase in GS activity observed in cultures treated with 1 μM dexamethasone for 24 hours was not due to cell proliferation or to an increase in protein because dexamethasone had no effect on the cellular content of protein or average cell-size (Table 3.2.2), and the number of cells per flask in cultures treated with dexamethasone for 24 hours was similar to that in untreated cultures (Table 3.2.2).

In rapidly growing cultures, the induction ratio represents the amount of new enzyme formed. The induction ratio of GS in cultures treated with glucocorticoids varied between four and six. VMDk P497 cells appear to have the potential for differentiation in culture. With appropriate inducers such as glucocorticoids, GS activity

can be increased six-fold.

3.3. Changes in cytosolic glucocorticoid receptor levels in VMDk P497 cells *in vitro*

To further investigate the apparent differentiation observed above, the binding of [^{3}H]-dexamethasone to cytosolic receptors and the changes taking place in the levels of specific binding during growth *in vitro* was studied.

3.3.1 Glucocorticoid receptors

The number of glucocorticoid receptors (specific binding) is the difference between total binding and non-specific binding. In Table 3.3.1 the cytosolic binding capacity of VMDk P497 cells for [^{3}H]-dexamethasone is shown. The specific binding in 5 day old cultures was 111.8, in 6 day old cultures 152.3, and in 8 day old cultures 141.4 fmol/mg protein. Therefore, glucocorticoid receptors were present in cultures at low and high cell density and VMDk P497 cells do not appear to lose these receptors during culture. The availability of these receptors during all the stages of growth *in vitro* means that these cells retain the potential to interact with glucocorticoids which are capable of inducing differentiation.

Having found that cytosolic glucocorticoid receptors were present in the VMDk P497 cells during all the stages

TABLE 3.3.1.

Binding of (^{3}H) dexamethasone in vitro to cytosol binding sites in developing mouse astrocytoma cells - VMDk P497.

Age in vitro (Days)	Total Binding Fmol/mg Protein	Non-Specific binding fmol/ mg Protein	Specific binding fmol mg Protein
5	813± 44.28	701.19± 54.73	111.81± 27.52
6	390.94± 38.34	240.62± 47.40	152.32± 23.83
8	201.28± 38.34	59.86± 47.40	141.41± 23.83
10	396.35± 38.34	333± 47.40	63.35± 23.83
5 Vs 6	$p < 0.05$	$p < 0.05$	NS
6 Vs 8	$p < 0.05$	$p < 0.01$	NS
8 Vs 10	$p < 0.05$	$p < 0.01$	$P < 0.05$

Footnotes:

Results are expressed in femtomoles of bound radio activity/mg cytosol protein. Each value is the mean of 4 independent experiments ± S.E.M. The results were analysed for statistical significance using ANOVA (one way). (NS = No Significant difference, $P > 0.05$)

TABLE 3.3.2

Effect of development *in vitro* on number of cells, cytosolic protein content and number of raceptors per cell in a mouse astrocytoma cell line.

Age *in vitro* (Days)	Cytosolic Protein µg/flask	Number of cells 10^6/ Flask	Number of receptors per 10^6 cells
5	106± 14	3.2± 0.085	3.86± 0.83
6	165± 12	8.4± 0.073	3.15± 0.71
8	176± 12	10.6± 0.073	2.32± 0.71
10	195± 12	10.9± 0.073	1.15± 0.71
5 Vs 6	P< 0.05	P< 0.0001	NS
6 Vs 8	NS	P< 0.0001	NS
8 Vs 10	NS	P< 0.05	P< 0.05

Footnotes

Each result is the mean of 4 independent experiments ± S.E.M. The results were analysed for statistical significance using ANOVA (one way)
(NS = No significant difference P>0.05)

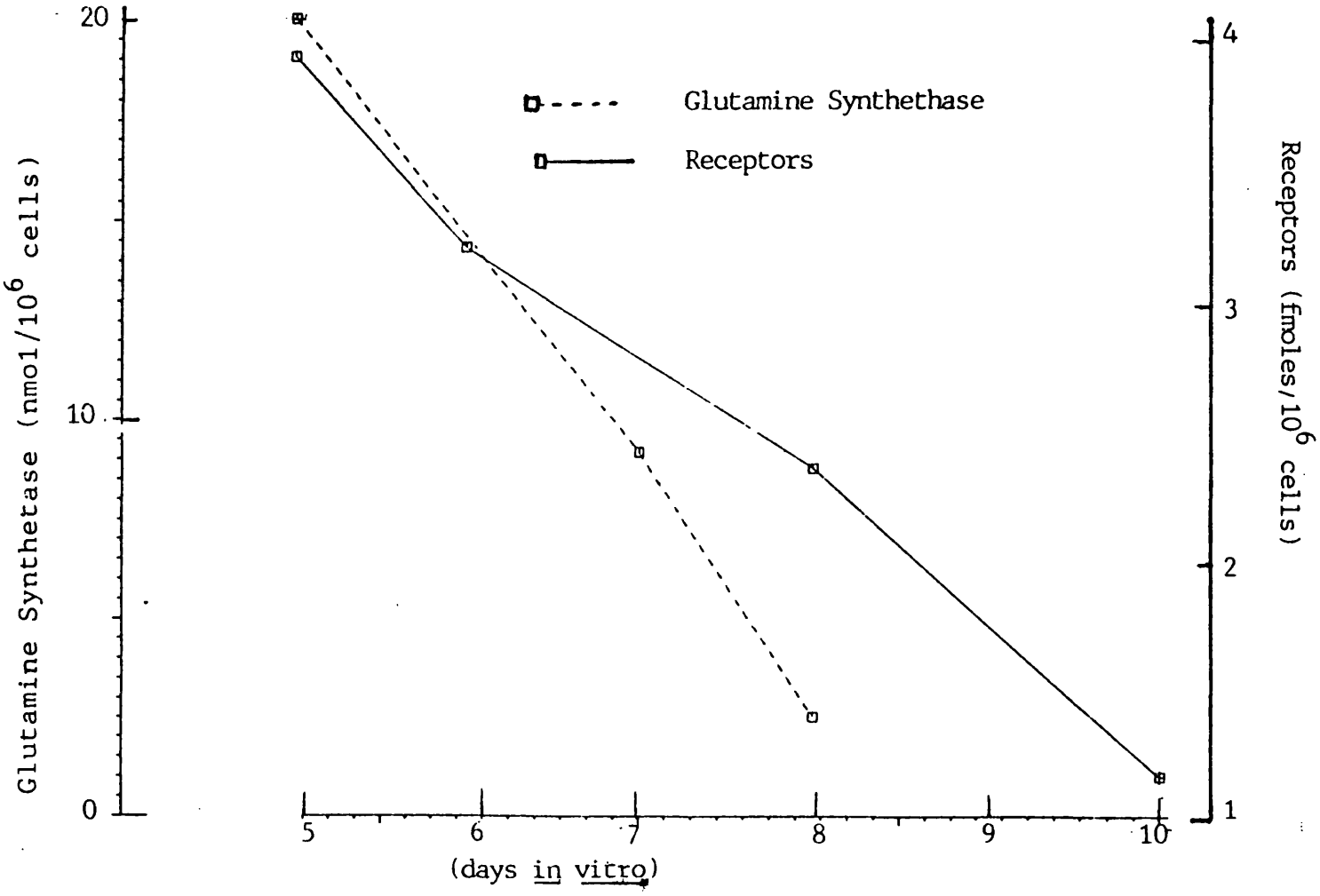

Fig 3.3.1

There is a fall in GS activity per cell during development in vitro in the glioma cell line VMdK P497. This is not accompanied by a total loss in glucocorticoid receptors and the cells retain the potential for induction by glucocorticoids.

of development *in vitro* a comparison was made of the changes taking place in GS activity during this period. Table 3.3.2 and Figure 3.3.1 show the GS activity and number of receptors per cell. The number of receptors per 10^6 cells in 5 day old cultures was 3.86 fmol and in 8 day old cultures was 2.32 fmol. Although GS activity in eight day cultures was much less than the activity in five day cultures the cells retained enough receptors to respond to glucocorticoids and an induction ratio of four could still be obtained in eight day old cultures (Figure 3.3.1) following treatment with dexamethasone. As expected, there was an increase in cytosolic protein and number of cells per flask (Table 3.3.2) during this period of growth *in vitro*.

In summary, VMDk P497 cells undergo de-differentiation during growth in culture with loss of GS activity and GFAP expression. However, these cells could be induced to differentiate by treatment with glucocorticoids during all stages of growth *in vitro* as evidenced by a sixfold rise in GS activity. This process appeared to be mediated through cytosolic glucocorticoid receptors.

3.4. Changes in ODC activity and polyamine levels in VMDk P497 cells *in vitro*

Having established that the VMDk P497 cells undergo de-differentiation during growth *in vitro* but retain

cytosolic glucocorticoid receptors through which they can be induced to differentiate, an investigation into the changes taking place in the intracellular levels of ODC was carried out.

3.4.1. ODC activity

ODC catalyses the conversion of ornithine to putrescine and this is the rate-limiting step in polyamine synthesis. Therefore, activity of this enzyme is an accurate reflection of intracellular polyamine levels. In Table 3.4.1 the changes taking place in ODC activity in VMDk P497 cells during growth in vitro are shown. ODC activity was not detected in lag phase VMDk P497 cells, two days after plating. In 4 day old cultures when cell division was taking place ODC specific activity was 1.17 fmol. This increase in specific activity from day 2 to 4 was associated with an increase in total protein per flask from 0.153 to 0.33 mg, and an increase in cell number from 0.84 x 10^6 to 1.05 x 10^6 cells per flask. In 6 day old cultures, ODC specific activity was 8.73 fmol. This increase in activity was associated with an increase in total protein per flask and number of cells per flask. The observed increase in ODC activity between days 2 and 8 was not associated with any changes in the protein/cell ratio. This was 0.322 on day 4, 0.446 on day 6 and 0.463 on day 8 after plating (Table 3.4.1). Therefore, the increase in ODC activity had no effect on the cellular content of protein or average cell size. The

TABLE 3.4.1

Developmental changes in the specific activity of ornithine decarboxylase in mouse astrocytoma cells in vitro.

Age in vitro (days)	Protein mg/Flask	No. of cells/ Flask (x 10^6)	Protein/cell Ratio	ODC S.A. fmol/mg Protein/h	ODC S.A. fmol/Flask
2	0.153	0.84	0.161	ND.	ND.
4	0.33	1.05	0.323	1.17	0.342
6	3.167	6.6	0.446	8.73	26
8	4.400	9.25	0.463	7.23	31
Residual Variance	0.0124	0.0097	0.0098	7.2×10^{-5}	3.4×10^{-4}
2 Vs 4	NS	$P < 0.05$	NS	-	-
4 Vs 6	$P < 0.01$	$P < 0.0001$	NS	$P < 0.05$	$P < 0.05$
6 Vs 8	NS	$P < 0.05$	NS	NS	NS

Footnotes:

Each result is the mean of 3 independent experiments. Results, were analysed for statistical significance using ANOVA (one way).

