PHENOTYPIC MANIPULATION OF NORMAL AND MALIGNANT BRAIN CELLS

by

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C O N T E N T S

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LIST OF ABBREVIATIONS

AAFM	amino acid free medium
BASG	β-alanine sensitive GABA uptake
BSA	bovine serum albumin
BSS	balanced salt sodium
CAM	chorioallantoic membrane
CEA	carcinoembryonic antigen
CNPase	cyclic nuclueotide phosphohydrolase
CNS	central nervous system
DAB	diaminobenzidine
dbcAMP	dibutyril cyclic adenosine monophosphate
DBSS	dissection BSS
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DX	dexamethasone
ECGS	endothelial cell growth supplement
ECM	endothelial cell mitogenesis
EDTA	ethylenediaminetetra acetic acid
ECTA	ethyleneglycol-bis-tetraacetic acid
EGF	epidermal growth factor
FCS	foetal calf serum
FGF	fibroblast growth factor
GABA	γ amino butyric acid
GFAP	glial fibrillary acidic protein
GMF	glial maturation factor
GS	glutamine synthetase
HCG	human chorionic gonadotrophin

HRPO	horse raddish peroxidase
IFN	interferon
Ig	immunoglobulin
IP	isoproterenol
LDM	lactate dehydrogenase
MCIF	melanocyte contact inhibitory factor
Mit.C	mitomycin C
MNU	methylnitrosourea
M.Wt.	molecular weight
NGF	nerve growth factor
NMA	N-methyl acetamide
PA	plasminogen activator
PBE	pig brain extract
PBS	phosphate buffered saline
PDD	phorbol didecanoate
PDGF	platelet derived growth factor
PL	placental lactogen
PU	plough units
Ret.Ac.	retinoic acid
RNA	ribonucleic acid
mRNA	messenger RNA
RSV	rous sarcoma virus
SGF	sarcoma growth factor
TAF	tumour angiogenesis factor
TCA	trichloroaceticacid
TPA	tumour promoting agent (12-O-tetradecanoyl phorbol-13-acetate)

SUMMARY

In this investigation several questions were posed about expression of differentiated and malignancy-associated properties in cell cultures derived from grades III and IV anaplastic astrocytomas. For comparison, cells derived from normal adult post-mortem brain and foetal brain, were also investigated.

Characterisation studies were complicated by the absence of the astrocyte-specific protein, GFAP from the normal adult cultures and many of the gliomas. Other groups of workers have demonstrated that both normal glia-(278) and glioma-(110) derived cultures can lose GFAP as a result of *in vitro* growth. In the case of the malignant cells, the loss of cellular differentiation could also be the result of *in vivo* neoplasia. GFAP positive glioma cultures were presumed to contain highly differentiated astrocytoma cells, whereas GFAP negative cultures probably contained less well differentiated or more anaplastic cells.

Biochemical investigation led to the hypothesis that the flat polygonal cells obtained in cultures from normal adult brain tissue were percursor glial cells or glioblasts. The malignant cell lines represented a gradation in states of biochemical, astroglial differentiation. The degree of differentiation exhibited by a particular cell line was not related to the pathological state of the tumour from which it was derived. The foetal cultures contained apparently mature, highly differentiated astroglia and were found to be phenotypically stable, relative to the normal adult and malignant cultures, in response to environmental changes. The accumulation of immunological and biochemical data for many cell

lines led to the postulation of a possible astroglial precursor pathway.

Investigating the relationship between differentiated and malignancy-associated properties, required the development of assays to represent marker properties. GFAP, high affinity GABA uptake and glutamine synthetase were chosen to represent expression of the differentiated astroglial phenotype and plasminogen activator and tumour angiogenesis factor (or endothelial cell mitogenesis), the malignancy associated phenotype.

The effects of varying the microenvironment of the cells in culture were investigated in a number of ways. Increasing cell density, dramatically increased the expression of GFAP in C_6 cultures and high affinity GABA uptake in many cell lines, at the onset of confluence. As these differentiated properties were stimulated, the production of PA in malignant cell lines was dramatically reduced; possible explanations for the observed effects with changing cell density were put forward in terms of the proliferative state of cells and the formation of cell-cell contacts. Experiments with heterologous co-cultures and high density perfusion cultures, further demonstrated the importance of cell-cell contacts in the expression of differentiation.

The effects of exposing neoplastic cells to various chemical agents were also investigated. Some of the agents upset the balance between differentiated and malignancy-associated properties. In particular dexamethasone, pig brain extract and interferon pushed the phenotypic expression of malignant cells in the direction of more mature, differentiated astroglia,

at the same time reducing expression of the malignancy-associated properties. The tumour promoting phorbol ester, TPA, effectively pushed the balance of phenotypic expression in the direction of malignancy, as determined by in vitro criteria. The differentiated properties were unaffected by this agent. The DNA-alkylating carcinogens,mitomycin C and methylnitrosourea, both used clinically in the treatment of cancer, stimulated expression of both differentiated and malignancy-associated properties. The relevance of these findings in considering the growth and spread of tumours after chemotherapy, and possible new treatment procedures for malignant disease, are discussed.

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Part I

The lack of understanding of the events and circumstances responsible for neoplastic cell behaviour is one of the most fundamental problems in cancer research. In order to gain deeper insight into the mechanism involved in initiating and maintaining cell transformation it is necessary to understand some of the elements of control of cell properties which are altered as a result of this transformation.

Cancer is often described as a disease in which cells have become faulty in one or more of the mechanisms controlling position, proliferation and differentiation. These abnormalities occur as a result of heritable change and lead to cell behaviour which deviates from that dictated by normal physiological events. Many "theories of oncogenesis" have been put forward and a great deal of biological experimentation has been carried out to try to throw light on the nature of the change and the stimuli which initiate and/or promote these events at the subcellular level. Whatever the exact nature of the genetic events and the oncogenic stimuli which produce them, some generalisations can be made about the behaviour of affected cells :

1. proliferation is no longer regulated i.e. cells divide in the absence of any physiological need for more cells, and
2. cancer cells are very often less mature than their normal counterpart i.e. some differentiated features of cells are no longer apparent and they often acquire properties associated with their malignant behaviour. In some cells re-expression of properties associated with embryonic phenotype is also evident.

The relationship between differentiation and malignancy is not well understood but is recognised to be extremely complex. This investigation represents an attempt to gain further knowledge of the relationship between expression of the differentiated phenotype and the malignancy associated phenotype, and to relate phenotypic expression with the proliferative state of cells. Use is made of the controlled environment tissue culture provides and a cell system chosen which allows direct comparison of cells derived from malignant brain tissue, normal adult brain and foetal brain. It is hoped that indentification of factors either exogenous or intrinsic, which regulate differentiation and/or malignancy in mammalian cells in culture, might lead to a better understanding of the processes by which neoplastic transformations occur and are maintained. Probing the link between proliferation, differentiation and malignancy might also be beneficial in considering possible new treatments for malignant disease.

1.1 Control of Cell Proliferation

Neoplastic cells by definition have lost their proliferation control. This uncontrolled cell multiplication leads to an ever increasing population of similarly unrestrained cells which have the capacity to divide under conditions that would block cell division in their normal counterparts. The mechanisms of normal growth control are not well understood but there is little doubt that elucidation of these mechanisms would contribute considerably towards the understanding of neoplastic growth.

Growth Control in vivo : Tissue Regeneration

Normal tissues and organs within the body grow to a particular size and then stop. Some, however, have the capacity to regenerate in response to injury or partial removal and this biological phenomenon has allowed normal growth regulatory mechanisms to be probed. Probably the most striking example of tissue regeneration occurs with liver, although other examples are provided in the compensatory growth of the kidney after unilateral nephrectomy, regeneration of thymus and wound healing. These systems provided some of the first evidence for the existence of blood factors which promote growth.

Surgical removal of two lobes of rat liver triggers a wave of mitotic activity in the remaining liver. Parenchymal cells attain their peak mitotic rate at around 24 hours after partial hepatectomy with the littoral and ductal cells peaking at around 48 hours (1). Two questions arise from these observations which have considerable relevance to theories of growth control; what stimulates the liver cells to divide and what factors control the extent to which regeneration occurs i.e. what are the stop signals once the correct tissue size has been reached?

In 1963 Bucher (2) showed that when pairs of rats were linked in parabiotic association, (one of which was subject to partial hepatectomy), the cells in the liver of the 'normal' partner showed increased DNA synthesis in response to partial removal of the liver of its partner. This implied that humoral factors were being released from the hepatectomised animal into the shared circulation, to promote growth of liver tissue in both animals. Further support for the

humoral nature of the elements of growth control comes from the observation that the first cells to be triggered into mitosis in regenerating liver are the parenchymal cells in the portal region (2,3,4). Cells in the central zone of liver are delayed in receiving the stimulus for growth. In this situation, at least, the mitogenic factors regulating growth are present not only in portal blood but distributed through the systemic circulation (5).

In 1971 Fisher et al (6,7) carried out experiments using a series-arrangement of transplanted and host liver remnant tissues in which they diverted portal blood through a partial liver transplant in a partially hepatectomised rat. The transplant receiving the portal blood responded in the same way as a liver remnant in a non-transplanted host. However, the response in the host liver remnant, which was in series with the transplant, was considerably reduced. It was concluded from these experiments that the transplant used up most of the mitogenic factor present in the portal blood and that little was available to stimulate the host liver remnant. These and related findings led to the view that promotion of mitogenic activity in liver was not the result of decreased concentration of an inhibitor, such as a chalone, in portal blood. Further evidence of a positive rather than a negative control system came from the in vitro experiments of Paul et al (8) when they found that foetal rat liver cells in culture responded to serum from partially hepatectomised rats by increased uptake of ^{3}H-thymidine and ^{3}H-leucine. Serum from the normal rats caused no such response. It seems unlikely that a negative control system such as a chalone mechanism, would result in this increased uptake of nucleic acid and protein precursors.

The humoral factor responsible for liver regeneration is not produced by the liver remnant itself (7) but probably by some "central" machinery which is controlling tissue and organ size within the body. Experiments by Czeizel et al (9), in which the effects of radiation and bone marrow injections on rat liver regeneration were studied, led to the observation that the injection of unirradiated syngeneic bone marrow into irradiated animals restored regenerative growth to normal and that an excess of bone marrow given by injection produced excessive regeneration i.e. bone marrow appeared to over-ride the normal hemeostatic control. These findings are consistent with the theory of Burwell (10) concerning the involvement of the lymphoid system in maintaining tissue size.

Although useful information has been derived about normal growth regulatory mechanisms from the study of regenerating rat liver, experiments by Rabes et al (11)have also led to an interesting observation on the relationship between normal growth control and the onset of neoplasia. They treated rats with diethylnitrosamine, a carcinogen which induces liver tumours, and studied the rate of mitosis in livers of treated rats. Initially when the mitotic index in the parenchymal cells remained at the control level the response to partial hepatectomy was normal. However, as the mitotic index in the parenchymal cells of carcinogen treated animals began to show and increase, as neoplastic growth began, the response of liver to partial hepatectomy was correspondingly reduced. These observations suggest that, in rat liver, carcinogen induced neoplasia is accompanied by loss of normal growth control.

Growth Control In Vitro

1. Growth Factors

Culture of most mammalian cells *in vitro* requires supplementation of chemically defined medium with serum. As a result, a great deal of effort has been put into the identification, purification and characterisation of macromolecular components which promote cell proliferation. The complex nature of serum and its many and varied effects on the behaviour of cells in culture has made elucidation of precise modes of action difficult and time consuming.

Under conditions of serum restriction, many cells become arrested in the Go/G1 phase of the cell cycle but these can be released from the quiescent state by the addition of fresh serum, which causes increased cell proliferation and higher final cell density (12). For many cell types, terminal cell density has been shown to be directly proportional to the concentration of serum in the culture medium (13). These findings imply that specific factors necessary for cell multiplication are contained in serum and the availability of these and related factors controls this process.

In recent years a number of mitogenic proteins, termed growth factors, have been isolated and purified from a variety of biological sources. One of the most potent mitogenic molecules present in serum is derived from blood platelets (14), whose prime function is in the process of blood coagulation. In 1974 Kohler and Lipton (15) demonstrated that mouse 3T3 cells require a Platelet Derived Factor for growth and it has subsequently been shown that glial cells (16) and primate dermal fibroblasts (17) are also stimulated by this factor. As well as Platelet Derived Growth Factor (PDGF) many other agents which promote cell proliferation

have been identified and isolated. These include many trophic hormones from the pituitary and other endocrine glands, the somatomedins (18), and insulin (19). A protein with multiplication stimulating activity (MSA) for chick fibroblasts has also been identified (20) which has insulin-like properties but whose activity is not reduced by insulin antibodies.

Growth factors have also been isolated which have a degree of specificity with respect to target cell type. One of these, Epidermal Growth Factor (EGF) was first isolated from mouse submaxillary gland by Cohen (21) and was found to cause premature opening of the eyes and eruption of incisors in new born mice. EGF was also found to stimulate keratinisation and proliferation of mouse epidermal tissue *in vivo* (22) and enhance the growth of chick epidermis *in vitro* (23). EGF has been shown to be mitogenic for a wide variety of epidermal cells in organ and cell culture, including mouse mammary epithelial cells and mouse mammary carcinoma (24), chick embryo cornea (23) and human foetal cornea (25). However, it has also been shown to be active in non-epidermal cells such as human foreskin fibroblasts (26), human diploid fibroblasts (27), 3T3 mouse fibroblasts (28) and human glial cells (29). The presence of EGF in cultures of chick embryo epidermal cells gives rise to an increase in DNA, RNA and protein synthesis (26, 30) as well as stimulation of polyribosome formation, accumulation of polyamines and induction of ornithine decarboxylase (28). Inhibition of the mitogenic effect of EGF can be achieved with dibutryl cyclic-AMP, theophylline and cholera toxin (30) implying that the growth factor might

exert its effect by decreasing intracellular cyclic-AMP levels, possibly by inhibition of adenyl cyclase. However, in some cases cholera toxin and other agents which increase cellular cyclic-AMP increase cell multiplication e.g. the addition of cholera toxin to human epidermal cells improves the growth of these cells in culture (31). These observations suggest that the link between cyclic-AMP and proliferation is complex.

Cells which respond to EGF possess membrane binding sites for growth factor and there appears to be a degree of specificity in that other peptide hormones do not compete for the binding sites (30). However, it has been shown that EGF binding to mouse skin epidermal cells in culture is considerably inhibited by low concentrations of the tumour promoters 12-O-tetradecanoyl phorbol-13-acetate (TPA) and phorbol didecanoate (PDD) but not by the non-promoting phorbol esters 4-O-methyl TPA and 4-O-PDD (32). In this in vitro system TPA has the effect of stimulating proliferation and inhibiting differentiation (33, 34). A group of transforming polypeptides, termed Sarcoma Growth Factors (SGF), which have been shown to compete with EGF for available membrane receptors, have the ability to reversibly confer the transferred phenotype on normal cells (3T3) in vitro (35). These SGF's have been shown to be immunologically and functionally distinct from EGF but require the EGF receptor to exert their biological effects (36). These observations imply that the effect of tumor promoters, such as TPA, and transforming factors might be mediated by interaction with membrane receptors for some naturally occurring regulatory factors. The endogenous production of polypeptide growth factors by cells that are

able to respond to their own products might represent a general mechanism for maintenance of cell transformation (37).

Another factor which may be closely related biochemically to EGF is Nerve Growth Factor (NGF). It has been isolated from its principal source, the adult mouse submaxillary gland in precursor form and it is thought to effect the morphological and metabolic differentiation of sympathetic neurones and the development of some sensory neurones (38, 39). Active NGF is essential for the growth and differentiation of neuroblasts in tissue culture (40) and the C_6 rat glioma has been found to synthesise NGF related polypeptides in culture.

A polypeptide hormone derived from brain and pituitary, Fibroblast Growth Factor (FGF), has been isolated and purified by Gospodarowicz (42). It has similar properties to PDGF. While FGF is mitogenic for most mesodermal cells *in vitro* there are exceptions, one being the fibroblast line W1-38. It is also mitogenic for some cells of non-mesodermal origin such as adrenal tumour cells (43) and glial cells (44). The mitogenicity of FGF is clearly not the same over a wide range of target cells and is dependent upon the concentration of serum in which the cells are maintained, as well as the presence of glucocorticoids (45).

Much interest has been focused on the fact that many transformed cells are relatively independent of the serum requirement for maintenance and growth in culture (46, 47, 48). *In vivo*, the types of growth control required can be subdivided into three broad classes. The first is growth

during embryogenesis and normal development; the second is growth in response to injury e.g. wound repair and liver regeneration; and the third is growth of neoplastically transformed cells. In the first type various naturally occurring growth factors such as insulin, EGF, FGF and many others may be important. Platelets and humoral factors are undoubtedly involved in the second type. In the third type, the autonomy of the neoplastic cell with respect to its proliferative capacity suggests that alterations in the cell have led to mechanisms of growth control which over-ride the normal regulatory factors. Studies of growth factors, their receptors, modes of action and degradation might provide useful information about normal growth regulatory mechanisms and the nature of the changes in neoplastic cells.

The effect of serum on cell growth and phenotypic expression of markers of differentiation and malignancy, was investigated in this project by withdrawing the serum (either partially or completely) at different stages of the growth curve i.e. log phase and plateau, and assaying for the appropriate markers. The rationale behind this was to determine, not only the degree of dependence of different cell lines on serum, but also how the growth factors and any other factors in serum to which the cells are responsive, might affect the state of differentiation and/or malignancy of the cells. Complete serum withdrawal is a useful way of considerably reducing cell division at any time and provides a tool in studying the link between cytostasis and phenotypic expression.

2. Density Dependent Inhibition of Cell Division

Cells grown as a monolayer on a two dimensional substrate in general follow the conventional growth cycle as shown in Figure 1,i.e. lag phase followed by exponential growth, culminating in stationary phase or plateau, where cell division is considerably reduced and mitosis occurs only as a means of maintaining steady state number. One example of a cell line which is extremely sensitive to "contact inhibition" or density dependent inhibition of cell division is the 3T3 mouse fibroblast line (49). At low cell densities 3T3 cells grow rapidly but as the cells become confluent (at around 1.5×10^5 cm^{-2}) proliferation is reduced. In this case the final cell density a culture will reach is directly proportional to the serum concentration in the medium, providing further evidence that serum contains a factor or factors required for 3T3 cell division (49). Virally transformed (49) and chemically transformed (50) 3T3 cells have greatly reduced requirements for serum factors and do not shown density dependent inhibition of cell division. Similarly, transformation of chick cells (which normally require an insulin-like serum factor for division (51)), by Rous Sarcoma Virus (RSV), results in a decreased requirement for this factor. RSV injected chick embryo cells also lose their requirement for anchorage dependent growth and are able to proliferate in soft gel suspension (52).

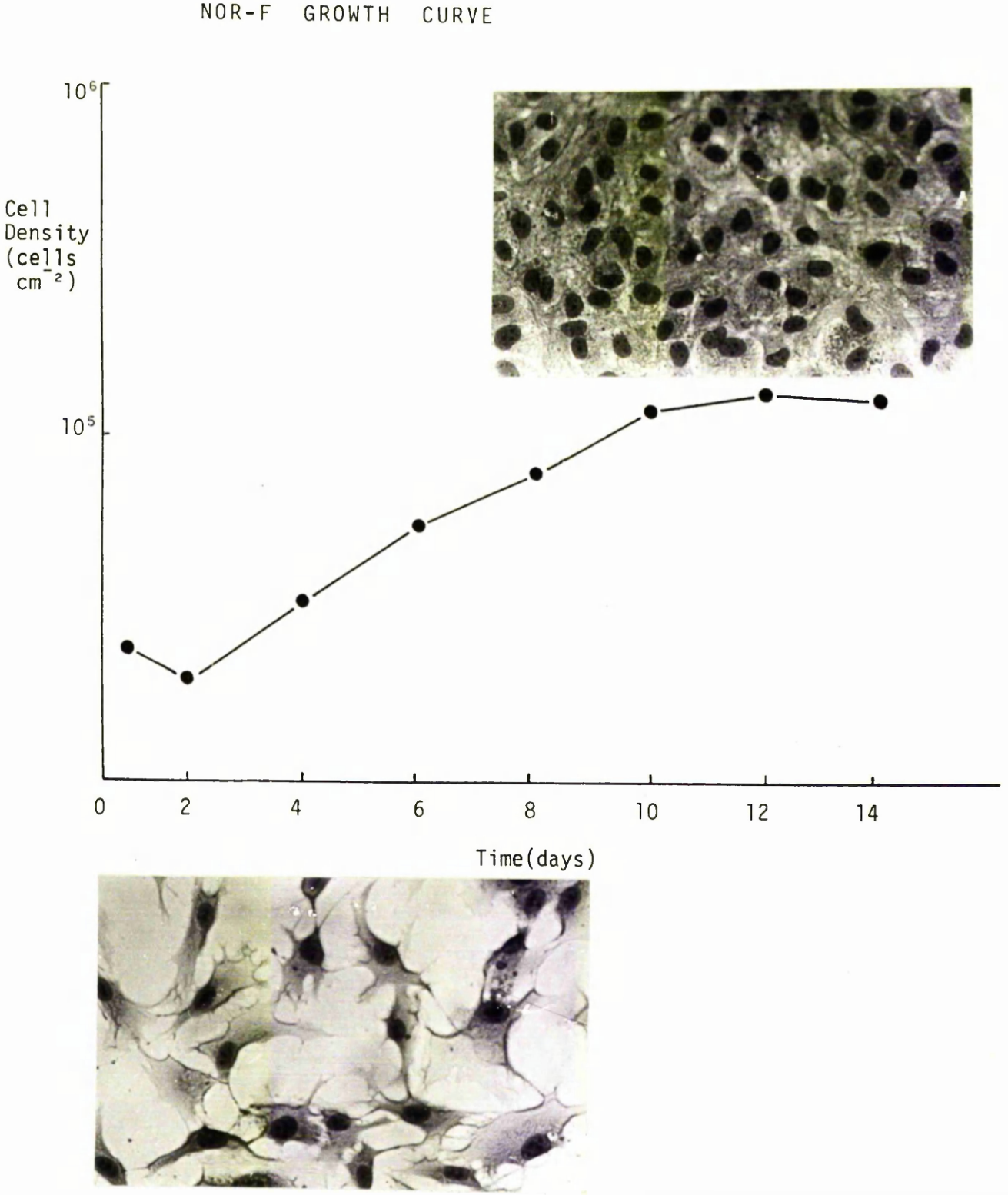
In a stationary culture the rate of cell division is low and the monolayer cell density remains relatively constant even under conditions of frequent medium renewal. If a small patch of cells is scraped away from the monolayer, the cells near the edge of the scraped region are stimulated to divide while the cells in the confluent regions remain

FIGURE I

Semi-logarithmic plot for NOR-F cells grown in 24-well plates. The cells were seeded at 2.5×10^4 cells cm^{-2} and the culture medium renewed every 2 to 3 days. At each time point the cells were trypsinised and Coulter counted. Each point represents the mean of duplicate counts.

The photographs show NOR-F cells at low density and at confluence. Giemsa stained. (x 125)

FIGURE I


NOR-F GROWTH CURVE
10^6
Cell Density (cells cm^{-2})
10^5
0
2
4
6
8
10
12
14
Time(days)

unstimulated (53). Once the 'scrape' has been filled by propagation and migration, these cells return to the low rate of cell division characteristic of the stationary phase. This is known as the "wound healing" phenomenon and gives rise to a situation where cells with a very high and a very low mitotic index co-exist side by side. The fact that confluent stationary phase cells can be triggered into division by removal of adjacent cells, leads to the conclusion that serum exhaustion is not the only factor involved in density dependent inhibition of growth. Cell-cell contacts are also vitally important and it might be that cell "crowding" is responsible for decreasing the availability of receptor sites on the cell surface for essential serum growth factors. This has been proposed as responsible for the density dependent regulation of growth of a monkey epithelial cell line, in conjunction with inhibitors produced by the cells and limiting concentrations of serum nutrients (54).

The "wound healing" phenomenon implies that the growth control mechanism is not the result of an inhibitor acting on all cells in the culture. This conclusion however, does not exclude the possibility that inhibitors do exist which might be necessary but not sufficient to effect the response of density dependent inhibition. A glycoprotein of molecular weight 160 000 has been isolated from contact inhibited hamster melanocytes and has been shown to restore some aspects of cell-surface mediated <u>in vitro</u> growth control to malignant cells (55). This protein, Melanocyte Contact Inhibitory Factor (MCIF) inhibits the growth of hamster, mouse and human melanoma cell lines as well as mouse neuroblastoma, rat mammary carcinoma, neurinoma or glioma, human colon carcinoma,

breast carcinoma and normal human epidermal cells (56). In all cell types growth inhibition is reversible, dose responsive and non-toxic at appropriate concentrations but at higher concentrations MCIF was selectively lethal to malignant cells. Experiments in which culture medium and whole cell homogenates for various tumour cell lines were looked at for presence of MCIF, suggested that at least some tumours are able to synthesise the protein but are not able to release it into the medium. Lipkin et al have suggested that MCIF might be important to normal surface interactions which are required for growth inhibition (56).

The behaviour of cells in tissues and in tissue culture implies that normal cell populations are inherently able to regulate their growth either directly or indirectly and that density dependent inhibition of growth is one in vitro manifestation of this ability (57). Cells derived from normal tissues grow in culture and reach a stationary density, with a greatly reduced mitotic rate soon after confluence is reached. In contrast to this, tumorigenic cells generally continue to divide after confluence, forming a multilayered mass, which only reaches a steady state cell number when nutrient depletion, loss of cells into the medium and partial necrosis balance the relatively uninhibited mitotic rate (57, 58). In some cases, malignant cells fail to achieve a stable density (59). Interesting examples of cells which fail to exhibit density-dependent inhibition of growth are provided by Marshall et al (60). They studied six epithelial cell lines including five derived from human bladder carcinomas and one from a colon tumour, and found that all these lines behaved in a manner resembling transformed fibroblasts i.e. they did

not exhibit density dependent growth inhibition and they were able to form colonies on 3T3 monolayers.

The regulation of proliferation of human-glia like and glioma cells in culture has been studied and the results are of particular relevance to this thesis. Pontén et al showed that astrocyte-like cells derived from adult human brain are subject to stringent growth control mechanisms in vitro (61). In a confluent monolayer only 1% of the cells were found to be synthesising DNA and the addition of fresh serum resulted in only a slight temporary increase in DNA synthesis. In experiments using human malignant glioma cells and virus-transformed glia-like cells in culture, Westermark has shown that all the neoplastic lines reached higher terminal cell densities than the corresponding normal glia-like cells (62). Although the proliferation rate decreased at high cell densities, an absolute resting state was not reached by the neoplastic cells with a significant amount of DNA synthesis still occurring. However, it was concluded from these experiments that neoplastic glial cells retain a degree of growth inhibition induced by high cell density. Human glioma cell lines have also been shown to be less serum-dependent than normal glial cells, and this has been postulated as part of the reason for the reduced density dependent inhibition of glioma cell growth, along with an inability to accomplish perfect physiological intercellular contacts (63). This hypothesis would explain the fact that a certain proportion of the cells in a high density monolayer are still able to grow and divide.

Although the exact mechanism which causes normal cells to stop dividing on reaching confluence, or soon after, is

not well understood, it is clear that some change occurs which blocks the cells in Go/G1 and reduces DNA synthesis. It is also known that inhibition of cell division is not the only alteration in cell behaviour which occurs at high density e.g. in B_{16} melanoma cells, tyrosinase activity reaches a maximum level soon after confluence has been reached (64) and in cultures of C6 rat glioma the percentage of cells which are positive for the astrocytic marker Glial Fibrillary Acidic Protein (GFAP) is dramatically increased at the onset of confluence (65). These findings lend weight to the possibility that after confluence, when cell division is considerably reduced, increased expression of specific differentiated cell functions might occur. It is also possible that the expression of other properties, such as those associated with rapidly proliferating cells, decrease after confluence.

These possibilities prompted an investigation into the expression of phenotypic markers, those associated with normal differentiated and neoplastic behaviour, in relation to different phases of the growth curve, to determine whether or not significant changes in glial phenotypic expression are evident at the onset of density dependent inhibition of growth. One particular question asked is whether or not the reductions in proliferation of neoplastic glial cells at high cell density is associated with changes in phenotypic expression from the malignancy associated phenotype to one more closely resembling mature, differentiated normal cells.

1.2 Cell Differentiation and Neoplasia

Differentiation

Cell differentiation is the result of precise gene

programming. It is based on selective activation of only a very small part of the genome with the vast majority of genes remaining silent in each cell type. From one genotype, many different cell phenotypes are thus generated, giving rise to the structural and functional specialisations that constitute differentiated cells. The many and varied biochemical and immunological differences distinguishing one cell type from another, are the combined result of qualitative and quantitative differences in gene expression. Environmental or extrinsic factors also play a part in determining exact cell phenotype and the way in which gene expression is controlled. The qualitative differences between cell types are obvious in many cases, with the presence or absence of specific proteins e.g. muscle cells produce actin and myosin, pigment cells produce the enzyme tryosinase and the β-islet cells of the pancreas produce insulin. These unique cell products provide dramatic evidence of cell differentiation. Many of the differences which exist between different cell types, however, are quantitative and these are equally significant for cell structure and function.

An example of specific genes which function to different extents is provided in the case of lactate dehydrogenase (LDH) isoenzymes. In most birds and mammals two different polypeptide subunits are the products of separate genes and it is the proportion of these two subunits in the LDH tetramer that determines the molecular form and thus the biochemical activity of the enzyme. These two genes are active to various degrees in different cell types and at different stages of cell differentiation (66, 67), implying that evolutionary divergence of these genes enables each to fulfill a different

physiological function. Comparative studies on purified LDH isoenzymes of human uterus, uterine myoma, uterine myosarcoma and cervical carcinoma, indicate a marked increase of one particular isoenzyme in cancerous tissue (68).

Enzyme adaptation is the ability of a cell to change enzyme levels in response to fluctuations in the chemical environment of the cell. The alterations in the levels of active enzymes resulting from increased gene expression, as opposed to metabolic fluctuation of enzyme activity, change the nature of cell metabolism and have significant physiological consequences. The process of differentiation must require a very large number of enzyme changes both qualitative and quantitative, occurring in a specific sequence leading ultimately to the production of a mature, highly differentiated cell.

Neoplasia

In most cases, neoplastic cells are also defined by the pattern of molecular gene activation and "silencing". With the exception of cells infected by oncogenic viruses, neoplastic cells produce no molecules which are not also found in some normal cells, either adult or embryonic. If this view is correct and neoplastic cells do not establish unique metabolic pathways, structural proteins or functions, then it is necessary to consider the differences between these cells and their normal counterparts in terms of the deletion of "switching off" of specific genes and the inappropriate expression of others. The observed effect is the loss of certain specific cell products and the appearance of others, which are not normally found in the particular cell type of interest at

that particular stage in its differentiation pathway. These latter products are often associated with neoplastic behaviour.

The apparent disturbed differentiation, which is evident in many neoplastic cells could, in theory, occur in two ways, each of which might operate in different circumstances:-

1.The oncogenic stimulus could act on a normal fully differentiated cell(s) changing its properties in such a way as to cause rapid proliferation. The resulting rapidly dividing population of cells might lose some differentiated features and so be less mature than the normal cell(s) from which they were derived.

2.The stimulus might affect immature stem cells giving rise to their propagation. Further development of these cells down the normal differentiation pathway might then occur with a possible block in a later step of the pathway, resulting in a population of cells which have not been able to achieve complete maturity. This "matuation-arrest" theory of carcinogenesis is the one thought to apply in the case of human leukaemia.

The Friend Cell System

A useful model for studying the regulation of differentiation in neoplastic cells is provided by the Friend Cell System (69). Friend cells are derived from the haemopoietic stem cells, transformed by the Friend virus complex after commitment to the erythroid line (70, 71), probably at the proerythroblast stage of erythroid maturation. After transformation, the committed stem cells are able to differentiate independently of the hormone erythropoetin (72), unlike

normal committed stem cells, by treatment with dimethyl-sulphoxide (DMSO) (69) or other inducing agents. The induction of differentiation resembles the normal process fairly closely and markers such as the synthesis of haemoglobin (73), membrane antigens (74), acetylcholine (76) and carbonic anhydrase (76) are useful in identifying various cells of the erythrocytic series, making this a useful model system for studying the relationship between differentiation and malignancy. Priesler et al (77) treated Friend leukaemia cells in vitro with chemical agents known to induce erythroid differentiation. The cultures were harvested and the degree of differentiation (as determined by haem levels and proportion of benzidine positive cells), in vitro clonogenicity and malignant potential in mice, were measured. The results of these experiments showed clearly that the induction of Friend cell differentiation is associated with a considerable decline in clonogenicity and a more modest decline in malignancy. These observations confirm that in some situations at least, induction of differentiation in neoplastic cells is associated with changes in other aspects of cell phenotype which are related to growth and malignant behaviour.

Metabolic Controls

When cells undergo neoplastic transformation, differentiated metabolic characteristics are very often diminished or lost. In some cases, however, certain functions are often not only retained but greatly enhanced. This is very often true of tumours of the adrenal gland or extra-adrenal chromaffin tissue, which secrete excessive amounts of adrenaline and nor-adrenaline and so carry out a lethal metabolic "attack" on the host, in

some cases while the tumour is still very small (78). Similarly insulin secreting islet-cell carcinomas can produce excessive amounts of insulin and this represents a serious breakdown in the control mechanisms regulating differentiated function.

Although neoplastic cells appear to have lost control of proliferation and expression of differentiated functions, it is clear that many controlled responses are not fundamentally different from those operating in normal cells. The operation of control mechanisms in neoplastic cells is subject to selection for the most beneficial metabolism to the rapidly proliferating cancer cell population. The metabolic patterns which result are often harmful to the host. In some cases,however, normal systemic controls are able to regulate neoplastic behaviour e.g. prostatic carcinomas show many different responses to exogenous hormone (79). Some can be kept in a state of arrested growth for a long period of time, showing that some tumours are still able to respond to hormonal regulation of cell division, while others are unresponsive to hormonal treatment. Hepatomas or another class of diverse tumours with some deviating only slightly from normal liver cells while others are highly malignant (80). These observations imply that, in making generalisations about the loss of growth control and differentiation in neoplasia, it is necessary to be aware of tumour diversity. Tumours are capable of exhibiting a fine gradation in metabolic and behavioural pattern from near normal to highly malignant.

Neoplasia Arising in Stem Cells : Teratoma

In viewing cancer as a disease of differentiation it is useful to consider the case of teratomas. These tumours are disorganised arrangements of several different differentiated cell types implying that they probably orginate from embryonic multipotential cells, such as the primordial germ cells. The differentiated tissues which are derived from teratocarcinoma stem cells usually divide slowly and are not malignant unlike the stem cells which often continue to proliferate rapidly to maintain the malignant stem line. Single cell cloning experiments from dissociated teratomas have shown that single embryonic carcinoma cells can give rise to teratocarcinomas composed of several different cell types (81). In this type of cancer the neoplastic event occurs at a very early stage in the differentiation process, in the primordial germ cell prior to embryonic development. This observation has profound implications for both developmental biology and oncology as it implies that specific regulation of gene function can proceed normally in highly differentiated cells, once the initial malfunction in gene programming has been overcome.

Oncofoetal Markers and Neoplasia

There are many examples of activation of developmental genes occurring in neoplastically transformed cells. Liver metastases of colon and breast carcinoma (82) and neoplasia of the gastrointestinal tract (83) have been shown to produce carcinoembryonic antigen (CEA), a glycoprotein, discovered in 1965 by Gold and Freedman (84), which is normally found in tissues of the human foetus of up to six months gestation.

CEA is present in the glycocalyx of tumour cells that produce it (85). Another protein, Alpha Foeto Protein (AFP)is found circulating in the foetal serum after the fourth week of gestation and was found by Tatarinov in the serum of a patient with primary hepatoma (86). Extensive studies have also shown it to be present in germ cell teratocarcinomas (87). During the growth of an AFP producing tumour the serum concentration of AFP maybe used as an indication of tumour growth (88).

There are many other tumour antigens which can be put into the category of re-expressed foetal antigens, including some melanoma surface antigens and beta oncofoetal antigen, found in several types of human cancer in concentrations significantly higher than in normal adult tissues (89). It is also generally recognised that embryonic isoenzyme patterns often re-appear in tumour tissues and differ both qualitatively and quantitatively from those found in the adult tissue of tumour origin (90). An example of this is found with the soluble esterases from virally transformed 3T3 cells which exhibit an isoenzyme pattern similar to that exhibited by primary mouse embryo cells but distinct from that seen in normal 3T3 cells (91).

Another group of antigens synthesised by cancer cells are the placental antigens, hormones such as human chorionic gonadotrophin (HCG) and placental lactogen (PL) (92).

It would appear that activation of embryonic genes, as seen by the detection of their protein products, is a genuine manifestation of neoplasia and in some cases provides useful markers for the clinician. It also provides support for the concept that neoplastic transformation can occur at

the early stages of cell differentiation.