(NS = No significant difference. $P > 0.05$)

(ND = Not detected)

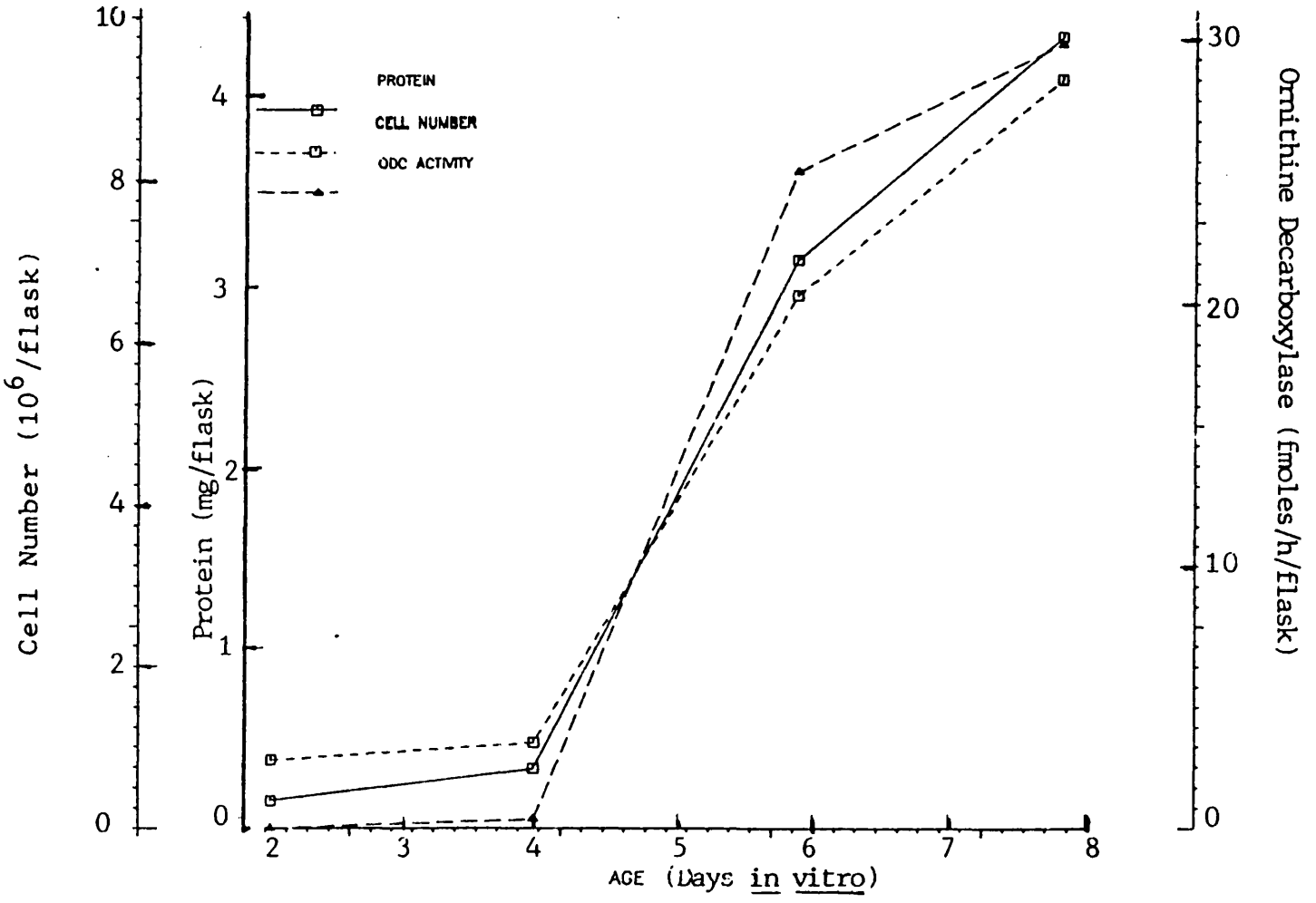

Fig 3.4.1

ODC activity in two days VM mouse astrocytoma cultures was undetectable. Transition from quiescence to proliferation at four days was associated with increase in ODC activity, cell numbers and protein.

changes taking place in ODC activity, cell number and protein content during growth *in vitro* are shown in Figure 3.4.1. The rise in ODC activity was progressive, indicating that there was a net accumulation of intracellular polyamines. It seems likely that this is responsible for an increased rate of DNA synthesis leading to cell proliferation (Figure 3.4.1). In VMDk P497 cells these changes are associated with de-differentiation and a decrease in GFAP levels and GS activity.

3.5. Effect of DFMO on cell proliferation and GS activity in VMDk P497 cells *in vitro*

3.5.1. Morphology

Treatment with 5 mM DFMO in culture medium did not have any effect on cell morphology.

3.5.2. Growth pattern

DFMO is an irreversible inhibitor of ODC enzyme and can be used to create intracellular polyamine deficiency. VMDk P497 cells grown in the presence of DFMO grew more slowly and reached a lower terminal cell density than cells grown in control growth medium (Table 3.5.1). The effect was more pronounced after 5, 6 and 8 days growth in medium containing DFMO ($p < 0.01$, $p < 0.01$ and $p < 0.05$ respectively). However, this did not affect the overall shape of the growth curve (Figure 3.5.2). DFMO

TABLE 3.3.1

Effect of ∝ - Difluoromethyl ornithine on proliferation of mouse astrocytoma cells - VMDk P497.

Age in vitro (Days)	Number of cells/ml ($x10^4$) Untreated controls		Difluoromethyl ornithine
2	1.43± 0.23	NS	1.60± 0.11
3	2.20± 0.15	NS	1.70± 0.14
4	4.23± 0.14	$p < 0.05$	3.03± 0.08
5	9.76± 0.15	$P < 0.01$	4.76± 0.14
6	18.50± 0.28	$P < 0.01$	9.70± 0.15
8	22.33± 1.45	$P < 0.05$	16.0± 0.57

Footnotes:

Each result is the mean of 3 independent experiments ± S.E.M The results were analysed for statistical significance using the paired Students t-test. (NS = No significant difference, $P > 0.05$).

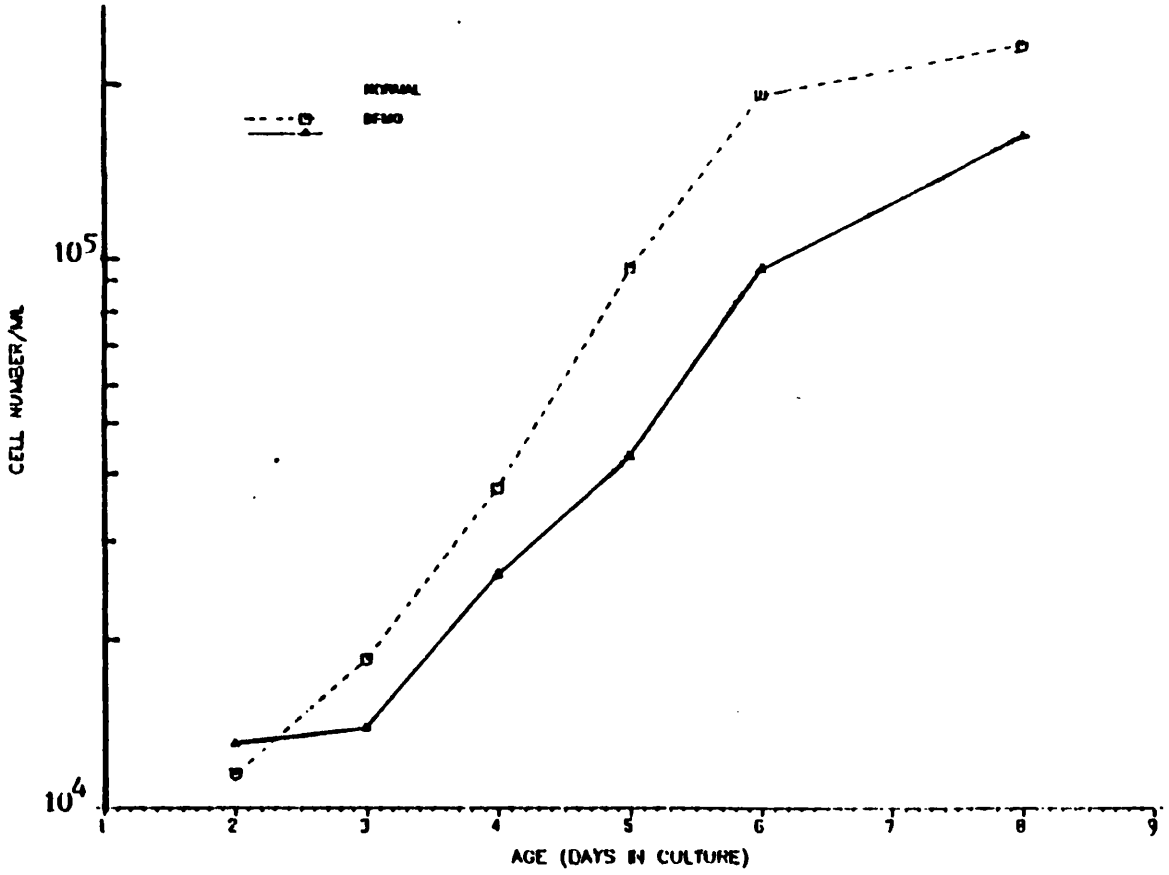

Fig: 3.5.2

VMDk1497 cultures grown in DFMO-treated growth medium have fewer number of cells/ml than control cultures. DFMO slows down cell proliferation.

did not cause complete arrest of cell proliferation but did slow it down. By inhibiting ODC activity, DFMO presumably, depleted intracellular levels of polyamines resulting in decreased DNA synthesis and cell proliferation.

3.5.3. ODC activity

The results of the time-course experiment of the effect of DFMO on ODC activity are shown in Table 3.5.2. After 12 hours treatment with DFMO there was no significant change in ODC activity in the cells. However, after 24 hours treatment there was a significant fall in ODC activity and this inhibition was maintained for upto 48 hours, confirming the irreversible effect of DFMO in this system. Treatment of cultures with DFMO for 24 hours decreased ODC activity from 19 fmol to 5 fmol. After 48 hours of treatment ODC activity was 4 fmol compared to 24 fmol in control cultures. These results are illustrated in Figure 3.5.3.