Differentiation in Cancer Therapy

The possibility of using differentiation of tumours in treatment of disease is intriguing. In 1978 Sachs proposed this possible approach to therapy of myeloid leukaemia when he studied induction of differentiation and phenotypic reversion of malignant cells using leukaemic cells (93). Certain tumours have also been known to differentiate spontaneously,e.g. neuroblastoma often differentiates to form a non-malignant ganglioneuroma (94). The differentiation of neuroblastoma cells in culture has been extensively studied and can be promoted by a number of agents, giving rise to well characterised phenotypic changes (95). Increasing intracellular cyclic AMP levels, by various means, induces differentiation as defined by neurite formation, membrane changes, increased levels of neurotransmitter metabolising enzymes such as tyrosine hydroxylase, acetylcholinesterase, choline-acetyl-transferase and catechol-o-methyltransferase. The exact relationship between differentiation and malignancy in neuroblastic cells is not clear but Prasad and Sinha (95) concluded that different differentiated functions are independently regulated and that no individual differentiated function is linked with malignancy. However, when several differentiated functions are maximally expressed, the tumorigenicity of such cells is abolished.

It would be of considerable value to identify drugs or chemical agents which promote differentiation in particular tumour types and to analyse changes in tumour cell phenotypic

behaviour which accompany this. For the cell system used in this project i.e. cells derived from normal adult brain, foetal astrocytes and cells derived from malignant astrocytomas, an attempt was made to identify agents which promote differentiation, as defined by cell products and functions known to be part of the specialised make-up of astroglia. Assay systems were developed to measure the levels of these markers under various conditions.

Acquisition of Malignant Potential by Cells

If non-virally induced neoplastic cells do not synthesise unique molecules or establish metabolic pathways which are not found in some normal cell, foetal or adult, at some stage in the developmental process, then clearly abnormal cell behaviour must be determined by incorrect combinations of gene products and functions not normally found in any one cell. For a malignant cell to survive and prosper it must acquire products and functions which enable it to grow and divide rapidly, invade surrounding host tissue and in many cases migrate, via the blood or lymphatic system to other sites in the host organism and establish new tumour growth at these sites. None of these processes are, in themselves, biologically abnormal. Rapid cell growth has already been described in the process of tissue regeneration in response to partial removal or injury. "Invasion" or migration of many kinds of cells occurs during embryogenesis and in the adult, leukocytes provide examples of cells which invade many tissues in carrying out their physiological role. Wound healing, also requires cell migration and local movement of cells occurs in the epithelial lining of the gut

and in formation of skin epidermis. Such behaviour is essential to normal development and survival. Invasion and migration, like rapid cell division, are not abnormal biological functions but become abnormal when they occur in the wrong cells at the incorrect times and places within the organism, and are not subject to normal regulation.

If these conclusions are correct, then the acquisition of malignant potential by cells arises by some selection procedure which selects new beneficial products and metabolic patterns enabling the cells to carry out efficiently the processes it requires to survive, grow rapidly, invade adjacent tissue and often metastasise to distant sites. The selection procedure is nearly always harmful to the host. In vitro criteria of malignancy of neoplastic cells may be obtained by the qualitative or quantitative detection of cell products or functions associated with their malignant behaviour, which are not normally found in that cell type.

In this project two such products were identified and assays developed to quantitate their levels of production. In phenotypic manipulation experiments, using chemical agents and cytostatic conditions, the levels of these two cell products were measured in conjunction with the levels of differentiation markers. While this provides useful information on the control of expression of differentiated and malignant properties in astroglia and the relationship between these, it is recognised that the two markers chosen to represent expression of the malignant phenotype are by no means the only two properties which determine the in vivo malignancy of the tumour cells. Thus, assessment of the degree of malignancy of neoplastic cells by these in vitro criteria

does not necessarily reflect accurately the malignancy of the tumour *in vivo*.

PART II

1.3 Normal and Neoplastic Glial Cells in Culture

The system used in this investigation is one which allows direct comparison of early passage cell lines derived from malignant astrocytomas (Grades III and IV, Kernohan and Sayre histological grading), normal human adult brain tissue and foetal brain tissue, by collagenase digestion. Malignant glial cells can be grown in tissue culture with relative ease and some of the cell lines derived from malignant brain tissue became established and appeared capable of indefinite proliferation. This confirms the observations of Pontén and McIntyre (96). The normal non-neoplastic cells derived from adult brain show slower initial growh in culture and have a finite *in vitro* life span of about 40-60 generations. Normal glial cells are stable in culture and do not undergo spontaneous transformation into continuous or morphologically altered lines (96). Foetal glial cells adapt very rapidly to growth in tissue culture although their *in vitro* life span has proved to be short, the cultures senescing around the 8th-12th generation in most cases.

The successful growth of normal and malignant glial cells provides one of the few model systems available with the potential for comparing cytology,growth kinetics, behavioural characteristics, biochemistry and immunology of malignant and normal cells of similar lineage. Most of the common solid tumours e.g. breast and colon carcinomas do not yield a large population of malignant cells and these are often overgrown by stromal fibroblasts. Growth of equivalent normal epithelial tissue has also considerable problems. Glioma-derived cultures are very rarely overgrown

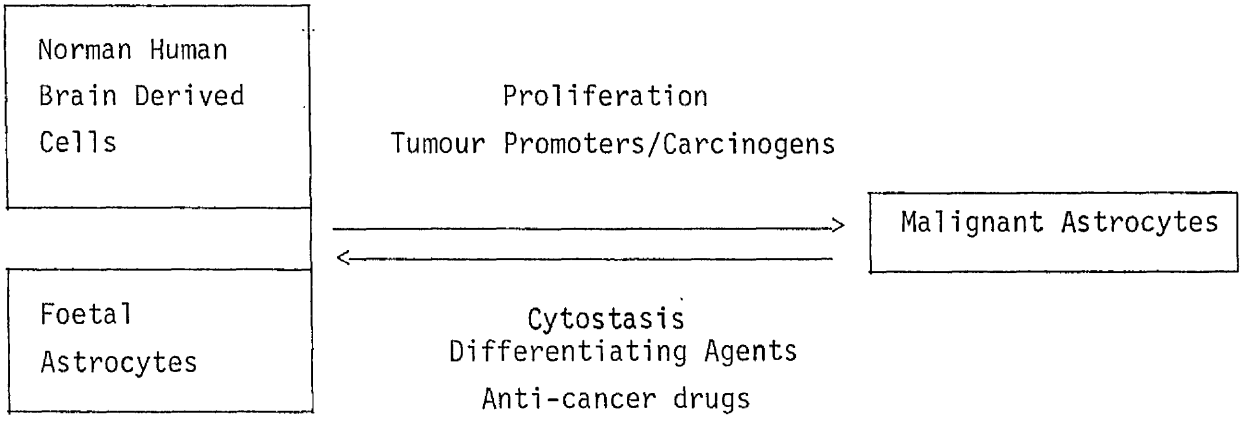
with fibroblasts although blood vessel endothelial cells are known to be a possible source of contamination. Only cell lines which have been shown immunologically to be negative for the specific endothelial marker, clotting factor VIII, were chosen for study. Neuronal cells are not able to divide and grow in culture and any neurones isolated in the primary cultures are subsequently lost.

A considerable amount of effort has been expended in recent years in the study of neoplastic human glial cells in culture. Pontén has shown that glioma cell lines in culture can vary quite considerably morphologically and generally fall into one of five types, i.e. astrocytoid, pleomorphic, epithelioid, bipolar and bimorphic (96). This is probably an *in vitro* manifestation of the fact that malignant gliomas *in vivo* are characterised by many different forms. The heterogeneity of genotypic and phenotypic characteristics of fifteen permanent glioma lines has been reported by Bigner *et al* (97). They looked at many different aspects of glioma phenotype and concluded that gliomas constitute a very diverse group of neoplasms showing extensive phenotypic variations. It is therefore necessary, when making comparisons between the phenotypes of malignant and normal glia, to use several different glioma lines, thus allowing for phenotypic diversity between gliomas.

In 1971 Mark carried out karyotypic analysis of fifty cerebral astrocytic gliomas, studied with conventional staining methods, and concluded that about threequarters of these were diploid or near-diploid (98). Pontén, however, has reported that most established glioma cell lines

are hypotetraploid with only one out of a large number of lines looked at being near diploid in chromosome number (99). Detailed karyotypic analysis has been carried out on only a few of the cell lines used in this study. However, analysis of early passage lines, derived in an identical manner from biopsies of anaplastic astrocytomas and normal adult post-mortem brain material, have previously been studied (100). The results showed that in general astrocytoma derived cell lines are predominantly hypodiploid, although diploid cells are also present. Analysis of normal brain cells has shown the diploid chromosome number of 46 (100). Two astrocytoma derived lines used consistently throughout this investigation have been analysed with respect to their chromsome numbers (101). One of these G-CCM is hypotetraploid with a modal chromosome number of 62. The other, G-IJK consists of a dual population of cells, some of which are near diploid and some near-tetraploid. Although early passage cultures were used for experimental purposes, these two cell lines are probably now established in culture. In general the aneuploid karyotypes exhibited by astrocytoma derived cell cultures are consisent with the view that they are predominantly neoplastic.

Model and Project Aims



The main aim of this project was to gain knowledge of the relationship between certain aspects of glial cell phenotype, in particular, aspects of the phenotype associated with differentiation and malignant behaviour. It was of particular interest to determine whether or not, in a group of malignant gliomas, there is a balance or equilibrium between the differentiated phenotype and the malignancy associated phenotype. The question was also asked as to whether or not cells can be pushed, on one hand, in the direction of differentiation by cytostatic conditions, differentiating agents or anti-cancer drugs and, on the other hand, in the direction of malignancy by conditions of rapid proliferation, treatment with tumour promoter or chemical carcinogen.

Marker properties have been chosen to represent the differentiated and malignant astroglial phenotypes. Biological, biochemical and immunological assays have been developed to demonstrate these properties and to quantitate their levels of expression under various conditions. An attempt was made to identify intrinsic or exogenous factors which were effective in manipulating phenotypic markers. One approach

was to examine the effect of cell density on phenotypic expression of differentiated and malignancy associated properties. Another was to treat cells with chemical agents, under conditions of low serum, to look at their effect in manipulating cell phenotype.

It is obviously essential to an *in vitro*, comparative study of malignant and normal cells, which are believed to be of similar lineage, that the identity of the cells is known. For this reason a considerable amount of effort has been put into the characterisation of cell lines used in this study. This was done by looking for specific, differentiated features characteristic of mature astroglia *in vivo*. Thus cell characterisation and the development of marker assays for differentiation overlapped almost completely.

1.4 Markers of Astroglial Differentiation

Properties associated with astroglial differentiation are closely concerned with the specific functions these cells perform in the brain *in vivo*.

Glial Functions

1. Structural The presence of glial cells in the brain is important to provide physical support for the more delicate neurones. In particular, within the CNS, fibrous astrocytes provide support for the axons which are not surrounded, as in the peripheral nerves, by the basal laminae of the Schwann cells.

2. Neurotransmitter Uptake and Metabolism The glial uptake of certain putative amino acid neurotransmitters by efficent, high-affinity processes and their subsequent metabolism, are processes which imply the involvement of glial cells in the possible regulation of extracellular concentrations of these substances. Uptake of amino acid neurotransmitters by glial cells is a possible means of reducing their active concentration at the synapse.

3. Nutritive Role Glial cells are also involved in the transfer of nutritive and regulatory substances from the bloodstream to the neurones.

4. Repair and Regeneration Glial cells in the brain are able to divide and this allows provision for another important function. As neurones are lost due to injury or ageing, glial cells proliferate and occupy the vacant spaces,forming a scar tissue.

5. Neuronal Organisation The hypothesis has been put forward that glial cells are able to define neuronal organisation (102). It appears that at some stages in the growth and development of the nervous system, neurones migrate along glial processes.

Three markers of differentiation have been selected for use in the investigation.

1. Glial Fibrillary Acidic Protein (GFAP)

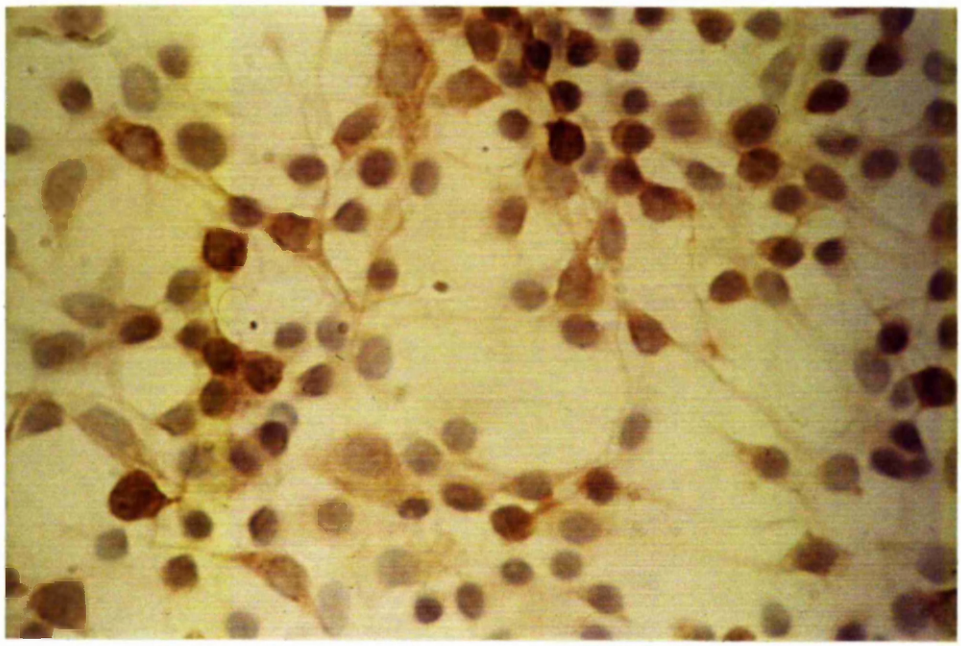
GFAP is a major constituent of astroglial fibres (103, 104) and is located predominantly in fibrillary astrocytes in mature brain and spinal cord. These astrocytes are thought to be important in maintaining the structural and metabolic integrity of the brain and they are characterised by the presence of large numbers of intermediate filaments (8-10 nm), containing GFAP in their processes and cell bodies. Dahl and Bignami (105) have proposed that the function of GFAP may be concerned with the support fibrillary astrocytes provide for central nerous system axons, which are susceptible to damage caused by trauma. The astroglial fibres, of which GFAP is a major component may provide this physical support.

Ontogenetically, the onset of glial differentiation in mice is accompanied by the appearance of a radial system of fibres containing GFAP (106). In the adult rat, these fibres are still present but they become widely spread and less prominent than in the immature animal (107). GFAP has also been observed in immature human astrocytes in primary culture (108) and is reported to be present in both primary and long term cultures of adult brain cells explanted from normal, neoplastic and non-neoplastic CNS diseases (109). Although GFAP is the most universally recognised marker for astrocytes it has been observed that only a few cell lines derived from human gliomas continue to express it (110). In the glioma heterogeneity studies of Bigner et al, they

observed that only two out of fifteen established glioma cell lines had readily detectable levels of GFAP (97) and work by Vivard et al has shown that many astrocytoma derived cell lines are entirely GFAP negative after repeated subculturing (111). The reason for these observations may lie in the state of differentiation and anaplasia of the cells derived from tumour material. Several groups of workers (112, 113, 114, 115) have shown that although GFAP can be detected in cells in most astrocytic gliomas, only the most morphologically differentiated cells express GFAP, while the more primitive and anaplastic cells do not express it. GFAP negative astrocytoma derived cell lines may therefore be composed of tumour cells which grew up from cell populations not expressing GFAP in the original tumour. The anaplastic GFAP negative component of astrocytoma tumours may arise due to the loss of cell specific, differentiated features which often accompanies neoplasia.

In a previous study by R. Shaw (116) on the GFAP content of a number of cell lines derived from human gliomas rat glioma and normal human brain, it was observed that only one rat glioma line (C_6) (Plate 1) and one human glioma line (G-IJK) had GFAP positive cells present. All the other lines looked at were negative for the astrocytic marker. (Some of the cell lines tested in this previous study were used in the present investigation). Induction of differentiation experiments in the rat C_6 glioma, as determined by increased proportion of GFAP positive cells in the culture, were carried out. It was found that as C_6 cultures reached confluence there was a dramatic increase

PLATE I



Rat C_6 glioma culture stained for GFAP by immunoperoxidase. Haematoxylin counterstain (x 420).

in the proportion of cells expressing GFAP from less than 50% to around 75% (Figure 2a). This was thought to be related to the onset of cell-cell contact formation, which occurs at confluence. A similar increase in GFAP positivity in C_6 cultures was seen by treatment with chemical agents, the most effective combination being dibutyrylcyclicAMP and dexamethasone (Figure 2b). These results showed clearly that in the case of C_6 rat glioma at least, expression of GFAP could be altered by environmental and chemical inducing factors.

In this investigation GFAP was detected visually in methanol or acetone fixed cell cultures, by immunoperoxidase or immunofluorescence. A more quantitative assessment of cellular GAFP content was also attempted using the same primary anti/GFAP antibody and a second antibody labelled with I^{125} isotope. These techniques were employed in both cell characterisation and phenotypic manipulation experiments.

2. High Affinity Amino Acid Uptake

It is well established that γ aminobutyric acid (GABA) functions as an inhibitory neurotransmitter in the mammalian brain (117). There is also strong evidence for the concept that glutamate exhibits exitatory effects on spinal cord and cerebral neurones (119). As discussed previously, if these amino acids function as neurotransmitters then there must be a mechanism for their inactivation at the synapse. This could be brought about by rapid uptake into satellite glial cells and their subsequent metabolism. These considerations have prompted studies by various groups of workers on the uptake of putative amino acid neurotransmitters into glial cells *in vivo* and *in vitro*.

FIGURE 2a

C_6 cultures were grown in 75cm^2 flasks and stained for GFAP by immunoperoxidase at the densities indicated. Between 200 to 500 cells were scored and the percentage GFAP positive cells determined. Control samples, without anti-GFAP antibody, were uniformly negative. Each point is the mean and standard deviation of 10 to 20 replicate fields from duplicate samples.

FIGURE 2b

GFAP determination as above. Cultures were treated with dexamethasone ($10\mu g ml^{-1}$) and dibutyrilcyclicAMP ($10^{-4}M$) (dbcAMP + DX) and compared with control cultures.

These experiments were performed by R. Shaw and have been reported (116).

FIGURE 2a

EFFECT OF CELL DENSITY ON GFAP PRODUCTION BY C_6 CELLS

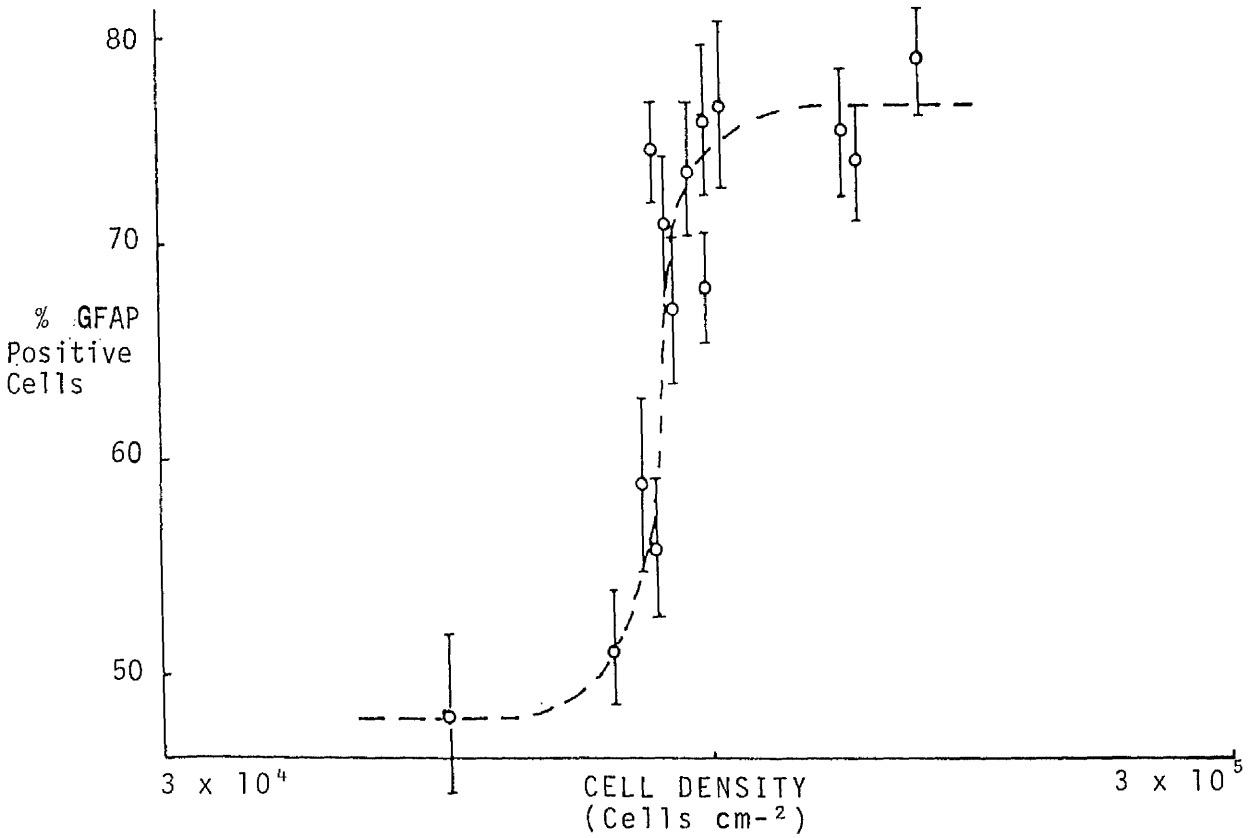
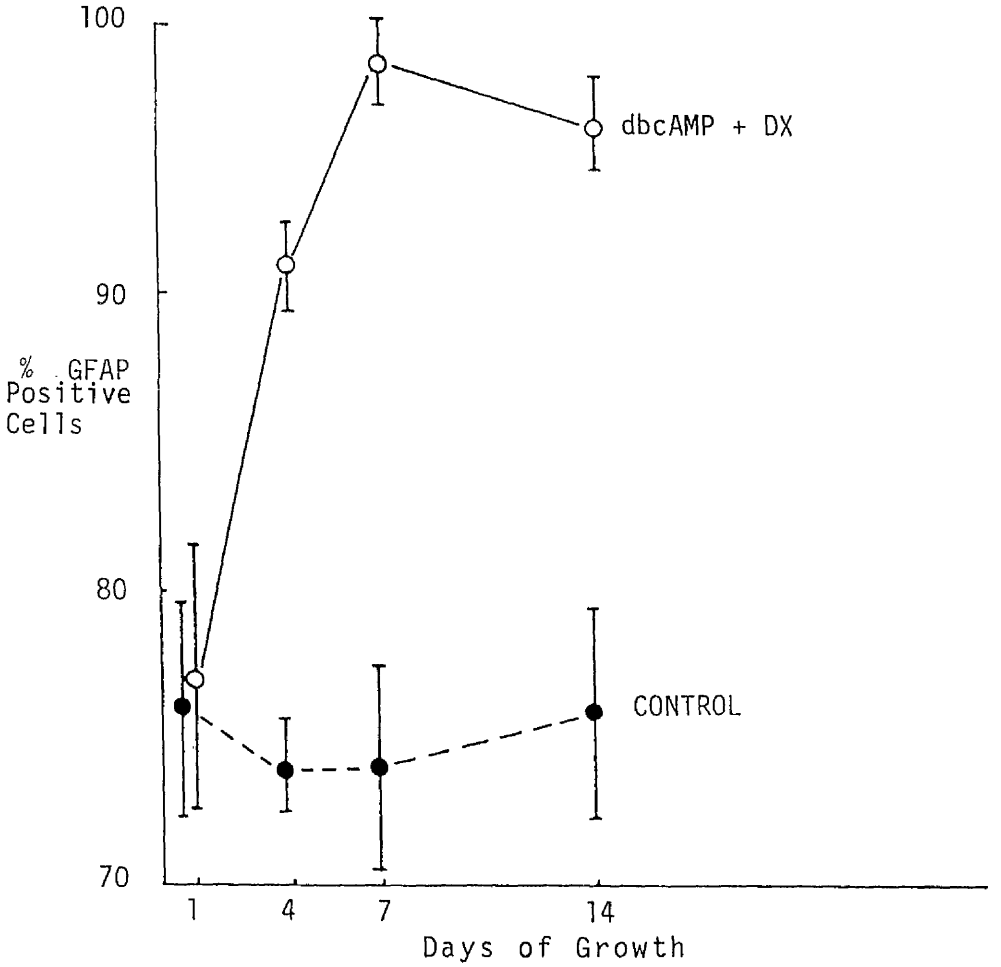


FIGURE 2b INDUCTION OF GFAP BY DIBUTRYRYL CYCLIC AMP AND DEXAMETHASONE IN C_6 CELLS



In 1974 Schon and Kelly reported the autoradiographic localisation of both [^{3}H]-glutamate and [^{3}H]-GABA in satellite glial cells surrounding the sensory neurone cell bodies in adult rat sensory ganglia, after *in vitro* incubation (120). More recently McLennan has shown, also by autoradiography, that glutamate is mainly accumulated into the glial elements in brain cortex (121), and Hösli and Hösli have confirmed that in rat cerebellum grown in tissue culture, [^{3}H]-GABA is taken up by glial cells (122) as well as by Purkinje cells and interneurones.

Glutamate Uptake Kinetics

Schousboe *et al* have studied the kinetics of uptake of glutamate in cultures of normal astrocytes obtained from dissociated mouse brain hemispheres (123). They reported the existence of a minor, unsaturable component together with an intense uptake which followed Michaelis-Menten kinetics. Glutamate transport into these cells is mainly via a Na^+ dependent, saturable, high affinity process which could be inhibited by asparate but not by GABA. A similar investigation by Logan and Snyder has shown that glutamic acid is taken into homogenates and slices of rat cerebral cortex by both a low affinity and a unique high affinity mechanism (124). This was demonstrated by a biphasic double-reciprocal kinetic plot which could be resolved into two distinct components (Km_{low} 1.5^{-4}M, Km_{High} $0.4x10^{-4}$M). Inhibition studies again showed that glutamic acid and aspartic acid, two chemically similar structures, share the same uptake system.

GABA Uptake

As with glutamate, GABA is taken up into cultured

astrocytes by an efficient, high affinity, sodium dependent, carrier-mediated mechanism (125). Other studies have confirmed the uptake of GABA by a high affinity process in glial cells separated from brain by a bulk gradient centrifugation technique (126). Similar high affinity uptake mechanisms for GABA have been reported in various glial tumour cells (rat glioma cells C_6 and C_2) maintained in tissue culture (127), and in glial cells in cultures of cerebellum tissue (128). As well as glial cells, GABA-inhibitory neurones in vivo can accumulate endogenous GABA and a useful way of distinguishing between these uptake systems is by use of β-alanine and, for example, amino-cyclohexane which differentially inhibit these processes (129). The glial uptake mechanism for GABA is preferentially inhibited by β-alanine which acts as a selective substrate for the high affinity GABA uptake sites in glial cells (130). Kinetic studies of [^{3}H] β-alanine uptake by brain slices have shown it to be taken up by a high affinity, Na^+ dependent mechanism with identical properties to the process described for the glial uptake of GABA (131).

The selective inhibition of glial high affinity GABA transport by β-alanine provides a potentially useful means of quantitating this high affinity process. β-Alanine sensitive GABA uptake is easily measured in monolayer cell cultures and was used as a means of detecting and quantitating another differentiated glial function.

3. Glutamine Synthetase

Glutamine synthetase (GS) catalyses the ATP-dependent formation of glutamine from glutamate and ammonia. The reaction catalysed by this enzyme provides a pathway for

the metabolism of glutamate while, at the same time, uses up free ammonia and produces glutamine. The latter two features of the reaction are favourable as ammonia, although a normal byproduct of brain metabolism, is toxic in high concentrations and glutamine is an important biochemical intermediate providing the amino groups for the biosynthesis of various important molecules. Glutamine is itself a building block for most proteins.

It is generally accepted that glutamate metabolism in the brain is compartmentalised (132). A large compartment has been assoicated with energy metabolism while a small compartment has been linked with glutamine synthesis, ammonia detoxification and metabolism of GABA. GS is an important enzyme of the small glutamate compartment and has been localised in the glial cells in rat brain by immunohistochemical techniques (133) while neurones, endothelial cells and choroid epithelium contain no GS. These observations provide more evidence for the role of glial cells in glutamate and ammonia metabolism in the brain. Further support comes from the effect of the GS reversible inhibitor methionine sulfoximine, which when administered to a number of animal species, causes convulsant activity (134, 135). This is thought to be due primarily to the effect on cerebral GS which results in increased ammonia levels and decreased metabolism of the neurotransmitter glutamate pool.

In embryonic neural retina GS can be induced by corticosteroids (136). Linser and Moscona have found that in the chick embryo retina, GS begins to rise sharply on the 16th day of development, after elevation of systemic corticosteroids. GS can be induced long before its normal rise in

the embryo, however, by injection of cortisol into the egg *in vivo* or by addition of the steroid to culture medium *in vitro* (136). This represents a developmental feature of retinal tissue and provides a useful model system for the study of neural cell differentiation and hormonal control of gene expression (137). The cellular localisation of the GS induction by cortisol was found by Linser and Moscona, to be confined to Müller fibers (retinoglia), in both retina *in vivo* and in organ cultures of retinal tissue (136).

The cellular localisation of GS in primary cultures from newborn mouse brain has been studied, using indirect immunofluorescence, by Hallermeyer *et al* (138). The enzyme was detected in about 40% of all cells including those of astrocytic morphology. Treatment with the glucocorticoid dexamethasone led to a marked increase in positively stained cells and the GS specific activity was considerably increased. A comparison of glial and neuronal cell lines, by the same authors, showed that although GS was present in both at a very low specific activity, it was induced to an elevated level in glial cell lines by dexamethasone, but not in neuronal cell lines (138). Studies on dexamethasone stimulation of GS have shown that in the C_6 rat glioma in culture there is a 7 - 10 fold increase in GS specific activity after treatment of the cells with 1μM dexamethasone for 96 hours (139). Both actinomycin D and cyclohexamide blocked this induction, showing that transcription and translation are necessary for the effect. The stimulation of GS by glucocorticoids is therefore a result of new enzyme synthesis rather than fluctuations in metabolic control of the enzyme.

Although it is widely reported that GS is present at

reasonable levels in only glial cells in neural tissue, the question of whether or not it is a reliable marker of differentiation is open to some doubt. Chinese hamster fibroblasts (CHO) in culture have been shown to have readily detectable levels of GS which are inducible by dexamethasone (140). Similarly V79 Chinese hamster lung cells (141) and HTC rat hepatoma cells (142) have GS activities which are also inducible by corticosteroids.These observations clearly demonstrate that many cells within the body other than glia, possess this enzyme with its capacity to be induced by glucocorticoids. However, with the exception of glia, it is not generally found in cells of neural tissue. This observation has led to its use as a marker of glial cell function, in cultures derived from brain tissue, with an appropriate degree of caution. In this investigation the GS specific activities of intracellular extracts prepared from a variety of normal, foetal and malignant cell lines, grown under varous conditions, were determined by biochemical means.

1.5 Markers of Malignancy

Two markers associated with malignant phenotype were used in this investigation.

1. Plasminogen Activator

Plasminogen activators (PA) are the group of proteases which catalyse the conversion of plasminogen into the active fibrinolytic enzyme plasmin, which effects the dissolution of blood clots. It is widely believed that PA plays a prominent role in malignant transformation and in the growth and spread of tumours *in vivo*. It has been implicated in the mechanism of invasion along with cell proliferation

and locomotion (143), and a possible mechanism for action of PA in enhancing the potential of a tumour cell to enter into the bloodstream, has also been postulated (144).

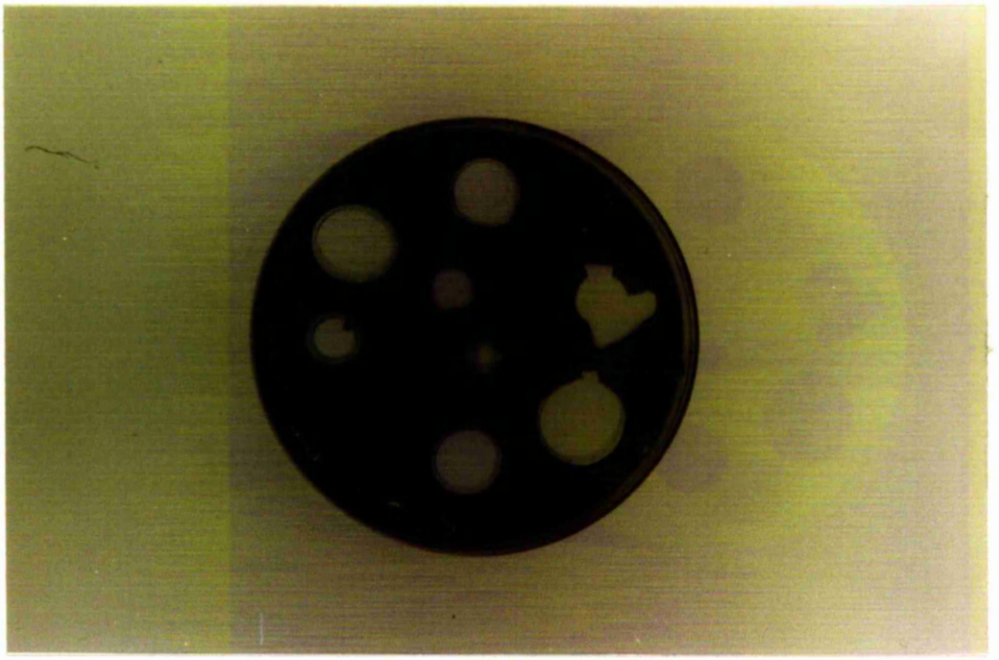
Cells transformed by oncogenic viruses (145) and cells treated with tumour promoters (146) both show increased cellular synthesis of PA. In a study by Corasanti et al of PA content in extracts of 23 pairs of surgically removed colon tumours and adjacent normal mucosa tissue, it was found that the tumour material had on average, more than four times the PA found in the corresponding normal tissue (147). They also noted that the enzyme content of a group of tumours showing invasive propagation or metastatic spread was higher than a group not showing these tendencies. Increased PA has also been correlated with expression of the malignant phenotype in a number of other experimental systems (148, 149, 150). As well as quantitative differences in PA between normal and neoplastic cells, qualitative differences in the molecular form of PA synthesised, have also been reported (151).

Differences in PA production by normal and neoplastic brain cells have also previously been investigated. In 1976 Tucker et al reported that lysates of brain tumour cell cultures contained fibrinolytic activity, while those from normal adult brain material did not (152). This could, in theory, be due either to increased PA production or decreased PA (or plasmin) inhibitor production. Natural inhibitors such as those found in serum (153) and the trypsin inhibitor found in brain (154) could play an important part in controlling the PA activity of brain cells. The net effect, however, is that glial tumour cells in vitro, are associated

with increased fibrinolytic activity. A similar study by Hince and Roscoe has demonstrated high levels of plasminogen-dependent fibrinolytic activity in cell lines derived from an ethylnitrosourea-induced rat glioma (155). Cell lines derived from normal adult rat brain showed only low levels of activity. In many of the cell lines derived from brain tissue after 111-112 days exposure to ethylnitrosourea, there was a close association of PA with growth in agar and tumourigenicity. These observations provide further support for the possible association of high PA activity, in some cases at least, with expression of the malignant phenotype.

These previous observations on PA activity in normal and neoplastic brain cells implied that PA might be a useful malignancy marker for the astrocytoma derived cell lines used in this study. A variety of assays exist for detecting cell PA, most of which involve the generation of plasmin from plasminogen and the subsequent monitoring of fibrinolytic activity. An example of these is shown in (Plate II). This shows the fibrinolysis of a preformed fibrin clot containing plasminogen, by a small amount of harvest medium containing no serum, from a variety of astrocytoma derived cell lines. Although this technique demonstrates the biological activity of PA in these medium samples, it does not take into account PA activity present at the cell membrane. Recently, chromogenic peptide substrates, which mimic the amino acid sequence at the scissile bond of natural substrates have been synthesised (156), and this has led to the development of a good quantitative assay system which allows measurement of total cell PA activity (157). In this investigation, a modification of the chromogenic assay developed by Whur (157)

PLATE II



Detection of Plasminogen Activator activity by fibrinolysis of a preformed fibrin clot.

was used to measure PA activity of the cells grown under various conditions. The PA activities were related to urokinase standards and expressed as units of urokinase activity.

2. Tumour Angiogenesis Factor

As the cells of solid tumours proliferate *in vivo* the tumour becomes vascularised to feed the growing cell mass. The growth of new capillary blood vessels, angiogenesis, is important in normal processes such as embryonic development, formation of the corpus luteum and wound healing, as well as being a component in some pathological processes, including neoplasia (158). Angiogenesis is a property of most solid tumours which, at a certain point in their growth, are capable of stimulating the growth of new capillaries from the vascular system of the host. This process is essential for the continued growth of a tumour (159).