3.5.4. Protein levels and cell numbers

It is thought that polyamines act on the DNA synthetic phase of the cell cycle probably as a cofactor in DNA synthesis. Cells are able to control their intracellular polyamine levels through regulation of ODC activity. Having confirmed the irrevesible inhibition of ODC activity by DFMO in the VMDk P497 cells the effect on protein content and cell number was investigated. Table

TABLE 3.5.2

Inhibition of Ornithine decarboxylase activity by Difluoromethyl Ornithine in VNDk P497 astrocytoma cells *in vitro*.

Treatment Duration (Hours)	Ornithine Decarboxylase S.A. Per Flask fmol/hr		Ornithine Decarboxylase S.A. Per cell (10^6)	
	Control	DFMO	Control	DFMO
12	12±1.0	11±1.0	5.6± 1.0	5.3± 0.9
	NS		NS	
24	19±2.0	5±1.0	5.1± 0.6	1.6± 0.5
	P< 0.05		P< 0.05	
36	13±1.0	3±1.0	3.6± 0.2	0.8± 0.3
	P< 0.05		P< 0.01	
48	24±4.0	4± 0.6	5.7± 0.6	1.2± 0.2
	P< 0.05		P< 0.05	

Footnotes:

Each result is the mean of 3 independent experiments ± S.E.M. The results were analysed for statistical significance using the paired Students t-test. (NS = No significant difference, P>0.05)

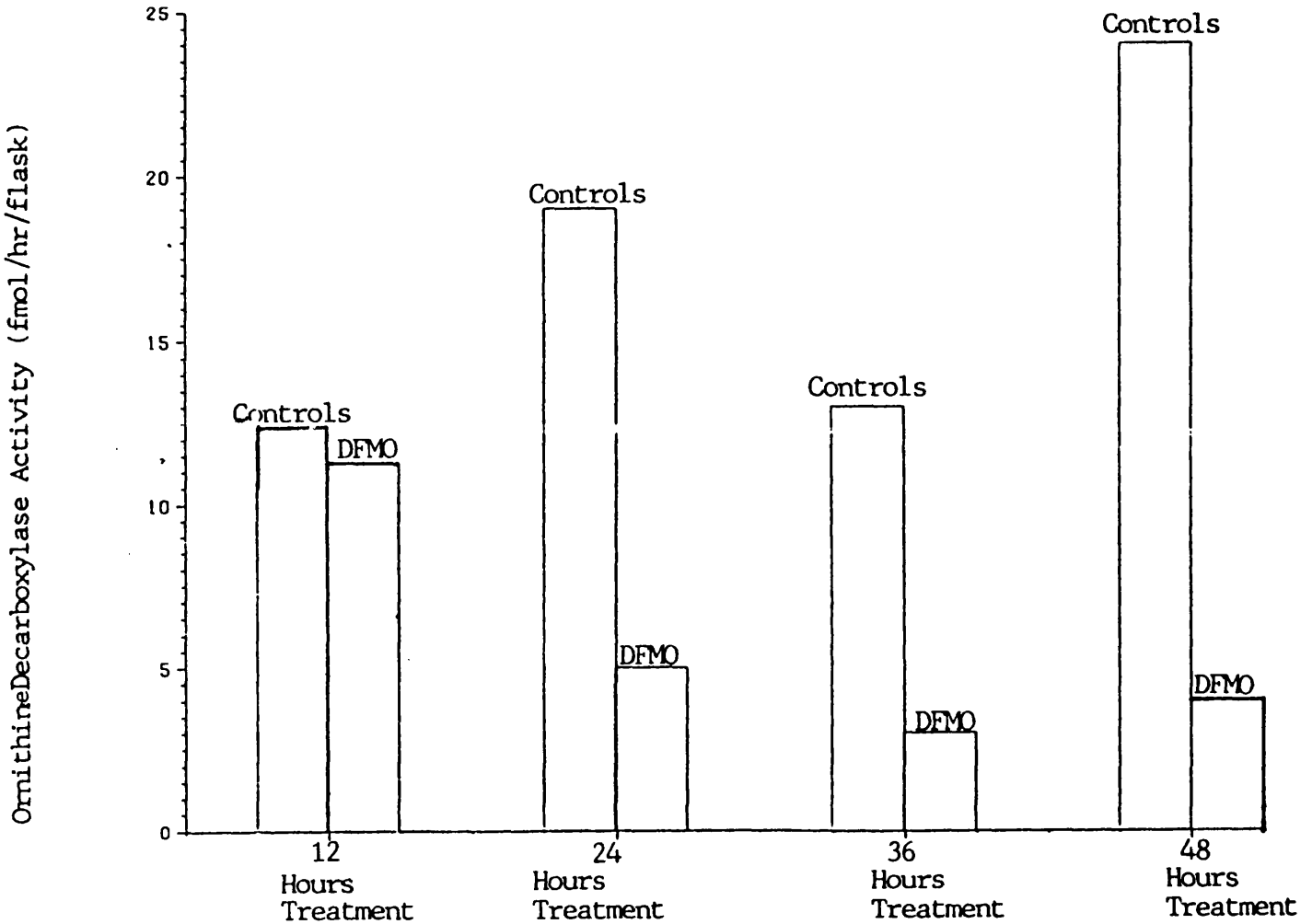

Fig: 3.5.3

Treatment of four days old VMdK P497 astrocytoma cultures with DFMO resulted in fall in ODC activity, more marked after 24 hours and lasting for longer than 48 hours.

3.5.3 shows the effect of DFMO on cell number, protein content and average cell size (ratio of protein/cell) in the VM cells. Treatment of VMDk P497 cells with DFMO decreased the number of cells per flask, although DFMO had no effect on the total protein content per flask. The protein per cell ratio did not change.

3.5.5. Glutamine synthetase activity

DFMO caused irreversible inhibition of ODC activity in VMDk P497 cells. This effect was associated with inhibition of cell proliferation, but had no effect on protein content or average cell size. The next step was to examine the effect of this on GS activity as a measure of cell differentiation.

In Table 3.5.4 the results of the time-course experiment on the effect of inhibition of ODC activity by DFMO on GS activity in VMDk P497 cells are shown. After treatment with DFMO for 24 hours, GS activity increased from 165 nmol in untreated cultures to 303 nmol in the treated cultures. Treatment of VMDk P497 cells for 36 hours increased GS activity from 203 nmol in untreated controls to 448 nmol. Inhibition of ODC activity by DFMO was associated with a decrease in cell proliferation and a significant increase in GS activity. Induction of GS activity was between 115% and 220% and was maintained for upto 48 hours. These results are further illustrated in Figure 3.5.4. It seems likely that as DFMO reduced cell

TABLE 3.5.3

Effect of Difluoromethyl ornithine on number of cells, protein content and protein/cell ratio in MDk P 497 astrocytoma cells in vitro.

Treatment Duration (Hours)	Number of Cells (10^6/flask)		Protein MG/flask		Protein/cell Ratio (Mg/10^6 cells)	
	Control	DFMO	Control	DFMO	Control	DFMO
12	5.17±0.063	4.09±0.063	2.43±0.31	1.93±0.35	0.46±0.067	0.47± 0.079
	P<0.01		NS		NS	
24	3.80±0.15	3.12±0.16	1.48±0.21	0.82±0.26	0.39±0.080	0.27± 0.075
	P<0.01		NS		NS	
36	3.73±0.12	3.40±0.10	1.32±0.28	0.90±0.16	0.35±0.015	0.26± 0.050
	P<0.01		NS		NS	
48	4.26±0.15	3.43±0.12	1.96±0.42	0.90±0.66	0.46±0.096	0.26± 0.0088
	P<0.01		NS		NS	

Footnotes:

Each result is the mean of 3 independent experiments ± S.E.M. The results were analysed for statistical significance using the paired Students t-test. (NS = No Significant difference, P>0.05).

TABLE 3.5.4

Induction of glutamine synthetase activity by difluoromethyl ornithine in mouse astrocytoma cells *in vitro*.

Treatment duration (hours)	Glutamine Synthetase nmol/h/mg Protein; S.A		Induction of Glutamine synthetase. (As a % of control)
	Control	DFMO	
12	144± 58	166± 11	115.3
	$p < 0.01$		
24	165± 74	303± 25	183.6
	$P < 0.01$		
36	203± 74	448± 18	220.6
	$p < 0.01$		
48	291± 61	343± 32	117.8
	$p < 0.01$		

Footnotes:

Each result is the mean of 3 independent experiments ± S.E.M. The results were analysed for statistical significance using the paired Students t-test. (Significant difference = $P < 0.05$)