Neovascularisation results in the formation of tumour blood vessels which do not usually mature and these represent "tubes" of undifferentiated endothelium which lack smooth muscle (160). The process of angiogenesis is thought to contribute considerably towards the kinetics of tumour growth. When a small tumour becomes vascularised initially, growth is exponential. However, in general, when the tumour reaches a certain size there is a gradual slowing in the rate of growth, resulting in a general pattern of diminishing growth rate with increasing size. Two possible explanations for this, both of which may be contributory, are the inability of endothelial cell proliferation to keep pace with tumour

cell proliferation (161), and the death of endothelial cells in the depth of the tumour due to vascular compression (162). Thus, angiogenesis initially releases a tumour from dormancy into exponential growth but later may be responsible for retardation of growth rate as the tumour reaches a larger size.

In 1970 Folkman reported that tumour cells release a diffusible factor (Tumour Angiogenesis Factor (TAF))which is mitogenic for capillary endothelial cells (163). TAF activity was found to be present both in the cytoplasm of Walker 256 carcinoma cells and in the hon-histone proteins associated with chromatin in the nucleus (164, 165). More recently Schor et al have isolated a low molecular weight compound which is capable of inducing neovascularisation in vivo (166) and Fenselau et al have also purified an angiogenic substance from the Walker 256 rat ascites tumour, which stimulates new blood vessel growth in vivo, as well as mitogenesis of foetal bovine aortic endothelial cells in vitro (167). This material has an apparent M.Wt. of 400-800 but its chemical nature is as yet undetermined.

There are a variety of biological assay systems available which demonstrate angiogenesis in vivo. These include the rat dorsal subcutaneous pouch, rabbit cornea and chick embryo chorioallantoic membrane (CAM), all of which show vasoproliferation in response to TAF containing material. The CAM is probably the most widely used bioassay for demonstration of angiogenic activity. Active TAF fractions implanted onto the CAM, exposed through a hole in the shell of a 9 day old embryo, result in the appearance of a radial system of blood vessels which grow in towards

the site of implantation.

In order to quantitate TAF more precisely, many groups of workers have cultured endothelial cells from various sources, and attempted to develop *in vitro* mitogenesis assays using these as target cells. Pure cultures of endothelial cells have now been successfully obtained from human umbilical vein (168), foetal bovine heart and aorta (169), bovine adrenal cortex and human foreskin (170) and rat brain (171, 172). Features specific to endothelial cells e.g. clotting factor VIII, Weibel-Palade bodies (rod-shaped organelles consisting of a bundle of fine tubules enveloped by a tightly fitting membrane, as seen by electron microscopy) (173), angiotensin converting enzyme and tight junctions (171), have all been useful in identification of endothelial cultures.

Stimulation of foetal bovine endothelial cells has been exhibited by crude cell extracts from Walker 256 tumours, while extracts from various adult normal tissues are ineffective (169). It was also observed that the tumour derived material was unable to stimulate mitogenesis in non-endothelial cells, under the same conditions. These results led Fenselau and Mello to conclude that this *in vitro* system might be a useful way of quantitating tumour-induced vascularisation. Schor *et al* have shown similar stimulation of mitogenesis of bovine brain derived endothelial cells by the low M.Wt., purified compound isolated from WRC-256 rat carcinoma, although they demonstrated a requirement, in this system, for growth of the endothelial cells on collagen.

In 1980 Folkman and Haudenschild reported that under appropriate culture conditions, capillary endothelial cells will form tubular network structures *in vitro*, which very

closely resemble capillary vascular beds *in vivo* (170). As growing colonies of human capillary cells reach a certain size, capillary tubes begin to form in the central, dense region of the colonies resulting in the eventual formation of a branched network of tubes which replaced the dense regions of colonies. This shows clearly, that the information necessary to construct capillary tubes, make branches and assemble an entire capillary network *in vitro*, can be expressed by a single cell type. Endothelial cells thus can be induced to build a complex three-dimensional structure which is a manifestation of their differentiated form. The successful culture of capillary endothelial cells might provide a suitable *in vitro* model for investigating the mechanism of angiogenesis and for identifying inducers and inhibitors of this process and their modes of action.

The endothelial proliferation and neovascularisation associated with CNS tumours is well established (174, 175, 176, 177). *In vitro* stimulation of primary human umbilical vein endothelial cells by conditioned medium from cultures of central nervous system tumours, has been demonstrated (178). Medium from a human astrocytoma tumour cell line and the rat C_6 glioma line increased the labelling index of the endothelial cultures from 2.1% to 30.1% and 96%. respectively, while medium from fibroblasts showed only a very slight increase. These results imply the production by brain tumours, of a specific chemical factor which increases mitotic activity of endothelial cells.

In this project the aims, with respect to using angiogenic activity as a marker of malignant phenotype in astrocytomas, were threefold:-

1. An attempt was made to demonstrate angiogenic activity in intracellular extracts of astrocytoma derived cells on the chick embryo CAM, and to quantitatively grade the responses.

2. An endothelial cell line was isolated and characterised from human umbilical vein for possible use in an endothelial mitogenesis assay.

3. An attempt was made to develop an in vitro assay system, using these endothelial cells, to quantitate the cellular levels of mitogenic factors in malignant astrocytes, foetal astrocytes and normal brain derived cells, and to determine whether or not endothelial mitogenesis activity relates directly to angiogenic activity on the CAM.

1.6 Manipulation of the Glial Phenotype

The three marker properties of differentiation (GFAP, high affinity GABA transport and glutamine synthetase) and the two properties associated with malignant behaviour (plasminogen activator and tumour angiogenesis factor) were used to investigate phenotypic expression and its manipulation by environmental and chemical factors.

Experimentally three approaches to phenotypic manipulation were considered:-

1. Cell Density

Monolayer Culture

Differentiation and malignancy marker assays were carried out at both the exponential and plateau phases of the growth curve. Differences between these could be due either to differences in the proliferative state of cells or cell-cell contact formation. These two possibilities can be distinguished by withdrawing serum from exponentially growing cultures, thus inducing cytostasis without contact formation. This allowed the effect of rapid proliferation, cytostasis and cell-cell contacts on glial phenotypic expression to be studied.

Three Dimensional Culture

Experiments were also carried out using a system of artificial capillaries which allowed cells to grow to very high, tissue-like densities. As shown in Plate III medium was continually perfused through the capillary bundles thus allowing electrolyte and nutrient diffusion to and from the cells, providing firstly a nearly constant micro-environment for cell growth and secondly a lattice on which cells can grow in three dimensions, so intensifying the effects of cellular interactions less apparent in monolayer culture. Two groups of workers have previously successfully used artificial capillaries. Knazek et al have shown that choriocarcinoma cells behave differently with respect to chorionic gonadotrophin production when grown to high densities on perfused capillaries (179). Similarly Rutzky et al have grown human colon adenocarcinoma in high density culture and found that the kinetics of CEA release were

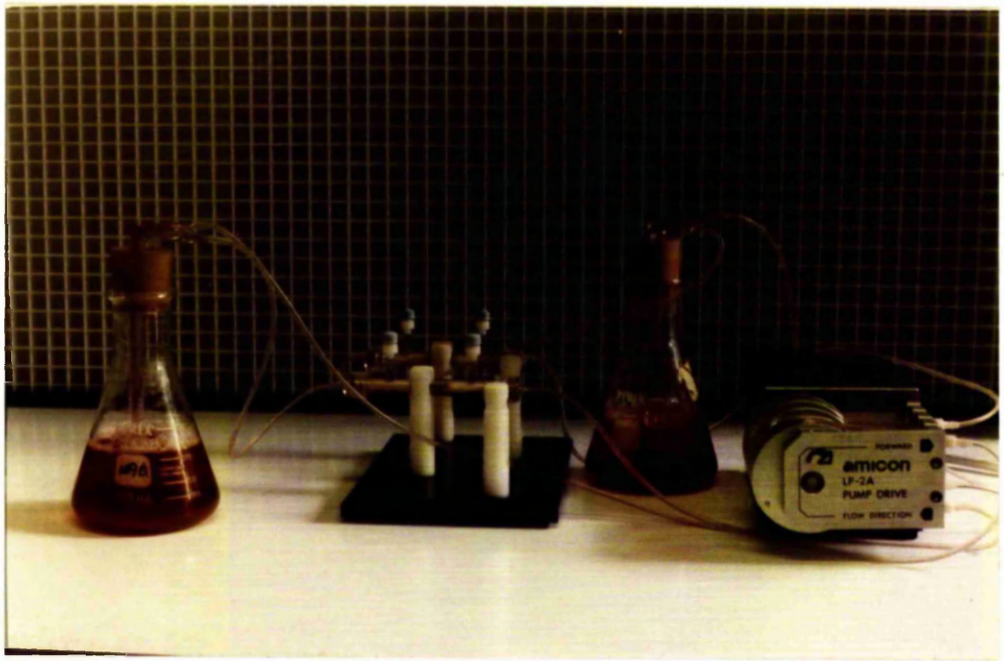
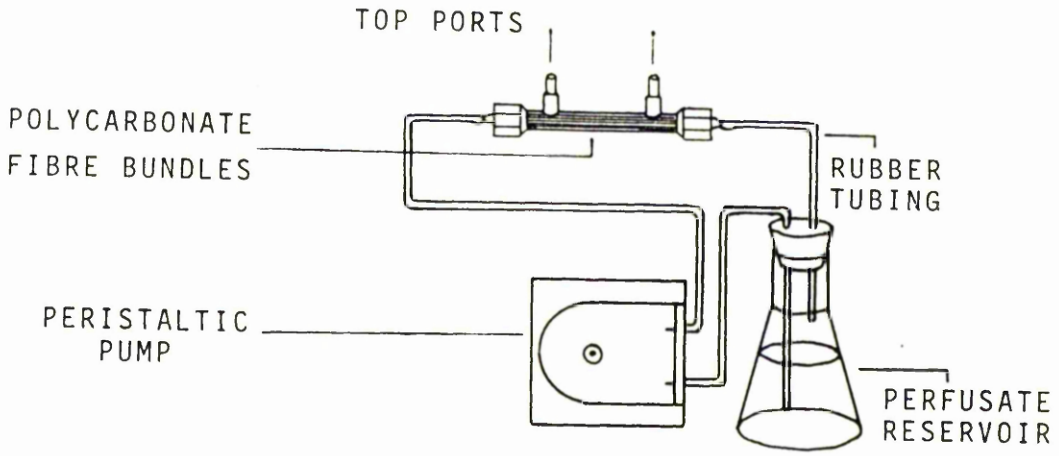


PLATE III Vitafiber Culture Units

DIAGRAMATIC REPRESENTATION OF VITAFIBER CIRCUIT



quite different from that seen in monolayer culture (180). Differences in cell behaviour could be due to organoid type growth, more physiological three dimensional contacts and possibly also the concentration of cell products (macromolecules) which control cell behaviour in the extracapillary compartment. Clearly a three dimensional, artificial capillary perfusion system might provide a better *in vitro* model, more accurately reflecting the true physiological behaviour of cells. Certain aspects of glial phenotypic expression were therefore investigated using cells grown in such a system, to determine in what way high cell density and three dimensional contact formation affects the phenotype.

2. Chemical Agents

The effect of cell treatment with various chemical agents, either alone or in combination, on phenotypic expression was also investigated. Low serum concentration (1%) was used in order to minimise serum factor effects. The compounds used were dexamethasone, isoproterenol, retinoid, interferon, mitomycin C, N-methylacetamide, pig brain extract, TPA and N-methylnitrosourea. The reasons for interest in their possible manipulatory effects on cell phenotype are discussed below.

Dexamethasone

Glucocorticoids are used as anti-neoplastic agents in the treatment of many types of malignant disease including leukaemia and breast carcinoma. Their effect on haemopoietic tumours is reported to be cytotoxic (181), while the effect on solid tumours is thought to be cytostatic (182,183).

The synthetic corticosteroid dexamethasone (DX) is frequently used in the treatment of patients with brain tumours. The primary effect of this drug is in relieving intracranial pressure by reducing oedema in the normal brain tissue surrounding the tumour (184). A secondary effect is thought to be a result of its growth inhibiting properties. The inhibitory effect of DX on glioma cells in culture is complex (185). It apparently stimulates cloning efficiency and cell proliferation in cloning experiments, with the cytostatic effect not evident until a certain colony size is reached. Treatment of monolayer cultures with DX, however, results in a lower terminal cell density (Figure 3) and reduced labelling index, than is achieved in its absence (185). DX has also been shown to inhibit growth of both human glioblastomas (186) and Syrian hamster melanoma (187) cells in culture. In the latter case i.e.RPMI 3460 melanoma cells, the presence of a DX-binding macromolecule has been located in the cytosol of these cells and this observation is consistent with the view that growth inhibition by DX is a receptor mediated event.

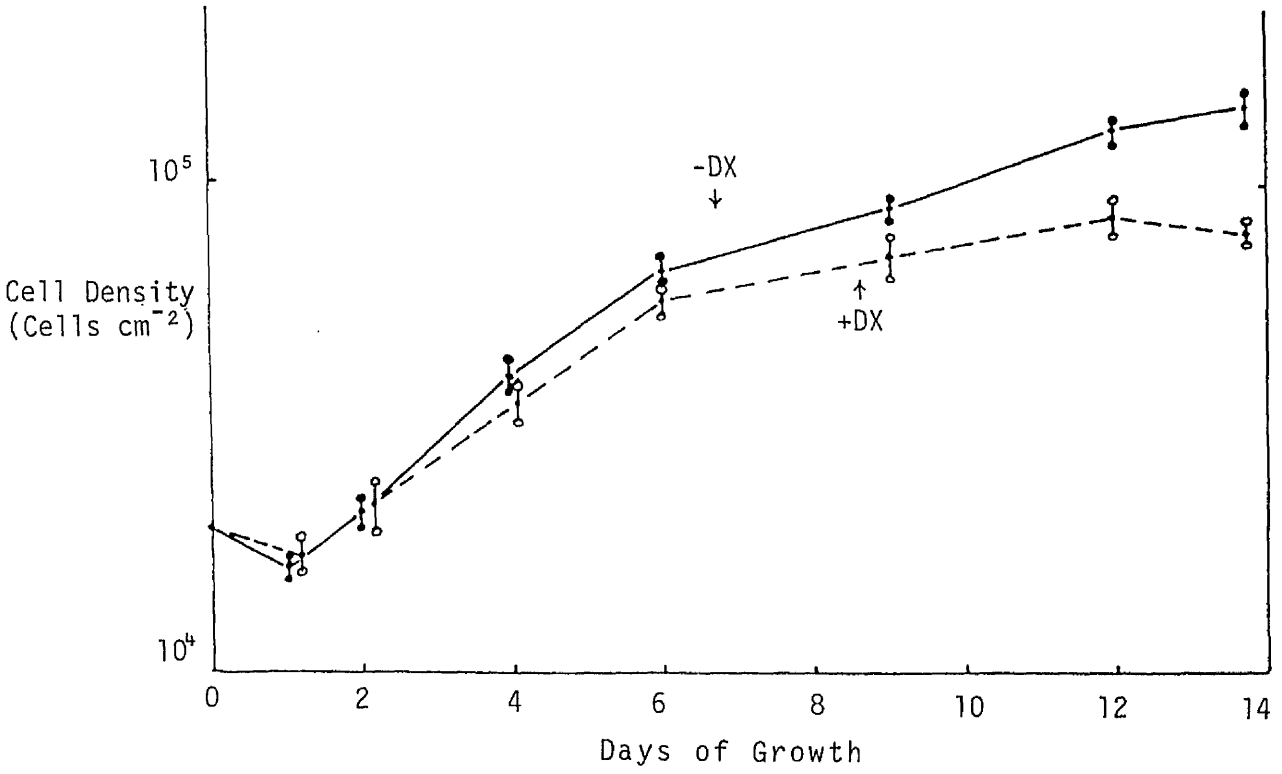
One of the well established biological effects of glucocorticoids is the induction of differentiation in many cell types including hepatoma (188), liver parenchyma (189), cerebral cell cultures (190) and the rat C_6 glioma (191). More specifically, DX induces glutamine synthetase production in Müller glial cells in neural retina (136) and in combination with cyclicAMP, it has been found to induce both high affinity GABA uptake in a number of human glioma lines and GFAP production in C_6 cultures (192).

FIGURE 3

Semi-logarithmic plot for NOR-F cells grown in 24-well plates in the presence O--O and absence ●--● of dexamethasone ($10 \mu g\ ml^{-1}$). Culture medium was renewed every 2 to 3 days. Each point represents the mean of duplicate cell counts. The duplicate values are shown.

FIGURE 3

EFFECT OF DEXAMETHASONE ON CELL GROWTH : NOR-F



DX has also been shown to be an effective inhibitor of plasminogen activator production by human embryonic lung cells (193) and rat hepatoma cells (194) in culture.

It is therefore clear from consideration of the biological effects of DX that it has profound effects on cell phenotype, including aspects of the phenotype associated with growth control at high density, differentiation and possibly aspects associated with malignant behaviour. The possibility therefore exists that steroids exert their cytostatic effect by promoting cells to differentiate and possibly "switching off" malignant phenotype.

Isoproterenol

Isoproterenol (IP) is a β-adrenergic agonist which acts through cell surface receptors and adenyl cyclase, resulting in increased intracellular cyclicAMP levels (195). The presence of a cyclicAMP-dependent protein kinase has been reported in glial cells (196) and phosphorylation of a 100,000 dalton protein in C_6 appears to be related to nor-adrenaline stimulation. Increased cyclicAMP levels has important biochemical consequences including increased synthesis of glial specific factors.

In studies using C_6 glioma, IP and dibutryl cyclic-AMP (dbcAMP) produced a change in cell morphology resulting in the appearnace of long, thin,branched processes after 24-48 hours exposure (116). Both IP and dbcAMP were equally effective in their ability to induce GFAP in C_6 cultures, while neither agent had any significant effect on terminal cell density. In this investigation IP was used to examine the reponse of cell phenotypic expression to raised intracellular cyclicAMP levels for two reasons.

Firstly,use of IP excludes any effects which might be caused by the butyric acid moiety of the dbcAMP and secondly, IP would be more feasible for practical, in vivo use than dbcAMP.

Most of the literature reports on induction of morphological and chemical differentiation in glial cells, however, have been on observations made using dbcAMP. In particular, mouse glioblastoma (197), human glioma cells in culture (198) and rat astrocyte precursor cells (199), all show morphological change on dbcAMP treatment, to a morphology characteristic of differentiated astrocytes in vivo. Biochemical differentiation of astroblasts also occurs on dbcAMP treatment e.g. LDH activity and isoenzyme distribution changes to resemble that of more mature cells (200). In C_6, induction of morphological differentiation is accompanied by increased content of the nervous system specific protein S100 and changes in the con A binding pattern, to one which is more characteristic of normal cells (201). These observations, together with the fact that cyclicAMP is present in adult rat brain at a much higher level than it is in foetal brain (202), suggest that cyclicAMP is probably an important mediator or "second messenger" in many aspects of the control of specific glial gene expression and function. IP, as a manipulator of intracellular cyclicAMP levels, might therefore be a useful agent in studying induction of differentiation in cell lines of glial origin.

Retinoids

Vitamin A and its derivatives, the retinoids,are essential to the growth, differentiation, and function of secretory epithelia. Their exact mode of action is unclear but it is thought to be via interaction with specific receptors.

In vivo experiments have shown the effective action of retinoids in reducing the development of squamous metaplasia, hyperplasia and carcinomas in rodents treated with carcinogenic cyclic hydrocarbons (203). Similar inhibition of tumour development in other tissues has also been observed (204, 205, 206), as well as therapeutic effects on established tumours. Lasnitzki has shown that *in vitro* carcinogen induced neoplasia in organ cultures of mouse prostate can also be inhibited or reversed by retinoids (207).

The prevention of hyperplasia caused by retinoids in these systems and the reduction of the growth and incidence of tumours in animals has led to speculation about the mode of action. Todaro *et al* have reported that retinoids inhibit the mitogenic action of a growth factor produced in cells transformed by mouse sarcoma virus (208), probably by blocking the interaction of the growth factor with its membrane receptor.

In tissue culture, retinoic acid has been found to inhibit growth and induce morphological differentiation in a human neuroblastoma cell line (201) . It also inhibited the ability of these cells to form colonies in soft agar and these observations are consistent with the view of Sidell (209) that retinoic acid is promoting differentiation in LA-N-I neuroblastoma cells and altering expression of the malignant phenotype. The direct growth inhibitory effects of retinoids have been demonstrated on a number of other human cell types in culture including breast, cervical, lymphoblastic and melanoma cells (210) and their action in inhibiting growth in suspension and

stimulating melanogenesis in melanoma cells in vitro (211, 212, 213), lends further support to the concept that retinoids might act by promoting differentiation as well as inhibiting the growth of cells.

In a limited number of cases, however, retinoids have shown effects that mimic these of tumour promoting phorbol esters (214, 215). In particular retinoic acid has been reported to induce plasminogen activator synthesis by cells of mesenchymal origin but not by cells of epithelial origin (216). Fibroblasts were used to show synergism between the effects of retinoids, TPA and Rous Sarcoma Virus in induction of PA synthesis (217). As a result of these observations it has been suggested that retinoids, while preventing tumours of epithelial origin, might function as promoters in mesodermal tissue.

Glial tumours, like melanoma and neuroblastoma, which have a favourable response to retinoids, are tumours of the neural crest. Retinoids might therefore prove to be useful in manipulating the differentiated and malignant phenotypes of glial cells in culture.

Interferon

In addition to inhibiting viral multiplication interferon (IFN) exerts a number of other effects on cells including inhibition of multiplication (218), enhancement of specialised functions and modification of cell surface (219). IFN has also been shown to exhibit a marked anti-tumour action and to influence the immune system (219). In experiments using mouse leukaemia L1210 cells, IFN was found to increase the intracellular concentration of cyclicGMP initially with a later increase in cyclicAMP (220). The transitory rapid increase in cyclicGMP in IFN

treated cells, has been postulated as the mediatory event for many of the diverse effects of IFN on cells, seen within a few hours of treatment e.g. inhibition of cell multiplication. The later rise in cyclicAMP, possibly a consequence of inhibition of growth (220), might then be associated with the longer term effects of IFN, such as switching on and off specific cell products. An example of such an effect is the inhibition of plasminogen activator release by SV40 transformed 3T3 cells, after 24 hour treatment with IFN.

IFN-β (provided by Beechams Pharmaceuticals) was used to study its long term effect on the phenotypic expression of both normal and malignant cells. Preliminary results have shown IFN-β to cause dose dependent growth inhibition of cells derived from malignant gliomas and normal brain, with the glioma cells more sensitive to its action. At concentrations used in the phenotypic manipulation experiments IFN-β was found to be cytostatic.

Mitomycin C

Mitomycin C is an antibiotic which acts as an alkylating agent of both DNA and RNA. It has demonstrable anti-tumour activity in a wide variety of animal tumours and is used often,in combination with other agents, in treatment of malignant disease.

Mitomycin C has been found to induce morphological differentiation in human neuroblastoma cells (NB-1) *in vitro* as seen by the extension of dendrite-like processes (221). A number of other agents including dbcAMP, N-butyric acid alone, cholera toxin, serum free medium and ascites of patients treated with interferon inducers, had similar

effects. At appropriate concentrations (as used in manipulation experiments) mitomycin C completely inhibits cell division of both normal and malignant glial cells, but allows the cells to survive and metabolise for several days after treatment. This enables its effect on the expression of differentiated and malignant phenotype to be studied.

N-Methylacetamide

Murine-virus-infected erythroleukaemia cells can be induced to differentiate along the erythroid maturation pathway by treatment with dimethylsulfoxide (DMSO). A number of other highly polar compounds, including N-methylacetamide (NMA) have similar effects in inducing erythroid differentiation of the virus-infected cells, as determined by the appearance of haemoglobin (222). NMA is generally less toxic to cells than DMSO. These agents are thought to act by changing the conformation of DNA or DNA-protein complexes, initiating transcription of the gene or genes that regulate expression of the erythroid cell programme (222).

The possible effect of DMSO and related compounds on the regulator gene controlling the expression of differentiated function in erythroleukaemia cells, makes these compounds of considerable interest in the possible induction of differentiation in other cell types. Another polar compound dimethylacetamide, which is effective in differentiating erythroleukaemia cells, has been found to cause nearly complete induction of morphological differentiation of a malignant mouse murine embryonal carcinoma into neuroepithelial and glandular derivatives,

when used in combination with retinoic acid (223). Differentiation was associated with decreased tumour growth rate, decreased mitotic index and increased surivival time of the hosts.

In the glial phenotypic manipulation experiments the polar compound selected for use was NMA, due mainly to the extreme toxicity of DMSO to cultured glial cells.

Pig Brain Extract

A crude, pig brain extract preparation (PBE) was used in the attempts to promote glial differentiation in normal and malignant cells in culture, as a result of the observations of Lim et al (199) on the effect of a protein factor present in brain extracts in promoting differentiation of dissociated foetal brain cells. This protein, Glia Maturation Factor (GMF), is capable of the reversible promotion of morphological and chemical maturation of glioblasts. The addition of GMF to a monolayer of glioblasts results in the formation of multipolar, branched processes giving an overall appearance of a network of cell processes. The change in cell shape, which is due to active extension of processes, rather than the cytoplasmic retractions seen with dbcAMP treatment, is correlated with a restructuring of cell components, as revealed by electron microscopy (224). Exposure of glioblasts to GMF for seven days leads to the disappearance of microfilaments (5nm) and an increased number of glial filaments (10nm). A ten fold increase in the neural tissue specific protein S100 occurs on treatment with GMF (225) as well as a concomitant increase in intracellular cyclicAMP level (225).

The reversibility of the GMF effect and the fact that

it is present in much greater amounts in adult brain than in embryonic brain (226), suggests that this factor has a role in maintaining glial differentiation in the brain. GMF is thought to be a protein (227) with an apparent molecular weight of 350,000 which is composed of smaller subunits. Although it is effective in stimulating cell division of cultured glioblasts (228) (as well as inducing differentiation), it appears to be distinct in physico-chemical properties from other growth factors reported. Lim et al (199) have postulated that GMF is not a humoral factor, by its absence from conditioned medium, but probably binds to cell surface membranes, occupying receptor sites which would be occupied in vivo. If glial cells do not produce and release GMF, it seems plausible that neurones might contain high levels of the factor which can promote and maintain the differentiation of glial cells in the brain. This would also explain the onset of gliosis following neuronal injury.

The PBE prepared and used in this project to manipulate glial phenotype was active in inducing apparent morphological differentiation of normal brain derived cells. The assumption was therefore made that it contains the GMF described above.

Tumour Promoter

The concept of two stage carcinogenesis i.e. initiation and promotion of tumours, arose from the observation that the application of two agents was often required to produce a tumour. Skin tumours in mice have been produced by a single subcarcinogenic dose of an initiator followed by repeated applications of a promoter, croton oil (229).

The tumour promoting compounds in croton oil have been identified as the phorbol esters (230). These molecules have both highly hydrophilic and lipophilic regions, enhancing their action as potent tumour promoters. Many model systems have now been studied *in vivo* (231), and the conclusions show that the concept of two stage carcinogenesis, as described in mouse skin, is not limited to that species and tissue. The number and diversity of experimental models which are subject to promotion suggest that environmentally induced human cancer might also involve similar stages in its development.

Most *in vitro* studies with tumour promoters have used 12-O-tetradecanoylphorbol-13-acetate (TPA), the most potent promoter in the phorbol diester series. Many attempts to demonstrate two stage transformation in culture systems have been reported and the effects of TPA on cell morphology and biochemistry have also been extensively studied (231). TPA induces considerable morphological changes in mouse 3T3 and WI-38 human fibroblasts (232). Growing cultures treated with TPA reach a higher density than control cultures (232, 233) and Diamond *et al* have suggested that this TPA induced increase in final cell density might be due to delayed onset of density dependent growth inhibition (232). In chick embryo fibroblasts, infected with Rous Sarcoma Virus, TPA accentuates the morphological alterations induced by viral transformation (234).

TPA has many and varied effects on the membranes of intact cells in culture (231). It affects the transport of substances, including glucose, into and out of cells, (Na^+,K^+)-ATPase, phospholipid acylation and has an effect

on hormone receptors on the cell surface. It has been postulated that tumour promoters may exert their effect by using the receptors for endogenous hormones e.g. TPA has been shown to inhibit the binding of epidermal growth factor to HeLa cells (235). However, decreased affinity of membrane receptors for their ligands is not a general property of TPA treated cells.

The effects of TPA on specific proteins include the induction of ornithine decarboxylase in mouse epidermal cells (236), decrease in fibronectin on the cell surface of chick embryo fibroblasts (237) and induction of plasminogen activator in a number of cell types including chick embryo fibroblasts, HeLa and HTC rat hepatoma cells (238). This induction requires de novo RNA and protein synthesis. Quigley has reported that PA itself is the protease responsible for the "supertransformed" morphology of TPA-treated, RSV infected chick embryo fibroblasts (239), by catalytically acting on an unknown cellular protein substrate. Elevated cyclicAMP levels inhibit enzyme induction by TPA (234).

Much of the recent interest in tumour promoters has come from consideration of the possibility that these compounds inhibit terminal differentiation of cells in culture. A positive correlation has been shown between the tumour promoting activity of a phorbol ester in mouse skin and the ability of the compound to inhibit spontaneous differentiation of Friend murine erythroleukaemia cell clones, which normally differentiate in the absence of inducing drugs (240). Similarly in mouse preadipose cells (241), mouse C1300 neuroblastoma (242), and primary cultures of

new born mouse epidermis (243) there is evidence for inhibition of differentiation by TPA. Rovera et al (240) and Diamond et al (244) have proposed that the inhibition of differentiation in self renewing tissues, such as skin, favours proliferation of a stem cell population in which the potential for malignancy may have been initiated by a carcinogen.

Whatever the exact mechanism of action of tumour promoters it is clearly a result of altered gene expression. In this study, the effect of tumour promoter on the expression of the differentiated and malignancy associated glial phenotypeswas investigated in both normal and neoplastic cells to determine what, if any, aspects of the phenotype were altered by treatment with tumour promoters.

Nitrosourea

Magee and Barnes, in 1956, reported the induction of neural tumours by the carcinogenic action of nitroso-compounds (245). Nitrosoamides e.g. N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea are strongly neurotropic, producing tumours selectively in nervous tissue after systemic administration. The neoplasms arising as a result of nitrosoamide induction are morphologically and biologically similar to naturally occurring tumours in man and animals The most common have been histologically identified as gliomas of the CNS and schwannomas of the cranial and peripheral nerves (246). A possible mechanism for the neurospecificity of these chemical carcinogens has been suggested by various groups of workers. Nitrosoureas result in the alkylation of DNA bases and Goth and Rajewsky have demonstrated considerable differences between the

"repair" rates of different tissues e.g. liver repairs O^6-ethylguanine much more rapidly than brain (247). Delayed repair of O^6-alkylguanine appears to be a major factor in sensitising the nervous system to carcinogenesis, at least in the rat. Nitrosoureas are also used in the clinical treatment of brain tumours as they constitute lipid soluble alkylating agents and can therefore be actively transported into the brain.

The susceptibility of the brain, and in particular glial cells to nitrosourea induced carcinogenesis, prompted inclusion of MNU in the glial phenotypic manipulation experiments. Very little evidence exists for any effect of nitrosoureas in altering cell phenotype. However, the influence of MNU on malignant transformation of mouse embryo fibroblasts has been reported (249) and there is evidence that *in vitro* treatment of rat liver cells with MNU results in some biochemical and antigenic changes in these cells (249).

In addition to the effect of these agents alone on the expression of differentiation and malignancy in glial cells, certain combinations of these agents e.g. DX and IP, TPA and MNU were examined for possible synergism.

3. Heterologous Cell Interactions

Finally, an attempt was made to look at the phenotypic expression of a normal brain derived and a glioma cell line which have been co-cultured with neuroblastoma cells. This type of co-culture recreates a mixture of both homotypic and heterotypic cell interactions, as found *in vivo*, and might be important for expression of normal differentiated cell function. Evidence for the close morphological relationship between neurones and glia has come from the observations

of Allin who reported that co-culture of rat glioma and mouse neuroblastoma cells results in reciprocal induction of differentiation of both neurones and glia, despite the species difference of the two cell lines (250).

Since neurones do not grow in culture, the human neuroblastoma cell lines TRK 14 and IMR-32 were used in the co-culture studies. After two weeks of co-culture the cells were trypsinised and separated by Percoll density gradient centrifugation, for determination of marker properties associated with differentiation and malignancy in the glial cells. Experiments were also carried out in which neuroblastoma and glial cells were cultured on separate coverslips in the same vessel, thus sharing the same medium. This allows the effect on cell phenotype of any diffusible factors produced by the neuroblastoma cells, to be distinguished from the effect of heterotypic cell-cell interactions.

MATERIALS AND METHODS

2.1 : Tissue Culture Methods

2.2. : Biochemical Properties

2.3 : Drug Preparations

2.1 Tissue Culture Methods

1 The Culture of Brain Tissue

(a) Biopsy Material Biopsies of tumour material were aseptically collected into holding medium[1] for transportation. The tissue was washed in dissection balance salt solution[2] (DBSS), finely chopped and the pieces allowed to settle before being transferred to a plastic culture flask (Falcon). Collagenase, at a final concentration of 200 units ml^{-1} in growth medium[3] was added for 24 hours at 37°C. The tissue fragments were centrifuged at 150xg for 10 minutes to remove collagenase and the pellet resuspended in fresh growth medium[3]. The suspension was incubated at 37°C in a culture flask and the progress of the primary culture monitored by phase-contrast microscopy. After a period of between 24 and 72 hours a monolayer primary culture was generally obtained.

(b) Normal Adult Post-Mortem Material Pieces of tissue were dissected from various regions of fresh post-mortem brain and transported in holding medium[1]. Monolayer cultures were obtained by fine dissection followed by collagenase digestion (200 units ml^{-1}) for 48-72 hours at 37°C. After removal of the tissue fragments from collagenase , a period of 3-4 weeks elapsed before proliferating primary cultures were obtained.

(c) Foetal Material Pieces of foetal brain were aseptically removed and transported in holding medium[1].The foetal material was handled in an identical manner to that described for the biopsies. Monolayer primary cultures were obtained between 24 and 72 hours after removal from collagenase.

2. Cell Maintenance

Monolayer cultures were fed with fresh medium[3] every 4 to 5 days and routinely passaged by washing in PBS/EDTA[4] followed by trypsinisation for 10 to 15 minutes at 37°C with 0.25% trypsin. The trypsinised cells were resuspended in growth medium[3] diluted and inoculated into new flasks or experimental culture dishes. Experimental cells in 24-well plates (Linbro) or microtitration plates (Linbro) were incubated at 37°C in a humidified, incubator with 5% CO_2.

3. Counting and Viability

Single cell suspensions were counted using a Coulter counter model ZB1 (Coulter Electroncis Ltd.) or a haemocytometer counting chamber. Cell viability was determined by exclusion of 0.1% trypan blue dye.

4. Cell Freezing

Frozen stocks of all cell lines were maintained in liquid nitrogen. Cell suspensions of greater than 1 x 10^6 cells ml^{-1} in culture medium[3] containing 10% DMSO were frozen with an approximate cooling rate of 1°C $minute^{-1}$.

5. Staining

Monolayer cell preparations were washed with PBS, fixed in methanol for 10 minutes and stained with 10% Giemsa in distilled water. Excess stain was removed with tap water and the stained preparations air dried. Morphological observations were generally made by light microscopy of giemsa stained cells or phase-contrast microscopy of living cultures.

6. Mycoplasma staining

Cultures fixed with 25% acetic acid in methanol, were

checked for mycoplasma by incubating with the fluorescent DNA-stain Hoechst 33258 at 0.05µgml^{-1} for 15 minutes at room temperature. The detection of large amounts of extra-nuclear DNA by fluorescence microscopy was indicative of the presence of mycoplasma infection.

7. Co-culture Experiments

Equal numbers of both cell types in co-culture experiments i.e. the normal cell line NOR-F or the malignant line G-UVW and the neuroblastoma lines TRK-14 or IMR-32 were seeded into tissue culture flasks or on to 15mm coverslips. After a few days the co-cultured cells were either fixed in methanol and stained or separated by Percoll density gradients for independent biochemical investigation.