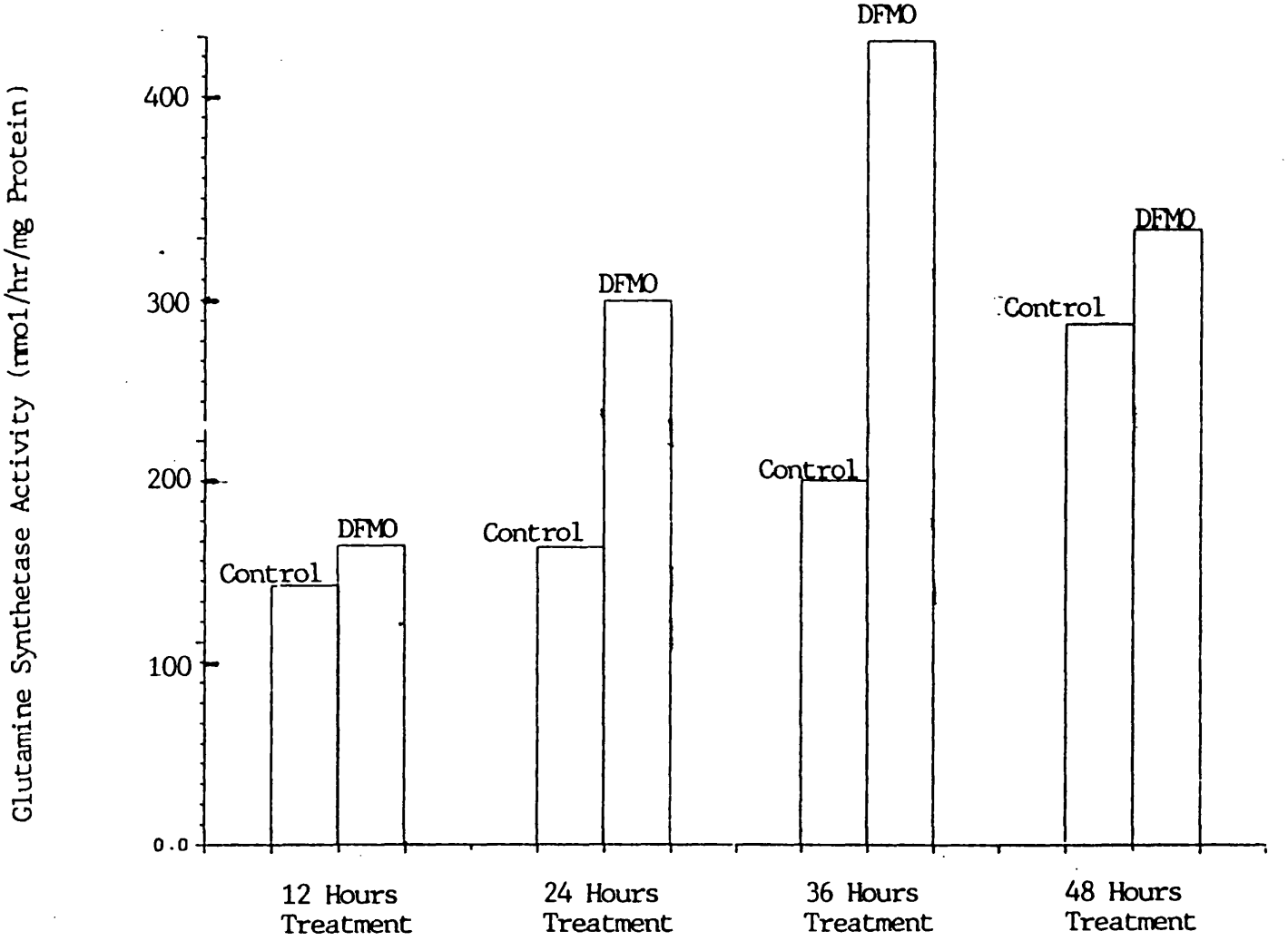

Fig: 3.5.4

Treatment of four day old VMdK P497 astrocytoma cultures with DFMO resulted in a fall in ODC activity and lower cell proliferation. This induced a biochemical differentiation with a rise in GS activity, maintained for more than 48 hours.

proliferation as a result of depletion of intracellular polyamine levels leading to decreased DNA synthesis this was associated with an increase in GS levels, one aspect of a more differentiated phenotype.

3.6. Effect of DFMO and BCNU on growth of subcutaneous implants of VMDk P497-(P1) cells

In the light of the decrease in cell proliferation and apparent induction of differentiation observed *in vitro* when VMDk P497 cells are treated with DFMO, an investigation was carried out to determine whether DFMO could potentiate the modest but significant effect of BCNU against subcutaneous implants of these tumour cells observed in previous studies (Bradford *et al*, 1990).

3.6.1. Tumour growth and morphology *in vivo*

Tumours were visible 13 days after implantation in 80% of the mice and attained maximum growth 30 days after implantation. The tumour volume doubling time (Table 3.6.1 and 3.6.2), increased with time following a Gompertzian type growth curve (Figure 3.6.1). Upon macroscopic examination following removal, the tumours were greyish white in colour and soft in consistency. Histological examination of the tumours was carried out to confirm that the specimens obtained were true tumours rather than granulomatous reactions or lipomas. Samples from all the five experimental groups of mice were confirmed to be of tumour origin (Dr. J. L. Darling,

personal communication).

3.6.2. Effect of DFMO on tumour growth

DFMO (1% solution in distilled water), was given as a sole source of drinking water. The mice tolerated this solution well with no obvious adverse reactions. DFMO had no effect on growth rate of subcutaneous tumour implants (Figure 3.6.1, Tables 3.6.1 and 3.6.2). The activity of ODC in tumour samples from the group given DFMO was not different from the activity in tumours from the control group (Table 3.6.3). The amount of DNA/g wet weight is a measure of cell size. There was no significant change in cell size in tumour samples from mice given DFMO compared to control mice (Table 3.6.3). The tumour weights in the group treated with DFMO were not different from those in the group given a normal diet (Table 3.6.4). The lack of response to DFMO was reflected in the final tumour weight and total tumour DNA (Table 3.6.4). There was no significant change in these parameters in the DFMO-treated group compared to control mice.

3.6.3. Effect of BCNU on tumour growth

Mice in group C were given a single intraperitoneal injection of BCNU (30 mg/kg body weight) on the tenth day after implantation. There was no effect on tumour growth three days after treatment (Tables 3.6.1 and 3.6.2). On the fifth day, tumour growth delay was observed (Figure 3.6.1). In Table 3.6.3 the results of the effect of

treatment with BCNU on cell size (DNA/g wet weight) and ODC activity in VM mouse astrocytomas are shown. ODC activity level per mg protein or per g wet weight was less compared to controls. There was no significant change in cell size in tumour samples obtained from mice in the group given DFMO compared to the vehicle treated control group. In Table 3.6.4 the effect of treatment with BCNU on tumour weight, volume, total tumour DNA and the ratio of volume per weight is shown. These results confirm the cytotoxic effect of BCNU which was evidenced by decreased tumour weight and volume and low levels of DNA in the tumour samples. The high ratio of volume to weight was due to the necrotic and cystic changes observed in the tumour samples from this group.

3.6.4. Effect of BCNU and DFMO on tumour growth

In group D, mice were given 1% DFMO as the sole source of drinking water. On the tenth day after implantation the mice were given a single dose of BCNU 30 mg/kg body weight i.p. Tumour growth was arrested three days after injection with BCNU (Table 3.6.1) and this effect was greater than when BCNU was used as a single agent (Figure 3.6.1). By the seventeenth day of treatment the tumours were smaller than they had been on the first day of treatment. In Table 3.6.3 the results of the effect of treatment of VM astrocytomas with both DFMO and BCNU on cell size (DNA/g wet weight) and ODC activity are shown. There was a slight change in cell size in

tumour samples obtained from this group when compared with the control group. ODC activity level in tumours from mice in the group given DFMO and BCNU was significantly lower compared to ODC activity in tumour samples from control mice.

In Table 3.6.4 the effect of treatment of mouse astrocytomas with DFMO and BCNU on tumour weight, total tumour DNA and the ratio of volume per weight is shown. DFMO potentiated the cytotoxic effect of BCNU. Tumour volumes in the group that received both BCNU and DFMO were significantly less than those in the group that received BCNU only, when compared with the control group ($p < 0.01$, and $p < 0.05$ respectively). This was also reflected in the weights of the tumours. Tumours in the BCNU/DFMO group were significantly lighter than those in the BCNU group, when compared with the control group ($p < 0.001$ and $p < 0.01$ respectively). The high ratio of volume/weight was due to the necrotic and cystic changes observed in these tumours.

TABLE 3.6.1

Effect of DFMO, BCNU and DFMO+BCNU on growth of mouse astrocytomas *in vivo*.

Day (Treatment)	Tumor volume (log) Group A[a]	Group B[b]	Group C[c]	Group D[d]
1	2.332	2.541	2.118	2.244
6	2.963	3.188	2.769	2.585
8	3.220	3.326	2.968	2.925
10	3.267	3.397	3.216	3.196
13	3.401	3.424	3.333	2.715
15	3.586	3.615	2.831	2.243
17	3.624	3.649	2.711	2.029
Residual Variance	0.037	0.015	0.093	0.11
1 Vs 10	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$
10 Vs 17	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.01$

Footnotes:

a: vehicle treated group (control), days 1-17 water + pellets

b; 1% DFMO + pellets days 1-17

c; BCNU i.p., day 10; days 1-17 water + pellets

d; BCNU i.p., day 10; days 1-17 1% DFMO + pellets

Each result is the mean of 4 to 5 independent experiments ± S.E.M. The results were analysed for statistical significance using ANOVA (one way) and unpaired t-test.
Number of mice per group n = 4 - 5.
(NS = No significant difference $p > 0.05$)

TABLE 3.6.2

Comparison of anti-tumour effect of DFMO, BCNU and DFMO + BCNU on mouse astrocytomas in vivo.

Day (Treatment)	Tumour Volume (Log) 1	6	8	10	13	15	17
Group A[a]	2.33± 0.17	2.96± 0.13	3.22± 0.12	3.27± 0.12	3.40± 0.14	3.59± 0.17	3.62± 0.17
Group B[b]	2.54± 0.13	3.19± 0.11	3.33± 0.09	3.39± 0.09	3.42± 0.11	3.62± 0.13	3.64± 0.13
Group C[c]	2.11± 0.15	2.77± 0.11	2.97± 0.10	3.22± 0.10	3.33± 0.12	2.83± 0.15	2.71± 0.14
Group D[d]	2.24± 0.17	2.58± 0.13	2.93± 0.12	3.19± 0.12	2.71± 0.14	2.24± 0.17	2.03± 0.17
Residual Variance	0.094	0.055	0.044	0.044	0.063	0.096	0.088
A Vs B	NS	NS	NS	NS	NS	NS	NS
A Vs C	NS	NS	NS	NS	NS	$P < 0.05$	$P < 0.05$
A Vs D	NS	NS	NS	NS	NS	$P < 0.01$	$P < 0.01$

Footnotes: a - d; see table 3.6.1

Each result is the mean of 4 - 5 independent experiments ± S.E.M. The results were analysed for statistical significance using ANOVA (One way) and unpaired t-test.