8. Cell Separation

Linear iso-osmotic Percoll gradients in F10 medium without serum (pH7.4) were formed in plastic universals (Sterilin) spanning the density range of 1.03 to 1.08 gm ml^{-1}. Solutions with densities of 1.08 and 1.03 gm ml^{-1} were mixed in a dual chamber gradient maker. Cells from 7 day co-cultures were trypsinised and resuspended in culture medium[3], spun at 400 x g for 5 minutes and resuspended in 0.5ml medium containing no serum. Suspensions were layered on to the top of the Percoll gradients and spun at 400 x g for 15 minutes. After centrifugation two distinct bands of cells were observed. 0.5ml fractions were carefully collected from the bottom of the gradients and placed in separate wells of 24-well plates with 1.5ml of culture medium[3]. The recovered cells were incubated at 37°C and 5% CO_2 for a few days, and were examined microscopically. Morphological identification of the two cell types showed that the neuroblastoma sedimented to a lower point in the density gradient and thus had a higher buoyant density

than the glial cells. After recovery from Percoll gradients both TRK-14 and IMR-32 neuroblastoma cell lines only loosely attached to the plastic substrate and did not proliferate. G-UVW and NOR-F were unaffected by Percoll.

From identical gradients NOR-F and G-UVW were recovered from co-cultures, seeded into 24 well plates and assayed for βalanine sensitive GABA uptake and plasminogen activator activity. Percoll had no effect on the cellular expression of these properties.

2.2 Biochemical Properties

1. GFAP

Fixation

For investigation of GFAP by immunoperoxidase or immunofluorescence cells were seeded on to 15mm diameter plastic coverslips (Thermanox) in 24-well plates, at a concentration of around 5×10^4 cells cm^{-2}. After 2 days the cells were washed with PBS and fixed either in acetone at 2°C for 20 seconds or methanol for 10 minutes at room temperature. The coverslips were mounted on to glass microscope slides with DPX mountant and left to dry overnight.

Immunoperoxidase

The source of the GFAP antigen used to raise the antiserum employed here was GFAP extracted from a fibrillary astrocytoma rich in glial filaments. The antiserum was raised in a rabbit and its specificity has already been reported (251). The control serum was normal rabbit serum. The fixed cell preparations were incubated for 30 minutes with a 1:300 dilution of anti-GFAP antiserum in tris/saline[5], previously absorbed with 100 $mgml^{-1}$ pig liver extract for 2 hours at 37°C to reduce non-specific background staining. Unbound anti-GFAP was

removed with several tris/saline[5] washes and the cells incubated with a 1:20 dilution of horse raddish peroxidase - conjugated swine anti-rabbit serum (IgG) for 30 minutes. Unbound swine anti-rabbit serum was removed with several tris/saline[5] washes and the cells were immersed in freshly prepared diaminobenzidine solution[6] (DAB) containining H_2O_2 for 5 minutes. Excess DAB was removed with tap water and the slides air dried. The presence of cellular GFAP was detected by an insoluble brown polymer occurring as a result of the electron transfer from DAB to H_2O_2 via the peroxidase enzyme.

Immunofluorescence

For flurorescence detection of GFAP the second antibody was a 1:10 diluted rhodamine-conjugated swine anti-rabbit serum. In this case GFAP was detected by fluorescence microscopy. Immunofluorescence has the advantage of not requiring DAB, believed to be a potent carcinogen, but has the disadvantage of the relative instability of the rhodamine label.

I^{125} Quantitation of GFAP

In order to attempt to quantitate GFAP, not possible by immunoperoxidase or fluorescence, the GFAP assay was modified and a radiolabelled goat anti-rabbit IgG employed as the second antibody. To investigate the effect of drugs on GFAP expression, cells were grown to confluence in microtitration plates, washed with PBS and fixed with methanol for 10 minutes. The fixed cells were incubated with a 1:100 dilution of the rabbit anti-GFAP antiserum for 1 hour, washed several times with PBS and incubated with the I^{125} labelled goat anti-rabbit IgG for 1 hour. After removal of unbound label with several PBS washes, the cells were dissolved in 1% SDS in 0.3 N NaOH

and the amount of I^{125} bound to the cells determined by gamma counting. The iodine counts were corrected for non-specific binding and cell number. Replicates of test microtitration wells were assayed.

2. Amino Acid Uptake

Cells for the kinetic analysis of glutamic acid and γ-amino butyric acid (GABA) uptake were grown in 24-well plates. After three PBS washes the cells were incubated in amino acid free medium[7] (AAFM) for 2 hours prior to assay to avoid interference from other amino acids. The velocity of uptake of various concentrations of GABA was determined in the following way. Duplicate wells were incubated for 40 minutes at 37°C (during which time the uptake was linear) with 0.5ml of a range of GABA concentrations from 25μM to 1mM in AAFM[7] containing a known amount of [^{3}H]-GABA (5μCi ml^{-1}). After the incubation, free GABA was removed by several PBS washes and the intracellular GABA extracted in the acid soluble cell fraction with 1ml of ice cold 10% TCA. The TCA extract was dissolved in 5ml instagel scintillation fluid and the [^{3}H] content determined by liquid scintillation counting. The velocity of uptake was calibrated in the following way:-

$$V_i = \text{cpm} \times \frac{100}{\%\ \text{eff.}} \times \frac{10^6}{\text{cell number}} \times 10^{-3} \times \text{Dil.Fac.}$$

$$\text{time} \times \text{Sp.Act.} \times 2.2 \times 10^{12}$$

V_i = initial velocity of uptake in moles $min^{-1} 10^6$ $cells^{-1}$

cpm = counts per minute

% eff = efficiency of scintillation counting

time (mins) = incubation time in minutes

cell number = total cell number per well

Sp.Act. = specific activity of [^{3}H]-GABA in Ci $mmole^{-1}$

2.2×10^{12} = number of disintegrations per minute per Ci

Dil.Fac. = $\frac{\text{[total GABA]}}{\text{[}^3\text{H-GABA]}}$

Lineweaver-Burk double reciprocal plots of $\frac{1}{V_T}$ against $\frac{1}{[GABA]}$ were constructed for various cell lines.

Glutamic acid uptake kinetics were carried out in the same way with the exception that the time of incubation with various concentrations of glutamic acid containing [^{3}H] glutamic acid was only 20 minutes.

To determine the effect of competing amino acids on the uptake kinetics of GABA and glutamic acid, the analyses were carried out using a range of amino acid concentrations with and without 2mM βalanine and aspartic acid respectively.

βAlanine Sensitive GABA Uptake

Inhibition of the uptake of 25μM GABA by 2mM βalanine was used as a measure of operation of the high affinity GABA uptake system. Duplicate wells of 24-well plates containing post-confluent cells were incubated for 40 minutes at 37°C with 0.5ml of 25μM GABA containing 5μCi ml^{-1} [^{3}H]-GABA in AAFM[7], with and without 2mM βalanine. After three PBS washes the [^{3}H]-GABA was extracted in 10% cold TCA and counted. Duplicate values for the uptake velocities in the presence of βalanine were substracted from the mean uptake velocity in the absence of βalanine. The substracted values represented a measure of GABA uptake by the βalanine sensitive pathway.

3. Glutamine Synthetase (GS)

Cells were grown to high density in 75cm^2 plastic flasks in the presence of either 2mM glutamine (standard culture conditions) or 2mM glutamic acid. The cells were harvested by trypsinisation and resuspended in growth medium[3], allowing the inactivation of remaining trypsin by serum components. After a final PBS wash the cells were stored for GS assay at -20°C as a frozen pellet.

The GS assay was a modified procedure of that described by Lehrer in 1971 (252). Cell pellets were taken up in 160mM tris malate buffer(pH 7.5) containing 1mM EGTA and 4mM MgCl, and homogenised in 1ml volumes. In the presence of 66mM sodium acetate and 13mM hydroxylamine, GS in the cell homogenates converts glutamine (present at 0.103M) to the active intermediate glutamic acid monohydroxymate.The incubation time is 15 minutes at 37°C. The addition of excess ferric chloride in 40mM HCl and 15% TCA results in the appearance of a red/brown colour. The optical density at 500nm is thus directly related to GS activity, expressed in terms of nmoles product formed min^{-1}mg protein $^{-1}$.

GS assays were carried out by Mr Andy Wilson, Department of Biochemistry, Glasgow University.

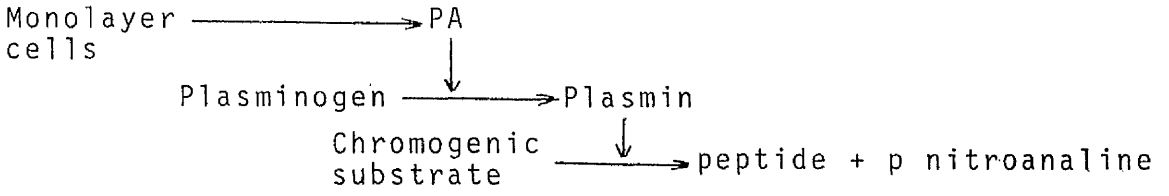
4. Plasminogen Activator (PA)

Fibrin Plate Assay

The fibrinolysis assay illustrated in Plate 11 of 1.5.1 was carried out by forming an *in vitro* fibrin clot. 10 mls of a 0.4% fibrinogen solution in sodium barbitone buffer[8] containing 0.05% plasminogen was stirred gently at 37°C for 15 minutes. 0.3ml of a 50 units ml^{-1} thrombin solution in 0.15M saline was placed in the centre of a 9cm plastic petri dish (Sterilin) and the fibrinogen solution pipetted rapidly onto the surface of the petri dish. The mixture was swirled filling out the plate uniformly and the gel allowed to set overnight. 10µl samples of cell spent medium (serum free) were placed on the gel and left overnight at room temperature for lysis to occur.

Chromogenic assay

The chromogenic assay used in this investigation was a modification of that developed by Whur et al (157) using SV40 transformed 3T3 cells and Lewis lung carcinoma cells. Cells grown in 24-well plates were incubated with a 0.5ml solution containing BSS (without phenol red)/glucose/vitamins[9], 1mM chromogenic substrate, 1 $C.U.ml^{-1}$ plasminogen and 0.15 $mgml^{-1}$ poly-D-lysine. The assay was a two step process:



The chromogenic substrates employed here were : S2302,H-D-Prolyl-L-phenylalanyl-L-arginine-p-nitroanilide. S-2251,H-D-Valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride. S-2238,H-D-Phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride.

The assay was terminated after 2 hours with the addition of 5% acetic acid. The OD at 405 nm was a mesure of the amount of paranitroanaline produced and hence directly related to the amount of PA released by the cells. The OD 405 nm was corrected for cell number and endogenous plasmin in the plasminogen preparation, either as a result of impurity or autocatalytic degradation. The proteolytic enzyme urokinase, which also activates plasminogen, was used as a standard. PA activities were expressed as Plough units ml^{-1} equivalents of urokinase/10^6 cells.

5. Tumour Angiogenesis Factor (TAF)

Chick chorioallantoic membrane(CAM)

The CAM was exposed through a small hole in the shell

of a 9 day old chick embryo and a small piece of Millipore filter paper (about 1mm^2), soaked in cell extract, placed on the CAM. The hole was sealed with tape and the egg incubated at 37°C under humid conditions for 6 days. The CAM was dissected out of the egg at day 15 and placed in formol saline for storage. The extent of vasoproliferation was examined using a dissection microscope.

Cultivation of Endothelial cells

Human umbilical cord was obtained at delivery, and processed within a few hours. A piece of cord (about 10cm in length) which was free from any damage was cannulated at both ends and the vein thoroughly washed by perfusion with PBS. The cord was suspended in a bath of saline at 37°C and the vein lumen filled with collagenase at 2000 units ml^{-1}. After 10-15 minutes the collagenase was discarded and the vein lumen rinsed twice with PBS, filled with culture medium and agitated gently for 1 minute. The cells stripped from the lumen of the vein were then transferred into culture flasks and fresh medium added with 100µgml^{-1} Endothelial Cell Growth Supplement (ECGS). Within 24 hours endothelial strips and single cells had attached and were proliferating.

HUV cell characterisation

Factor VIII

Monolayer cells on 15mm plastic coverslips or plastic culture flasks were thoroughly washed with PBS and fixed for 2 minutes in a 5% acetic acid/95% methanol mixture. Coverslips were mounted on glass slides using DPX and allowed to dry overnight. Immediately prior to staining the fixed cells were incubated for 5 minutes with a freshly prepared 0.005% trypsin solution (Sigma Type III) in PBS at room temperature. The cells were thoroughly washed with PBS or tris/saline[5]

and incubated for 30 minutes with a 1: 200 dilution of rabbit anti-human factor V111 antiserum in tris/saline. After removal of excess antibody with tris/saline, a 1: 10 dilution of HRPO conjugated swine anti-rabbit serum was applied to the cells for 30 minutes. Excess second antibody was removed by tris/saline washes after which the cells were immersed in fresh DAB[6] solution containing H_2O_2. The stained cells were thoroughly washed with tap water and air dried.

Electron Microscopy

Transmission electron microscopy was carried out on post confluent early passage cultures of HUV cells in conjunction with Dr D.I. Graham and Shahida Abraham (Southern General Hospital, Glasgow). Growth medium[3] was removed and the endothelial cell sheet, which detached from the plastic culture flask with agitation, was fixed in 2% glutaraldehyde in PBS for 30 minutes at room temperature. The samples were rinsed in PBS and placed in a 1:1, 2% osmium tetroxide:PBS solution for 15 minutes. After PBS washes the cell layer was dehydrated using 2 changes each of 25%, 50%, 75% and 95% alcohol,followed by 4 to 6 changes of 100% absolute alcohol. The cells were transferred into propylene oxide in glass centrifuge tubes for 5 - 7 minutes. The propylene oxide was replaced by 1:1 propylene oxide:araldite resin and spun for 1 to 2 hours. This mixture was replaced by pure araldite resin and spun for 1 to 2 hours. The samples were finally transferred to moulds which were filled with araldite resin and polymerised at 60°C for 16 hours. Sections (about 100nm thick) were cut and stained with uranyl acetate and lead citrate. The preparations were examined in a Philips 201 Transmission Electron Microscope.

2.3 Drugs employed in Phenotypic Manipulation Experiments

The drugs used to chemically modulate the astroglial phenotype are listed in Table 8 and the reasons for interest in their effects were discussed in 1.6.2. The drug concentrations employed were as follows:

1. Dexamethasone (DX)

DX was used at a final concentration of $10\mu g ml^{-1}$, the previously determined optimum concentration for stimulation of cloning efficiency of glioma cells and reduction in terminal cell density.

2. Isoproterenol (IP)

IP was used at $1 \times 10^{-5}M$, the concentration previously used to induce GFAP in C_6 cultures (116) and that reported to be optimal for activation of adenylate cyclase and elevation of intracellular cyclicAMP (274).

3. Retinoic Acid (Ret.Ac.)

Ret. Ac. was used at a final concentration of $1 \times 10^{-5}M$, the concentration at which the cloning efficiencies of two astrocytoma derived cell lines G-ATA and G-CCM, were reduced by 50%. Ret.Ac. was made up at $1 \times 10^{-2}M$ in dimethylsulphoxide and stored at 4°C. The concentrate was diluted 1:1000 with culture medium for experimental use. The exposure of post confluent cells to 0.1% DMSO alone, had no effect on cell number or any of the parameters measured.

4. Interferon (IFN-β)

IFN-β was used at 100 $Iuml^{-1}$, the concentration at which the cloning efficiencies of three out of four astrocytoma derived lines tested i.e. G-ATA, G-CCM and G-RAT were reduced by 50%. Freeze dried IFN was reconstituted at 10000 $Iuml^{-1}$

in 10mM glycine/HCl buffer (PH 3.5)containing Human Serum Albumin at 10mgml^{-1} and stored at 4°C.

5. Mitomycin C (Mit.C)

Mit.C was used at 0.05µgml^{-1}, a concentration which completely inhibited cell division while continuing to allow cells to metabolise.

6. Pig Brain Extract (PBE)

PBE was prepared by homongenising fresh pig brains in tris-saline[5] and spinning at 23,000 x g for 15 hours. The supernatant was filtered using 0.2µ Millipore filters and stored at -20°C. For experimental purposes the crude extract was diluted 1:32 in culture medium, the titre found to be optimal for the morphological transformation of NOR-T and NOR-F cells.

7. N-Methylacetamide (NMA)

NMA was used at a final concentration of 15mM, the concentration previously used for inducing erythroid differentiation in virus-infected erythroleukaemia cells *in vitro* (222).

8. 12-Tetradecanoyl phorbol-13-acetate (TPA)

TPA was used at 4µgml^{-1}, the concentration which reduced the cloning efficiency of G-ATA cells by 50%.

9. Methylnitrosourea (MNU)

MNU was used at 15µgml^{-1}, the concentration which reduced the cloning efficiency of G-ATA cells by 50%.

Unless otherwise stated the drugs were made up as 100 X concentrates in culture medium containing no serum or glutamine and stored at -20°C. Stock solutions were thawed and diluted immediately prior to use. After exposure of post-confluent cultures to the above drug concentrations for 6-7 days, more than 90% of the cells remained viable as determined by trypan

blue dye exclusion. Cell metabolism continued throughout the period of drug exposure as observed by the drop in pH of the culture medium.

RESULTS

3.1 : Cell Characterisation and Assay Development.

3.2 : Cell Density : effect on phenotypic expression.

3.3 : Heterologous Co-culture.

3.4 : Chemical Agents : effect on phenotypic expression.

3.1 Characterisation and Assay Development.

The cell lines used in this project and their derivation are listed in Table 1. The characteristics of many of these cell lines are presented below.

Morphology

Cells cultivated from normal brain tissue had, in general, a flattened, polygonal morphology (Plate IVa) forming a continuous cell sheet at high density. Treatment of these cells with dibutryl cyclicAMP (dbcAMP) or pig brain extract (PBE) resulted in the appearance of multipolar branched processes giving a morphological appearance more reminiscent of mature astrocytes *in vivo* (Plates IVb and IVc). However, the changes in morphology in response to dbcAMP and PBE differed in their exact nature. PBE induced active extension of processes as well as retraction of cell bodies while the dbcAMP effect was mainly due to cytoplasmic retraction alone. Plates Va, b and c respectively show the morphology of low density untreated NOR-T cells, dbcAMP treated NOR-T cells and PBE treated NOR-T cells at higher magnification.These findings are similar to the observed effects of these agents on cultured rat glioblasts (202). The extent to which cells responded to PBE treatment varied between cell lines e.g. in NOR-F and NOR-T cultures, more than 75% of the cells were morphologically altered whereas in GDU-T cultures less than 30% of the cells responded.