Number of mice per group n = 4 - 5

(NS = No significant difference, $P > 0.05$)

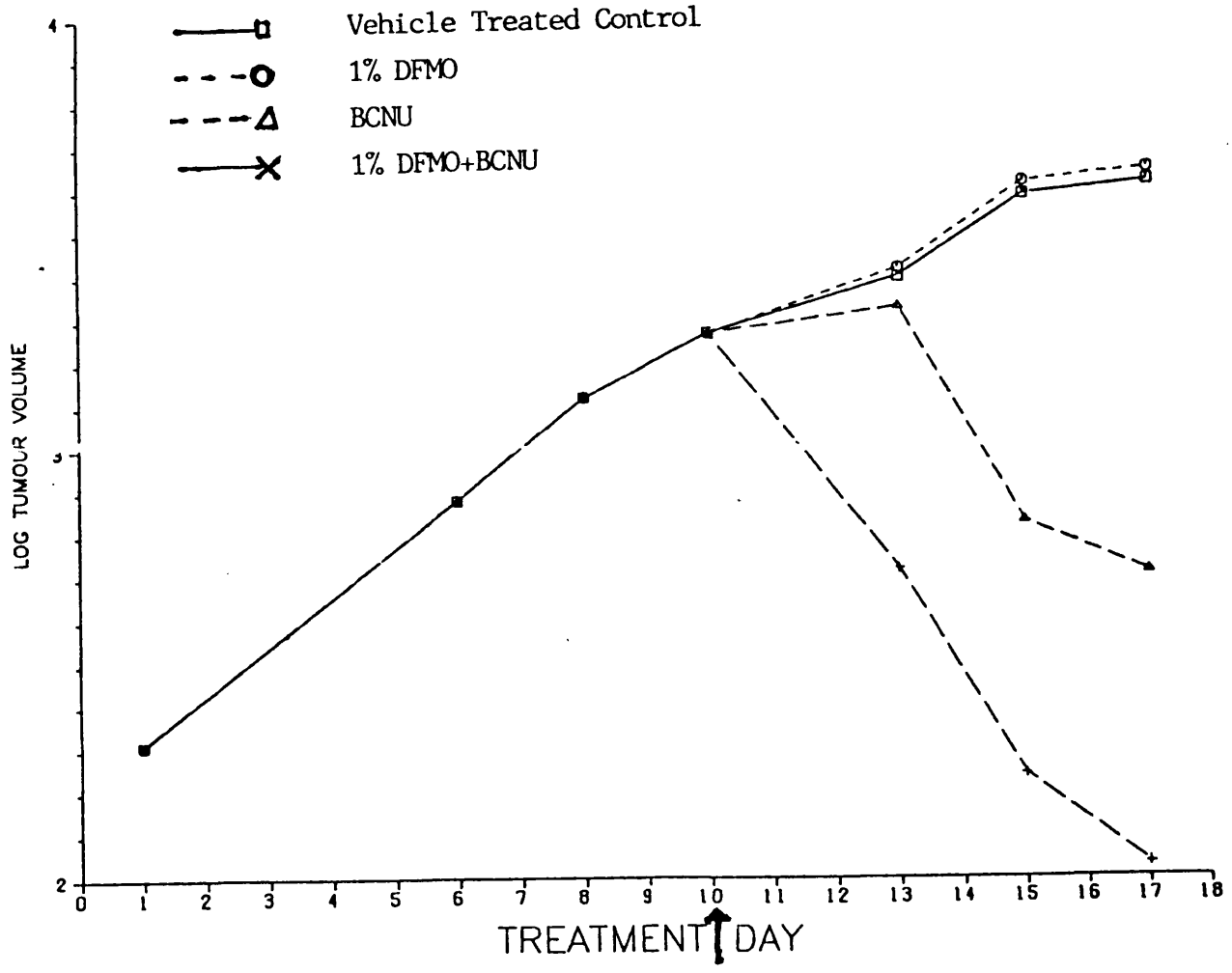

Fig: 3.6.1

Growth of VM mouse astrocytomas in vivo follows a Gompertzian growth pattern and tumour volume doubling time increases with time. DFMO alone has no effect on tumour growth. DFMO potentiates the cytotoxic effect of BCNU.

TABLE 3.6.3

Effect of DFMO, BCNU and DFMO + BCNU on DNA levels and ODC activity in mouse astrocytomas in vivo.

Group	DNA/g wet weight	Ornithine decarboxylane S.A fmols/Gm wet weight	fmols/mg pr/hr
A[a]	0.66	0.230	2.53
B[b]	0.58	0.165	1.83
C[c]	0.52	0.086	0.74
D[d]	0.42	0.042	0.35
Residual variance	0.0028	0.00586	1.1×10^{-5}
A Vs B	NS	NS	NS
A Vs C	NS	$P < 0.05$	$P < 0.05$
A Vs D	$P < 0.05$	$P < 0.05$	$P < 0.05$

Footnotes: a - d see tab. 3.6.1.

Each result is the mean of 3 independent experiments. The results were analysed for statistical significance using ANOVA (one way) and unpaired t-test.
(NS = No significant difference $P > 0.05$)

TABLE 3.6.4

Effect of DFMO, BCNU and DFMO+BCNU on weights, volumes and volume/weight ratios of mouse astrocytomas in vivo.

Group	Tumour weight (Gm)	Tumour Volume (Logn)	Volume/weight ratio	Tumour DNA
A[a]	4.26	3.62	0.85	2.81
B[b]	4.52	3.64	0.81	2.62
C[c]	1.36	2.71	1.99	0.71
D[d]	0.76	2.03	2.66	0.31
Residual Variance	0.52	0.088		0.021
A Vs B	NS	NS		NS
A Vs C	$P < 0.01$	$P < 0.05$		$p < 0.05$
A Vs D	$P < 0.001$	$P < 0.01$		$p < 0.01$

Footnotes: a - d see tab. 3.6.1.

Each result is the mean of 4 - 5 independent experiments. The results were analysed for statistical significance using ANOVA (one way) and unpaired t-test.
(NS= No Significant difference, $P > 0.05$)

CHAPTER 4. DISCUSSION

An historical review of patients with gliomas treated surgically at The National Hospital For Nervous Diseases, Queen Square, London has shown that most patients with poorly differentiated malignant gliomas, in the absence of adjuvant therapy, are unlikely to survive for longer than six months, while those with well differentiated gliomas may survive for twenty to thirty years (Thomas, 1983). This has led to the hypothesis that induced differentiation in tumour cells may limit cell proliferation *in situ* and improve patient survival rates (Patel *et al*, 1986).

In order to examine this hypothesis, a programme of experiments was designed. The purpose of these studies was to investigate the effect of agents known to produce apparent phenotypic differentiation in other tumour cell types either alone or in combination with BCNU, in cells from a murine astrocytoma model.

The model system that was chosen was the VMDk murine astrocytoma. The VM astrocytoma model fulfills many of the criteria of an ideal animal model (Wilson, 1978). It was of spontaneous origin, glial in composition, uniformly fatal within a reasonable time period, and has the therapeutic sensitivity of human malignant glioma (Bradford *et al*, 1989, 1990). It is also capable of *in*

vitro growth and is transplatable intracranially or subcutaneously in syngeneic animals.

Pilkington et al (1985) obtained a 100% incidence of tumours with P497 cells at passage 65 by injecting cells intracerebrally and subcutaneously into syngeneic VM mice. The cells gave rise to malignant glial tumours with a uniform latency and growth. These findings differ slightly with those of Serano et al (1980). They injected P497 cells at passages between 22 and 37 into 10 animals and obtained an 80% incidence of tumours. The apparent increase in the take rate between these two studies may indicate that through serial in vitro passage the cells have adopted a more malignant phenotype.

Glial filaments provide a framework for proliferation and extension of astrocytic processes and gives them stability. Gliofibrillogenesis begins during radial glial formation very early in neurogenesis and occurs again in astrocytes just before myelination and continues throughout life as a response of the central nervous system (CNS) to injury.

The cytoskeleton of foetal rat astrocytes in primary culture contains vimentin, glial fibrillary acidic protein (GFAP) and actin. Vimentin is an intermediate filament protein present in fibroblasts and in the cytoskeleton of immature astroglia in the cerebellum

(Dahl et al, 1981). Dahl et al (1982) demonstrated that vimentin was a major cytoskeletal component of the new born rat central nervous system but it decreased to undetectable levels in mature brain. This decrease was accompanied by an increase in GFAP. Such an association between vimentin and GFAP has not been shown in gliomas.

Work presented in this thesis indicates that growth in vitro was associated with a fall in GFAP levels in VMDk P497 cells. The level of GFAP in confluent cultures (8 days old) was less than in younger cultures. These changes were associated with an increase in cell numbers and total protein per flask. These results are not in agreement with those of Liao et al (1977) who found that GFAP was present only in a minority of C_6 cells when these were maintained in sparse monolayer or suspension culture but present in most cells when grown in organ culture. When an organ culture system was employed there was a two-fold increase in GFAP in cells harvested during logarithmic growth and a six-fold increase in cells harvested during stationary phase. This led to the suggestion that the organ culture system provides a more favourable atmosphere for cell differentiation. The cell-to-cell contact occurring in the foam matrices of the organ culture system probably plays a major role in the increased production of the protein. Another factor is the more favourable rates of cellular oxygenation found in the organ culture system (Liao et al, 1978). It

is also possible that the variation in GFAP production in the various culture systems could be due to differences in solubility rather than to differences in the total amount of protein. It has been shown that GFAP is only partially extracted with water from human brain tissue and this has led to the suggestion that the water-soluble GFAP represents the unpolymerised form of glial filaments while the water-insoluble (detergent-soluble) GFAP represents the polymerised glial filaments (Liao et al, 1978).

The amount of GFAP in gliomas has been associated with the degree of differentiation of the tumour (Duffy et al, 1982). Duffy (1982) studied the differentiation of astrocytic cells in vitro in relation to the formation and intracellular localization of GFAP. Differentiation of astrocytoma cells was induced by altering the growth medium. Differentiation only occurred at low serum concentrations in Minimum Essential Medium (MEM) not in Eagle's basal medium (BME). Using an immunoperoxidase method and microspectro-photometry in serum deprived cultures of cells derived from human benign and malignant astrocytomas Duffy (1982) was able to show that GFAP was abundant and uniformly distributed in spindle-shaped cells in early explants in culture but became predominantly perinuclear as the cells assumed a flat "irregular" shape. In cells grown in serum-free MEM GFAP moved from the perinuclear site to the periphery of the cells and into the developing processes. There was no

apparent translocation of GFAP in cells grown at low serum concentrations in BME. In spontaneously differentiated cells in medium with 10% serum GFAP was distributed throughout the cytoplasm and processes and was more abundant than in cells which had been induced to differentiate.

The primary phenotypic expression of GFAP remains unaltered by the malignant transformation (Bigbee et al, 1983). These workers were able to translate messenger RNA, (mRNA), extracted from a human glioma cell line grown in culture or as a solid tumour, in an mRNA-dependent reticulocyte lysate system. The translation products were labelled with [^{35}S]-methionine and immunoprecipitated with antiserum specific for GFAP. These were separated by one and two dimensional polyacrylamide gel electrophoresis and analysed fluorographically. They found that immuno- precipitates from both cell culture and tumour mRNA translations had a molecular weight of 49,000, which was similar to GFAP extracted from human tissue. From these results, Bigbee et al (1983) concluded that GFAP produced by the glioma cell line was chemically and immunologically similar to normal human GFAP and the primary phenotypic expression of GFAP in the tumour cell line was not altered by neoplastic process.

The changes in GFAP levels observed in the VMDk P497

cells during culture were presumably the result of de-differentiation. Several workers have demonstrated changes in GFAP expression during the development of normal astrocytes. GFAP was present in the mouse brain on the first postnatal day at a level corresponding to half the adult value (Jacque et al, 1976). The amount of GFAP increased rapidly until 12 days of age and reached a value double that of the adult level by 18 days of age. The rapid increase in GFAP during the first 12 days of postnatal life was associated with a rapid proliferation of glial cells and occurred just before myelin synthesis started. Similar results were reported by Bock et al (1980) working with cultures of normal astrocytes. In primary cultures of dissociated cerebral cells from the foetal rat brain GFAP increased continuously, reaching by day 38 in culture, a level 18-fold higher than that seen in adult forebrain.

Work done by Chiu and Goldman (1984) has provided some insight on the rate of GFAP turnover in rat astrocytes. Primary cultures were exposed to [^{14}C]-leucine to label GFAP and the turnover of filaments was then studied by following the decay of radioactivity. High levels of synthesis were detectable by the third day of culture in the early log phase of growth, and the pattern of labelling at day 3 was similar to that at 14 days when the cultures had reached confluency. In short-term incorporation experiments, GFAP was labelled within

five minutes after exposure of the cultures to radioactive leucine. There was no saturation of labelling upto six hours of incubation. The turnover of GFAP, studied by following the decay of radioactivity from pre-labelled GFAP, displayed biphasic decay kinetics. In the initial phase a fast-decaying pool with a half life of twelve to eighteen hours contributed about 40% of the total activity. A major portion of the protein decayed slowly and had a half-life of about eight days. There are several possible methodological problems in studying and interpreting kinetic data for protein turnover. For example, the accumulation of protein biases the measurement of turnover toward a shorter half-life, while re-utilization of radioisotope tends to prolong the apparent half-life of labelled protein. Re-utilization may be minimised by ensuring that the concentration of leucine in the incubation medium is less than that in the chase medium which reduces the re-uptake of released label from the chase medium. Re-utilization may also be minimised by replacing the chase medium every twelve hours during the initial phase of decay.

The turn-over rates for GFAP in vitro obtained by Chiu and Goldman (1984) are in contrast to the turnover rates obtained in vivo by De Armond et al (1986). Twenty-day old mice received a single tail vein injection of [guanido-^{14}C]-arginine. The cytoskeletal material was extracted from the spinal cords at various times after

injection. GFAP formed a band that was distinct from other protein bands in one dimensional polyacrylamide gels. The specific activity of GFAP was determined by eluting the protein from the band on a preparative one dimensional gel. Specific activity reached a peak two hours after injection with [^{14}C]-arginine. Forty percent of the incorporated radioactivity was still present in cytoskeletal GFAP at nine weeks, indicating that a significant proportion of glial filaments turn over relatively slowly *in vivo*. The heterogeneity of astrocytes in the CNS with their potential for differential growth *in vitro* may in part explain the differences between the *in-vivo* and *in-vitro* GFAP turn-over rates. Furthermore, the *in-vitro* studies of Chiu and Goldman (1984), utilised astrocytes from neonatal rodents, raising the possibility that GFAP half-life in mature astrocytes is longer than in immature astrocytes.

Growth *in vitro* was associated with a progressive fall in GS activity in VMDk P497 cells. These changes were associated with a rise in cell numbers and protein. These results are similar to those reported by Nicklas and Browning (1978), using C_6 glioma cells. Nicklas and Browning (1978) investigated the amino acid and carbohydrate metabolism of confluent cultures of C_6 glioma cells. They observed that the presence of glutamine in the incubation fluid was essential to

maintain high glutamine levels in the cells during a twelve-hour incubation. When cells were incubated in a cerebrospinal fluid-like medium glutamate, glutamine, aspartate and GABA levels were comparable to those occuring in the forebrain of adult rat. The cells released significant amounts of the neuro-inhibitory amino acids, GABA and glycine into the medium and rapidly cleared the medium of the neuro-excitatory amino acids glutamate and aspartate. The "small" compartment of glutamate which rapidly labels glutamine from non-glycolytic precusors is located in glial cells. The C_6 cells contain little GS and depend entirely on exogenous glutamine to maintain intracellular glutamine levels. Lack of this enzyme may reflect the anaplastic nature of these cells.

These observations, however, are in contrast to those of Parker et al (1980), who reported an increased production of GS in C_6 cells with increasing passage and number of days in culture. Our observations with VMDk P497 cells are more in agreement with those of Nicklas and Browning (1978).

GS has been associated with the differentiation of astrocytes during development in the rat brain in the chicken retina (Moscona et al, 1980). The retina is a simple system to study because functionally and developmentally it is an extension of the brain. Moscona

et al (1980) observed that GS levels were low in the retina of the early chick embryo but began to rise sharply on the sixteenth day of incubation and increased by 100-fold in 5 to 6 days. This increase occurred several days after growth and cell replication in the retina had ceased and was triggered by the levels of adrenal corticosteroids which are produced one or two days earlier (Moscona et al, 1980).

Patel et al (1983a; 1983b) found that the increase in GS in developing rat brain occurred long after total cell acquisition had ceased and was therefore associated with the differentiation of astrocytes. Their work also emphasized the role of glucocorticoids in the regulation of GS activity in the developing rat brain. Pilkington et al (1983) showed that treatment of VMDk P497 cells with dexamethasone increased cytoplasmic differentiation. Cells were treated with fresh medium containing 1 μM dexamethasone, a non-toxic dose, for 24-72 hours after which the cells were examined by transmission and scanning electron microscopy. Cells treated with dexamethasone exhibited surface structural differentiation and had rounded perikarya and surface projections with numerous mitochondria and rough endoplasmic reticulum. These features were more pronounced after prolonged treatment with dexamethasone. Immunofluorescence staining indicated that GS was distributed throughout the cell body and processes of

untreated cells but that the staining was intensified after treatment with dexamethasone. High concentrations of dexamethasone (above 100 μg/ml) however, were severely toxic to glioma cells in culture (Pilkington et al, 1983). A similar dose of dexamethasone was used in the present study. Exposure of VMDk P497 cells immediately after plating to growth medium containing 1 μM dexamethasone apparently slowed cell proliferation. This effect was not observed when cultures already in exponential growth were treated with dexamethasone. There was a four to sixfold rise in GS activity in dexamethasone treated cells. This effect was apparent in both low and high density cultures.

Continuous exposure of VMDk P497 cells to dexamethasone resulted in a lower terminal cell density. Similar observations have been reported by Freshney (1980) in human glioma cell cultures, where glucocorticoids at low concentrations (10 μg/ml) are cytostatic and promote cell differentiation (Freshney et al, 1980). Morgan and Freshney (1980) observed that when low density cultures were treated with dexamethasone, cell growth was stimulated an effect which they attributed to an alteration in cell surface glycoproteins. It is possible that these biochemical changes are related to the cell surface structural changes observed in P497 cells treated with dexamethasone by Pilkington et al (1983).

The findings reported in the present study on regulation of GS activity in murine astrocytoma cultures by corticosteroids are similar to those of Vaccaro et al (1979) and Hallermayer et al (1981). Vaccaro et al (1979) studied GS activity in vivo and in primary cell cultures of rat hypothalamus. GS activity in vivo increased steadily from the eighteenth day of gestation to the twentieth postnatal day. Cortisol treatment of hypothalamic cultures also enhanced GS activity. Hallermayer et al (1981) determined the cellular distribution of GS in cultures of dissociated brain cells from new born mice using indirect immunofluorescence. The enzyme was detected in 40% of unstimulated cells but treatment with dexamethasone produced a marked increase in the number of positively stained cells. Quantitative assay indicated that GS activity was elevated about sixfold after 36 hours of treatment with 0.1 μM dexamethasone. The basal activity of GS in these cultures was near that found in brains of newborn mice but far below the activity in adult brains. The elevation by glucocorticoid of GS specific activity appeared to be due to induction of enzyme synthesis rather than activation of pre-formed enzyme molecules. Hallermayer et al (1981) also showed that in monolayer cultures of brain cells the inducibility of GS did not depend on cell to cell interaction within tissues. They also observed a gradual but steady increase of GS

activity from low levels in the new-born animal to high values in the adult mouse and suggested that this increase was mediated by endogenous corticosteroids.

The work presented in this thesis has established quantitatively that GS activity in VMDk P497 cells can be induced by dexamethasone. Pishak and Philips (1980) examined the regulation of GS biosynthesis in C_6 glioma cells. The addition of 1 μM dexamethasone caused a 7-10 fold increase in GS specific activity over a 96 hour period. Both actinomycin D and cycloheximide blocked the corticosteroid induced stimulation of GS activity, indicating that both transcription and translation are necessary for this to occur. This strongly suggests that the mechanism requires de novo enzyme synthesis rather than activation of pre-formed GS molecules.

The induction of GS involves differential gene expression elicited by the corticosteroid (O'Malley, 1978). Dexamethasone, a lipophilic molecule, enters the cell by passive diffusion through the cell membrane. In the cytoplasm it binds to a high affinity receptor protein, forming a steroid-receptor complex. Each cell has only a limited number of receptor molecules. The steroid-receptor complex is translocated to the cell nucleus where it associates with chromatin and elicits transcription of GS mRNA.

The present study with VMDk P497 cells confirms that glial-neuronal cell interaction is not a pre-requisite for the induction of GS activity, in agreement with the work of Hallermayer et al (1981). Linser and Moscona (1979) however, investigated the cellular localization of GS induced by cortisol in the neural retina of chicken embryos by immunostaining with GS-specific antiserum and indirect immunofluorescence. In the retina in vivo and in organ cultures of retinal tissue hormone-induced GS was found to be solely confined to Muller fibers. In mature chicken retina, the enzyme was also detected only in the Muller fibers. There was no corticosteroid-induced increase in GS activity in short-term monolayer cultures of dispersed embryonic retina cells. However, when the dispersed cells were reaggregated, GS could be induced but only in the Muller fibers. The authors suggested that the induction of GS in embryonic retina required contact-dependent interactions between Muller glial cells and retinal neurons. Linser and Moscona (1979) thought that signals generated by conditions at the cell surface were relayed within cells affecting gene expression. GS-induction involves hormone receptors and differential gene expression. Therefore it could depend on cell surface signals which are disturbed by dissociation of the tissue into single cells and their maintenance in monolayer culture.

In this study, the presence of cytosolic

glucocorticoid receptors has been demonstrated in VMDk P497 cells. These receptors were detected on cells in low and high density cultures. The distribution of nuclear receptors in the rat brain is different for corticosterone, the naturally occurring glucocorticoid hormone, and dexamethasone, a synthetic compound. [^{3}H]-corticosterone binding was greatest in the hippocampus and septum, whilst [^{3}H]-dexamethasone binding was uniform across all cell regions (De Kloet et al, 1975). This suggests that there are two classes of glucocorticoid receptors, one for dexamethasone on glial cells and the other for corticosterone on neuronal cells (Krozowski and Funder, 1983). Ontogeny of cytosolic glucocorticoid-binding macromolecules has been investigated in rat hippocampus, hypothalamus and pituitary (Olpe and McEwen, 1976). The concentration of corticosterone binding sites was lowest in all three areas around day one after birth and then increased by a factor of two or three, reaching adult levels around day 32. A similar pattern was found for dexamethasone. The presence of cytosolic glucocorticoid receptors during all stages of growth in VMDk P497 cells supports the view that they retain the potential to interact with glucocorticoids during all phases of growth in vitro.

ODC catalyses the conversion of ornithine to putrescine and this is the rate limiting step of polyamine synthesis (Janne and Raina, 1968; Pegg and

Williams-Ashman, 1968; Russell and Snyder, 1969). ODC has a short half-life of between 15 and 45 minutes. Cells have membrane receptors that are differentially responsive to extracellular polyamine concentration. The intracellular ODC activity is related to the sensitivity of these receptors. In tumours, the membrane receptors are poorly responsive to polyamines and therefore tumour cells have higher intracellular ODC and polyamine levels (Canellakis et al, 1978). The regulation of ODC activity is therefore associated with these membrane receptors, the decay of enzyme activity and ODC antienzyme. The antienzyme is activated when cellular polyamine levels reach a threshold level, and it has the effect of neutralising cellular ODC. Control mechanisms operating intracellularly and at the cell membrane help maintain polyamine intracellular concentrations at levels required for metabolic processes. Irreversible inhibitors are therefore necessary in experimental situations aimed at creating intracellular polyamine deficiency. One such synthetic inhibitor is DFMO (Metcalf et al, 1978).

The present study has shown that the activity of ODC in VMDk P497 cells increases from low levels immediately after plating at low cell density to very high levels after eight days in vitro. This increased activity presumably causes accumulation of intracellular polyamines, resulting in increased DNA synthesis and cell proliferation. These changes were associated with a fall

in GFAP levels and GS activity. This is in agreement with data from Mattson et al (1984) who showed that stimulation of human SH-SY5Y neuroblastoma cells by FCS-supplemented medium induced ODC which resulted in cell division. Cells grown for four days had basal ODC activity. An increase in ODC activity was detected within two hours of a change to fresh medium and maximum activity was reached four to six hours after the medium change. This appeared to be due to the presence of FCS in the medium, since only slight activation of the enzyme was detected when the cells were stimulated with serum-free medium. FCS produced a dose-dependent stimulation of ODC activity with the maximum stimulation occuring in 10% FCS. This stimulation was inhibited by the presence of actinomycin D or cycloheximide indicating that this process was dependent on RNA and protein synthesis.

ODC catalyses the formation of putrescine from ornithine, the initial reaction in the biosynthesis of the polyamines, spermidine and spermine. The activity of this enzyme and polyamine levels are highest during cell replication, differentiation and enlargement although each tissue appears to have its own ODC/polyamine developmental pattern. In the rat, regional ODC activities correspond to periods of maximal cell proliferation and differentiation. The enzyme level in regions which mature relatively early (such as the midbrain and brainstem) reaches a peak before birth,

whilst ODC activity in late-maturing regions, such as cerebellum, peaks postnatally (Slotkin and Bartolome, 1986).

The activity of ODC was shown by Bachrach (1980) to be markedly elevated in tumour cells. Marton (1981) showed that the increase of putrescine and spermidine levels in the CSF of patients with medulloblastoma correlated with the progression of this tumour and elevation of CSF polyamines were often the earliest indicator of tumour recurrence. Elevation of CSF polyamine levels has also been reported by Marton (1981) in patients with glioblastoma multiforme and anaplastic astrocytoma. The potential of using CSF polyamine measurement as a brain tumour "marker" has been proposed by Marton (1981). Early detection of brain tumours, although likely to be a rare clinical event, might facilitate therapy and cure. Measurement of CSF polyamines may also help in the long-term monitoring of patients with tumours and short-term monitoring of the efficacy of a specific course of therapy. The mechanisms responsible for polyamine release into CSF are unknown although tumour tissue contains higher levels of polyamines than normal brain tissue and these may be released during rapid growth or necrosis of the tumour.

In malignant glioma putrescine levels have been correlated with the degree of histological malignancy

(Harik et al, 1978). Levels of spermidine and spermine and their precusor putrescine were measured in normal brain and tumour obtained from patients undergoing craniotomy. Tissue from grade II astrocytomas had significantly higher levels of putrescine than normal brain. In grade III and IV astrocytomas, the putrescine level was further elevated to more than three-fold normal values. Levels of putrescine in slowly growing "benign" brain tumours did not differ from levels found in normal brain.

Selective uptake and metabolism of radiolabelled putrescine has been demonstrated in experimental and human brain tumours by Volkow et al (1983). Seidenfield et al (1981) observed that treatment of 9L rat gliosarcoma cells with between 1 and 25 mM DFMO effectively blocked the synthesis of putrescine and spermidine. Treated cells grew at the same rate as controls for the first 24 hours, but thereafter significant growth inhibition occurred. Flow cytometric studies showed that the fraction of cells in G_1 increased and that entry of cells into S phase depended on an adequate intracellular pool of polyamines. Cytostasis caused by 10 or 25 mM DFMO could be reversed by the addition of exogenous putrescine. Cells pre-treated for 48 hours with DFMO and then re-plated in fresh medium did not enter exponential growth until polyamine production resumed. Addition of exogenous putrescine at the time of

replating allowed pre-treated cells to resume exponential growth at the same time as controls. Seidenfield et al (1981) concluded that the presence of a minimum threshold level of polyamines were necessary for initiation of DNA synthesis, which was then followed by a lag of about one cell cycle time before cell growth could be observed.

Treatment of cultures with DFMO slowed down the rate of cell proliferation of VMDk P497 cells. At a concentration of 5 mM DFMO was found to be maximally effective and least toxic to the cells in vitro. Inhibition of ODC was observed within 24 hours of treatment and the effect lasted for more than 48 hours. This cytostatic effect was accompanied by a two-fold rise in GS activity.

Other workers have reported similar results on the effect of DFMO on ODC (Slotkin et al, 1982). A single dose of DFMO given to neonatal rats inhibited ODC almost completely for a 24 hour period. This was accompanied by a decrease in the rate of RNA and protein synthesis, although the major effect was on DNA synthesis and cell replication (Slotkin et al, 1982).

The inhibitory effect of DFMO on cell proliferation has been demonstrated with many other cell systems (Tabor and Tabor, 1984). In addition, several workers have been able to show that DFMO has an effect on induction of cell

differentiation. Jetten and Shirley (1985) induced ectodermal differentiation in F9 murine embryonal carcinoma cells after treatment with DFMO. This was quantitated by determining the number of cells producing plasminogen activator or expressing keratin filaments. The action of DFMO on F9 cells was compared with the action of retinoic acid. Retinoids induce differentiation and inhibit cellular proliferation in many mammalian cell lines, although the mechanism of action is not known. Treatment of F9 cells with DFMO induced morphological changes identical to those of retinoic acid treated cells (Jetten and Shirley, 1985). These changes were accompanied by an induction of plasminogen activator secretion and expression of keratin intermediate filaments. Treatment with DFMO caused a rapid reduction in ODC activity resulting in a decreased level of intracellular polyamines and cell proliferation.

Human SH-SY5Y neuroblastoma cells can be induced to differentiate morphologically and biochemically in the presence of TPA, retinoic acid or a combination of these two substances. Cells treated for 4 or 8 days with TPA or a combination of TPA and retinoic acid had low ODC activity which could not be induced by fresh medium (Mattson et al 1984). These workers suggested that TPA affects the binding of growth factors, present in serum, to SH-SY5Y cells, thus blocking the induction of ODC.

Growth of prostatic or mammary cancers can be affected *in situ* by hormonal manipulation, and agents such as retinoic acid can reverse pre-malignant differentiation of prostatic cells *in vitro* restoring them to an apparently normal phenotype (Schmidek, 1987). The retinoids can modify the expression of peptide growth factors and their receptors, as well as control the synthesis of numerous cell surface glycoproteins. A number of small molecules are highly active in inducing differentiation in susceptible cell lines; substances such as DMSO, actinomycin D, anti-metabolites and cAMP. These agents work by binding to DNA in committed, but not fully differentiated, cells and by activating the genes required to have the cells enter G_1. The replication of DNA and synthesis of histone proteins occurs only in S phase (Schmidek, 1987). During G_2 a cell contains two copies of each of the DNA molecules present in the G_1 phase. The cell divides during M phase when identical copies of cellular DNA are distributed to each of the daughter cells. In this phase the cell is unable to initiate a new cycle of growth and division and undergoes terminal differentiation into a specialised cell. Neoplastic cells are presumably blocked prior to achieving their fully differentiated state and maintain the capacity to divide that most normal cells lose as they mature. Unfortunately this blockage is not totally reversible and although some types of cancer cells can be

induced to adopt more differentiated phenotypes by exposure to various factors, most such agents do not appear to stop malignant growth *in vivo*.

This study has shown that DFMO potentiated the cytotoxic effect of BCNU on subcutaneous astrocytoma implants in VM mice. Analysis of the changes taking place at the molecular level in these tumour samples has shown a greater inhibition of ODC activity in tumours receiving combined therapy than in those receiving either BCNU or DFMO alone. It has also been shown in the present study that tumour samples from mice treated with combined therapy contained less DNA than samples from mice receiving either BCNU or DFMO alone. In the present study, DFMO had no effect on the growth of subcutaneous tumour implants in mice when used as a single agent. The average cell size in tumour samples from mice given DFMO was similar to the average cell size in tumour samples from vehicle treated controls. Pre-treatment of mice with 1% DFMO orally for 10 days before receiving a single dose of BCNU intraperitoneally, potentiated the cytotoxic effect of BCNU. When BCNU was used as a single agent, the cytotoxic effect was apparent on the fifth day after injection with BCNU. In mice pre-treated with DFMO the antitumour effect was observed earlier, three days after injection of mice with BCNU, and the observed effect was significantly greater than when BCNU was used as a single agent.

Studies done by other workers have shown that DFMO potentiates in-vivo cytotoxicity of BCNU in animals bearing the 9L rat gliosarcoma (Marton et al, 1981b). Marton et al (1981b) used DFMO alone and in combination with various single doses of BCNU to treat rats with intracerebral 9L gliosarcoma. Used as a single agent, DFMO had little or no effect against these tumours. However pre-treatment with oral DFMO before intraperitoneal administration of BCNU potentiated the effect of BCNU without increasing toxicity.

Biological response modifiers may have at least three potential uses in the clinical treatment of tumours. They may be used as cytotoxic agents in their own right in single drug or combination protocols, as growth inhibitors in the interval between conventional therapies or as modifiers of the effect of other therapeutic agents. Polyamine depletion modifies DNA structure and conformation. Therefore, depletion of cellular polyamine levels with appropriate inhibitors might not only sensitize cells to nitrosoureas but to variety of chemotherapeutic agents which have DNA as their target site. In the present study, it has been possible to demonstrate some of the changes taking place at the molecular level when BCNU and DFMO are combined and these changes may explain the observed tumour response.

There are three reasons which may explain the greater antitumour response obtained by combining DFMO with BCNU. Firstly, the combination may cause a greater degree of destabilization of DNA structure. In rat neonates, studies with [^{3}H]-thymidine indicate that reduction in DNA synthesis precedes the decrease in DNA content and fall in cell numbers (Bell et al, 1986). These workers examined the regional selectivity of the effects of DFMO administered either post-nataly or during gestation, in order to determine whether specific phases of maturation are particularly sensitive to polyamine depletion. In the cerebellum, in which cells undergo major phases of replication and differentiation after birth, postnatal DFMO administration caused a profound and progressive deficit in tissue weight gain as well as in DNA, RNA and protein content. As mentioned before Seidenfield et al (1981) have shown that DFMO causes inhibition of cell proliferation in the 9L rat brain tumour cells in vitro and this effect can be reversed by addition of exogenous putrescine. They have found that polyamines are necessary for initiation of DNA synthesis in the 9L rat brain tumour cells.

Secondly, it is possible that DFMO synchronises tumour cells, making them more sensitive to phase-specific cytotoxic drugs. Many cytotoxic drugs produce lethal-age responses, that is, cells vary in their sensitivity to the lethal action of drugs according to

which phase of the cell cycle the cells are in at the time of treatment (Madoc-Jones and Mauro, 1974). Although BCNU is capable of killing cells in all phases of the cell cycle the cytotoxic effect is greatest in the G_1/S phase of cell cycle (Barranco and Humphrey, 1971). Seidenfield et al (1981) have shown that treatment of 9L gliosarcoma cells with DFMO synchronises the cells at the G_1/S boundary of the cell cycle, which should potentiate the cytotoxic effect of BCNU. Of course, this effect may not be so clear cut in vivo. Flow cytometry was used to follow the cell-cycle progression of 9L rat brain tumour cells pre-treated for 48 hours with DFMO and then harvested and re-plated in the absence of the inhibitor. Visual inspection of the DNA distributions revealed that by 12 hours after re-plating there was a large increase in the number of control cells in S and G_2+M. The fraction of control cells in these phases of the cell cycle continued to increase over the first 36 hours, after which a decline was observed. For cells pre-treated with DFMO there was a decrease in the S and G_2+M fractions of the population. This decline continued for at least 120 hours after re-plating, after which an increase in the S and G_2+M portions of the DNA distributions was observed. Computer fits of the visual interpretations confirmed that cells pre-treated with DFMO appeared to gradually accumulate in the G_1 peak to a maximum of 70% of the population at 96 hours. These cells did not initiate DNA synthesis as readily as

controls until the polyamine content was replenished. It has been shown in the present study that pre-treatment of VM mice with 1% DFMO orally for ten days before administration of a single dose of BCNU intraperitoneally, potentiates the cytotoxic effect of BCNU. It is possible therefore that pre-treatment with DFMO synchronised the cells in the G_1/S phase of the cell and this resulted in the greater cytotoxic effect observed when BCNU was administered. Goldstein et al (1976) have shown that infection of primary cultures of mouse kidney cells with polyoma virus causes a biphasic increase in the activities of ODC and SAMD as well as in the levels of the polyamines, putrescine, spermidine and spermine. An early peak occurs during the period when early viral mRNA is synthesized and prior to the onset of virus-induced synthesis of host cell DNA. The late peak coincides with the virus-induced synthesis of cellular DNA. Infection of primary mouse kidney cultures induces some metabolic changes which cause a progression from G_1 (or G_0) phase to the S phase of the cell cycle.

Finally, BCNU may block the message required for synthesis of a proliferation factor resulting in the cells being more sensitive to the action of agents like DFMO which induce differentiation. According to Scott et al (1982), the differentiation of most mammalian cells is preceded by growth arrest in the G_1 phase of the cell cycle. They have shown that the growth arrest preceding

the differentiation of BALB/c 3T3 mouse proadipocytes occurs at a distinct stage in G_1 which they have designated G_0. The G_0 arrested cells differentiated in the absence of DNA synthesis.

Growth factors are substances that stimulate cell proliferation and may promote cell differentiation of specific target cells. Some tumours can produce their own growth factors and are said to be under autocrine control, whilst other cells produce growth factors which are effective only against other cell types. The presence of a proliferation factor within the cell which responds to outside signals operating at specific receptors of the cell membrane has been proposed (Scott et al, 1982). In continuously proliferating cells, the proliferating factor levels are elevated, its response to modulation by growth factors is altered or its response to differentiation factors is altered. Lotem and Sachs (1979) have shown that a decrease in growth factor dependent events enhances the cells' sensitivity to the action of differentiation factors. The control of cell proliferation and differentiation by TPA was studied with different clones of mouse myeloid leukaemic cells, a line of human myeloid leukaemic cells and normal mouse bone-marrow myeloblasts. TPA treatment induced normal cell differentiation in one of the leukaemic clones. In the human leukaemic cell line, TPA also induced differentiation. The ability of the phorbol esters to

produce these effects on normal myeloblasts and myeloid leukaemic cells paralleled their ability to act as tumour promoters. A tumour promoter such as TPA can induce the production of, and increase cell susceptibility to, a normal regulator of cell multiplication and differentiation. It may act as a tumour promoter by increasing cell multiplication in initiated cells, it can induce differentiation in some cells or inhibit differentiation in others depending on which molecules are being regulated in the TPA-treated cells. Takeda et al (1982) have shown that only 2-4% of ML-1 myeloblastic leukaemic cells differentiate spontaneously to intermediate stages of maturity, while in the presence of DNA specific inhibitors, TPA, DMSO and ara-C, a majority of viable cells undergo differentiation. Myeloblastic leukaemic cells acquired morphological characteristics of monocytes or of macrophages. An appropriate dose sufficient to cause differentiation induction but minimal cell death is necessary with these DNA specific inhibitors. Such a dose would decrease the expression of proliferation-associated information without blocking subsequent events related to differentiation.

DFMO pre-treatment of 9L cells in vitro potentiates the cytotoxic effect of BCNU in all the phases of the cell cycle, particularly in G_1 phase (Oredson and Marton, 1984). DFMO potentiates the cytotoxic effect of BCNU on subcutaneous astrocytoma implants in the VM mouse. Since

DFMO synchronises the cells in G_1/S phase, it is possible that dexamethasone, a drug known to induce the phenotypic differentiation of human and rodent glioma cells in vitro, may further potentiate the effect of BCNU and DFMO.

It is uncertain which of these mechanisms are responsible for the potentiation of the cytotoxic effects of BCNU. Further studies are necessary to establish the mechanism of action of agents capable of inducing differentiation and how interactions with conventional cytotoxic agents could be therapeutically applied in the chemotherapy of human glioma.

SUMMARY OF ACHIEVEMENTS AND SUGGESTIONS FOR FURTHER WORK

This study has described several features which change during in vitro culture of VMDk P497 murine glioma cells. It has been shown that during culture there is a fall in the expression of astrocytic markers, GFAP and GS. There also appears to be a concomitant rise in ODC activity over this period and this is accompanied by an increase in cell number and total protein. This can be interpreted as an apparent de-differentiation of the cells upon prolonged in vitro culture.

It has been shown for the first time, that dexamethasone induces GS activity in VMDk P497 murine glioma cells. It has also been shown for the first time

that this effect is mediated at the transcriptional level through cytosolic glucocorticoid receptors which are present in both low and high density cultures of these cells.

In addition, treatment with DFMO *in vitro* reduces the activity of ODC which has the effect of reducing the level of intracellular polyamines and cell proliferation. Such cells also appear to have markedly higher GS levels indicating an increase in phenotypic differentiation. In mice bearing subcutaneous implants of VMDk P497-(P1) cells, DFMO alone administered in their drinking water does not appear to have any effect on tumour growth. BCNU has been shown previously in the VM model system to modestly but significantly affect the growth of intracranial and subcutaneous implants. Work presented in this thesis confirms these observations. However, in mice bearing subcutaneous implants of the VM tumour who have been pre-treated with DFMO in their drinking water, the anti-tumour effect of a single intraperitoneal dose of BCNU is greatly potentiated. Tumours regress sooner than tumours treated with BCNU alone and do not regrow so quickly. Biochemical studies on excised subcutaneous tumours indicate that ODC activity is suppressed in animals receiving combination treatment and that the total DNA content of these tumours is reduced.

One obvious avenue of study would be to examine the

effect of DFMO alone and in combination with BCNU on the growth of intracranial implants of the murine glioma cells. Previous studies have shown that BCNU is capable of producing increased life span in treated mice (Bradford et al, 1990).

Further work will need to be done to determine the effect of other biological response modifiers, like retinoic acid and TPA on the growth and differentiation of VMDk P497 cells in vitro and in vivo. This may be approached in similar manner by using the model system described in this study. Further work may also be done to study the effect of combining dexamethasone, DFMO and BCNU on the growth of murine glioma cells in vitro and in vivo.

Certain cancers such as acute childhood leukaemias and testicular carcinoma are curable with chemotherapy (Porter and Suffrin, 1986). However most solid tumours, including malignant glioma appear not to be particularly chemosensitive. There is considerable interest in improving the available chemotherapy for malignant glioma. Polyamines offer an interesting approach as a target site for cancer chemotherapy and much work has been carried out looking for more potent irreversible inhibitors of polyamine biosynthesis.

There is a pressing need for more potent, less toxic

and tumour specific agents in the chemotherapy of gliomas. DFMO, a relatively non-toxic agent, may be used as a potentiating agent so that the cell kill obtained from a given dose of BCNU can be increased or BCNU may be given at a less toxic dose but with the same therapeutic effect. Much work needs to be carried out to identify the cellular targets of DFMO and related compounds as well as to define optimal schedules for administering combination therapy with nitrosoureas as well as other cytotoxic drugs and DFMO. The VM model, which therapeutically resembles human malignant glioma, provides an ideal system to carry out such studies.

The logical extension of these studies is to start to examine polyamine levels and metabolism in established and short-term cultures of human malignant gliomas as well as other human brain tumours. The biochemical effects of DFMO on the growth, differentiation and synergistic effects with cytotoxic drugs have not been well documented using human material but may provide useful insights which will improve the prognosis of patients with malignant brain tumours.

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