The cell lines derived from anaplastic astrocytomas fell into several categories with respect to morphology. An example of each of these categories i.e. large astrocytic, small astrocytic, flattened polygonal, elongated polygonal and

Table 1 CELL LINES AND DERIVATIONS

CELL LINE	SPECIES	TISSUE OF ORIGIN
NOR-T	Human (Adult)	Brain (Temporal Lobe)
GDU-T	"	" "
NMB-C	"	" (Corpus Callosum)
NMB-F	"	" (Frontal Lobe)
NOR-F	"	" "
NAB-P	"	" (Parietal Lobe)
NBL	"	"
NMN	"	"
NFF	Human (Foetal)	Brain
NFH	"	"
NFJ	"	"
NFM	"	"
NFO	"	"
NFP	"	"
NFQ	"	"
NFR	"	"
NFS	"	"
NFT	"	"
NFU	"	"
G-CCM	Human (Adult)	Anaplastic Astrocytoma
G-IJK	"	" "
G-RAT	"	" "
G-ATA	"	" "
G-UVW	"	" "
G-MCN	"	" "
G-EME	"	" "
G-ARY	"	" "
G-JPT	"	" "
G-ROG	"	" "
G-VAG	"	" "
GMS	"	" "
C_6	"	" "

CELL LINE	SPECIES	CELL TYPE
HUV	Human	Umbilical Vein Endothelial
WRC-256	Rat	Carcinoma
HES	Human (Foetal)	Skin Fibroblasts
MRC-5	Human	Fibroblasts
FHI	Human (Foetal)	Intestinal Epithelial
M-Bro	Human	Melanoma
M-ERS	"	"
M-AVO	"	"
CHB	Human	Colon Carcinoma
CSM	"	" "

PLATE IVa

NOR-F control culture. Giemsa stained
(x 275)

PLATE IVb

NOR-F culture treated with 0.1mM
dibutyrilcyclicAMP for 48 hours
Giemsa stained (x 275)

PLATE IVc

NOR-F culture treated with 1:32 solution
of Pig Brain Extract for 48 hours
Giemsa stained (x 275)

PLATE IVa

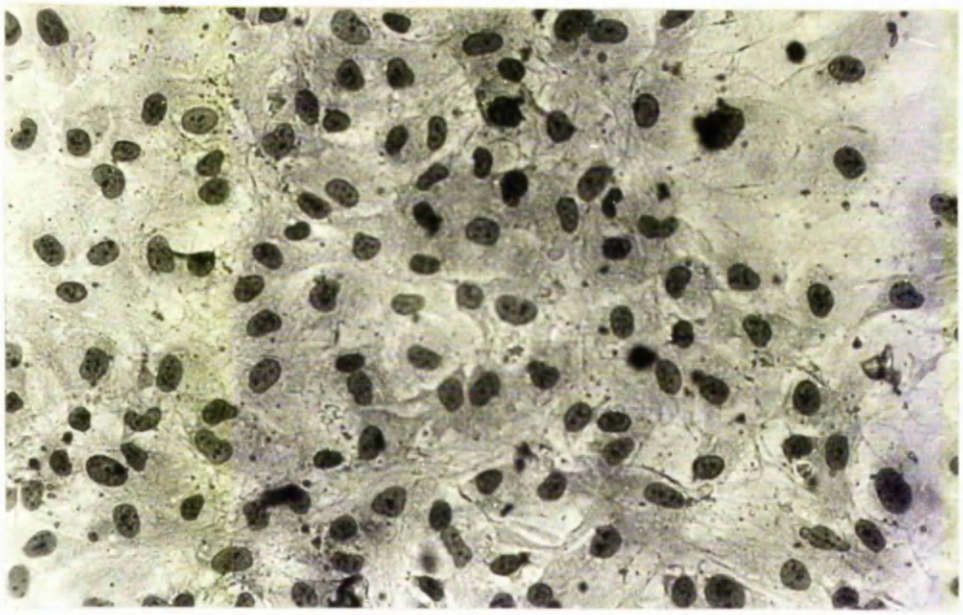


PLATE IVb

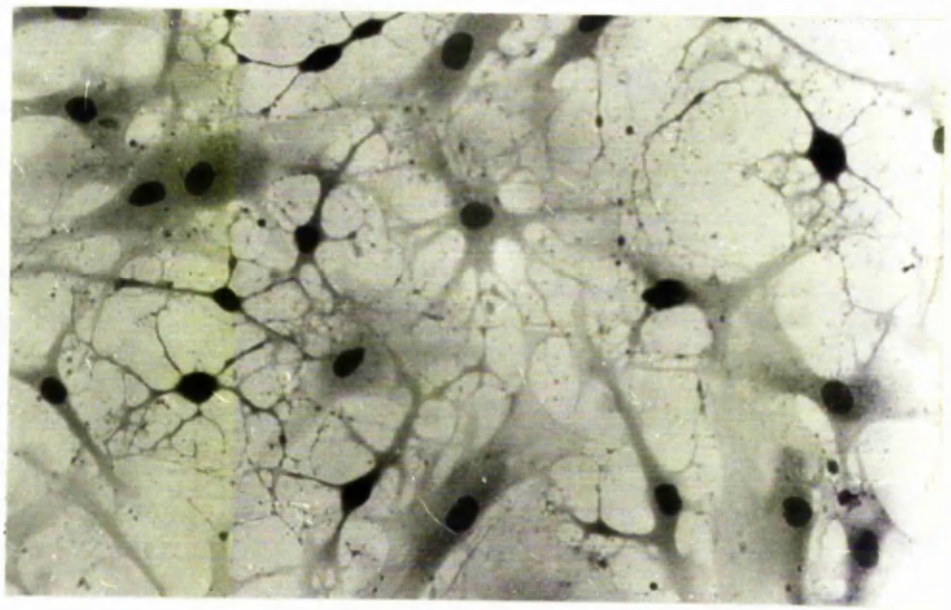


PLATE IVc

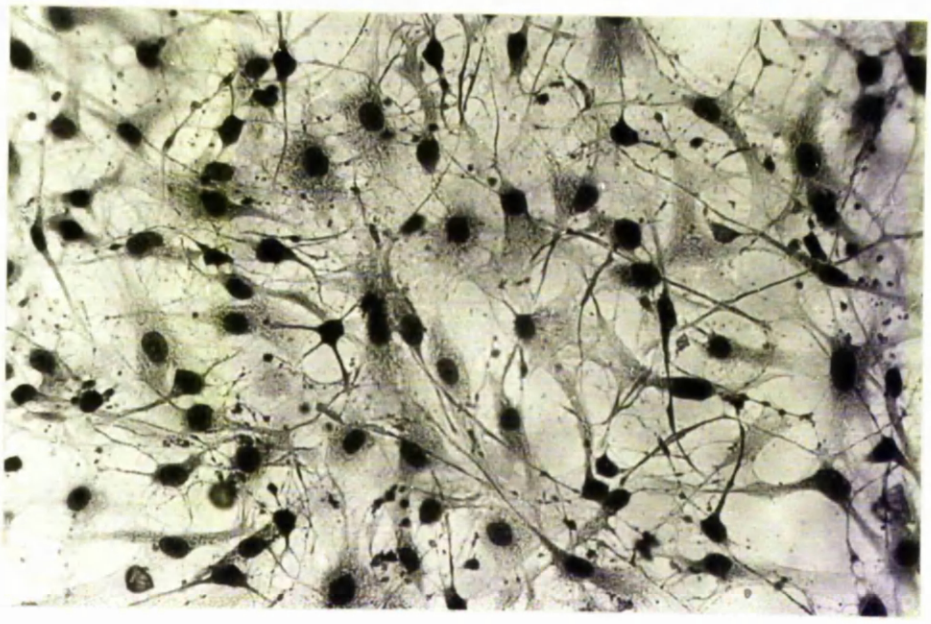


PLATE Va

Low density control NOR-F. Phase contrast (x 540)

PLATE Vb

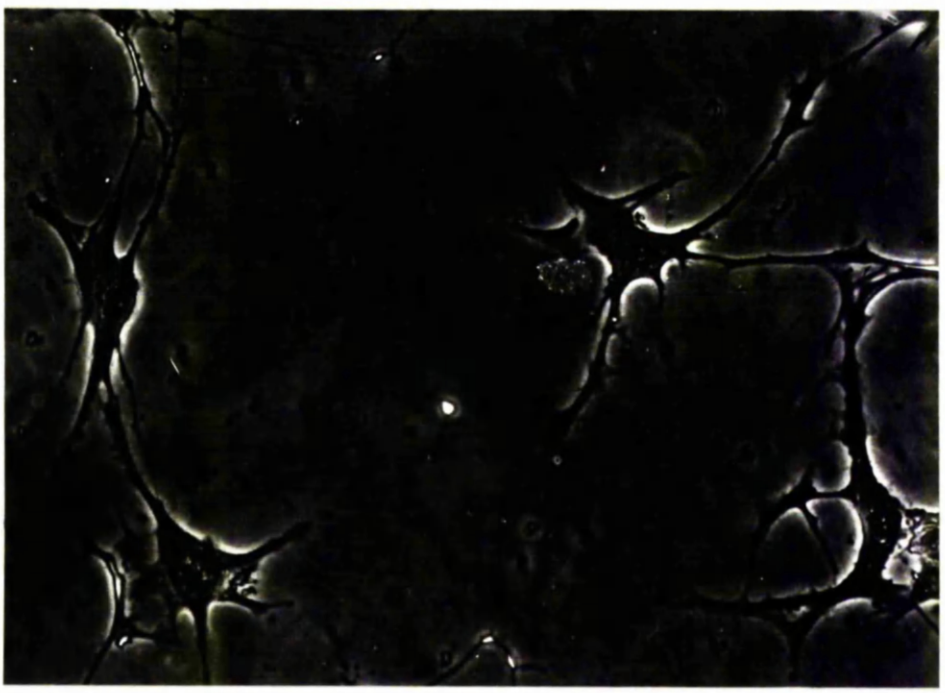
Low density NOR-F cells treated with 0.1mM dibutyrilcyclicAMP for 48 hours. Phase contrast (x 540)

PLATE Vc

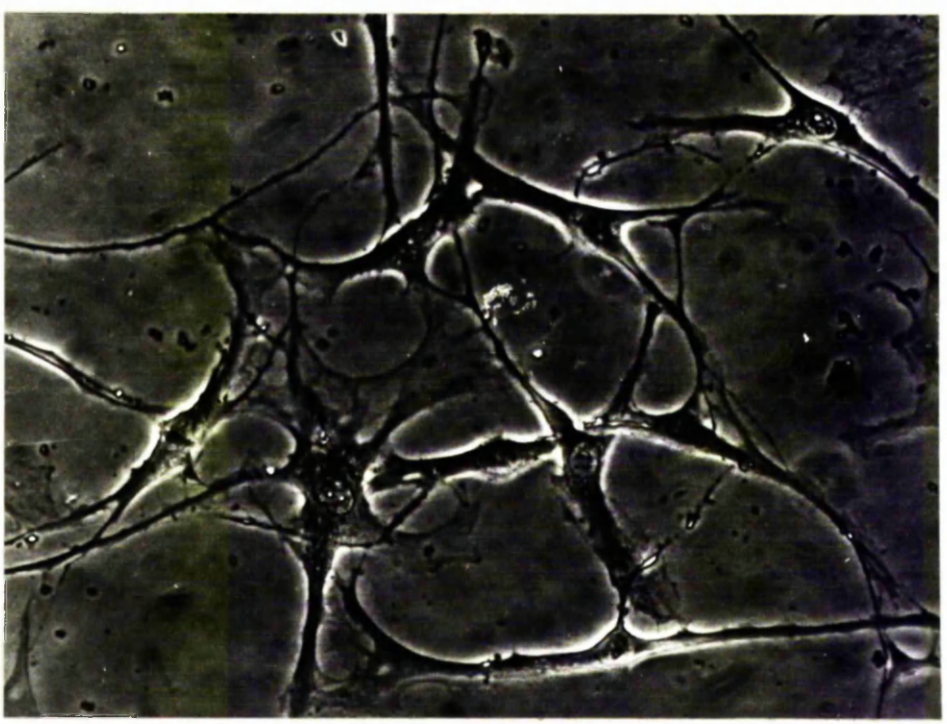
Low density NOR-F cells treated with 1:32 dilution of Pig Brain Extract for 48 hours. Phase contrast (x 540)

Va

Vb



Vc



fibroblastic is shown in Plates VIa, b, c, d and e respectively and the types of morphology exhibited by a range of glioma lines, at high cell density, are listed in Table 2.

The foetal brain derived cells were generally composed of one or both of two cell types. One was "astrocytic" and the other "elongated polygonal". These cells were only successfully cultured to the third or fourth passage, by which time, the latter cell type nearly always predominated.

Glial Specific Markers

GFAP

GFAP was consistently visualised in only four of the glioma lines investigated. Of these, G-CCM was 100% GFAP positive (Plate VIIa), G-ROG was about 80% positive, C_6 was 50-60% positive under standard culture conditions (Plate 1) and G-IJK had only a minor component of GFAP positive cells. Of the remaining astrocytoma derived lines, some had GFAP positive cells present in primary culture, which were subsequently lost on subculturing. All the foetal brain derived cell lines were GFAP positive, either partially, as in the case of NFM (Plate VIIb), or entirely, as in the case of NFQ (Plate VIIc). The GFAP persisted in these cultures over the first few generations, during which time they were used experimentally. The normal brain derived cultures were GFAP negative and despite apparent morphological and biochemical induction of differentiation in these cells, GFAP was never detected by immunoperoxidase or immunofluorescence. This latter observation and the presence of GFAP negative cells in the foetal and astrocytoma derived lines, prompted further biochemical characterisation of the cells.

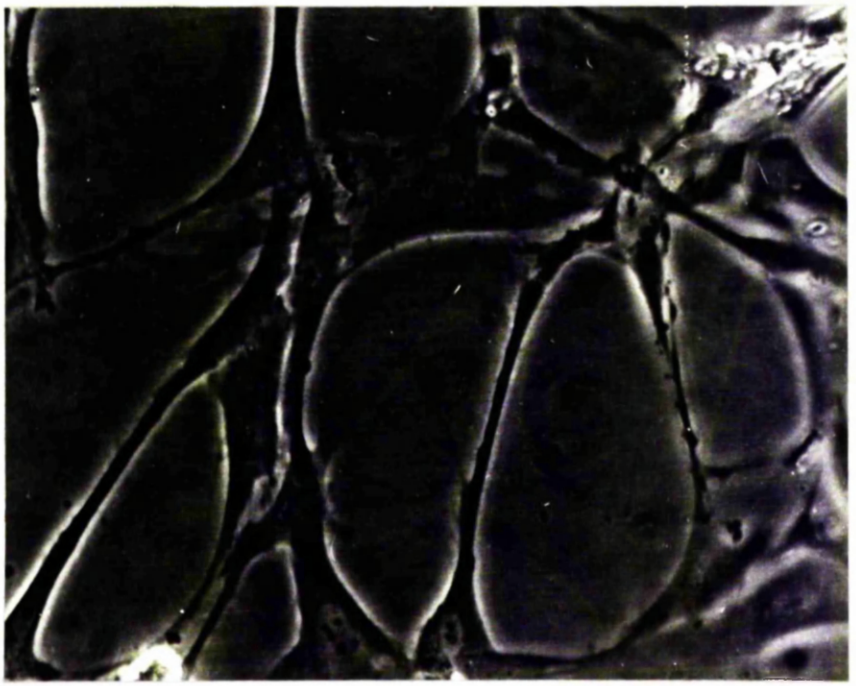
PLATE VIa

"Large astrocytic", G-ARY cells. Phase contrast (x 300)

PLATE VIb

"Small astrocytic", G-IJK cells. Phase contrast (x 300)

VIa



VIb

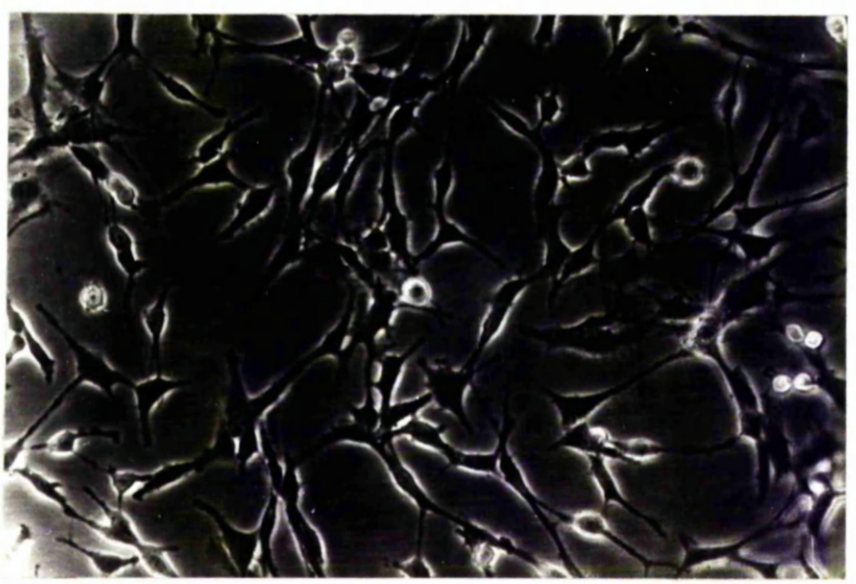


PLATE VIc

"Flat polygonal", G-ATA cells. Giemsa stained
(x 300)

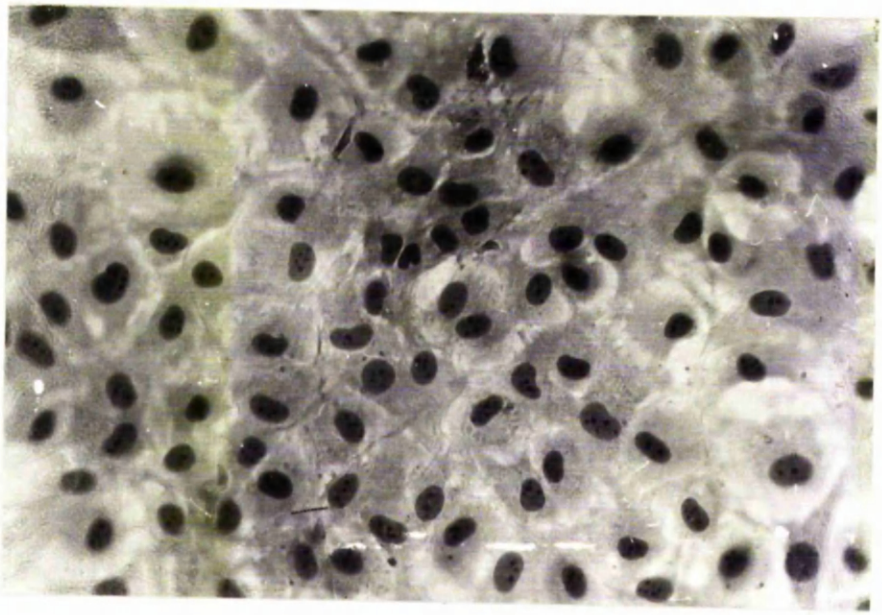
PLATE VId

"Elongated polygonal", G-EME cells.
Giemsa stained (x 300)

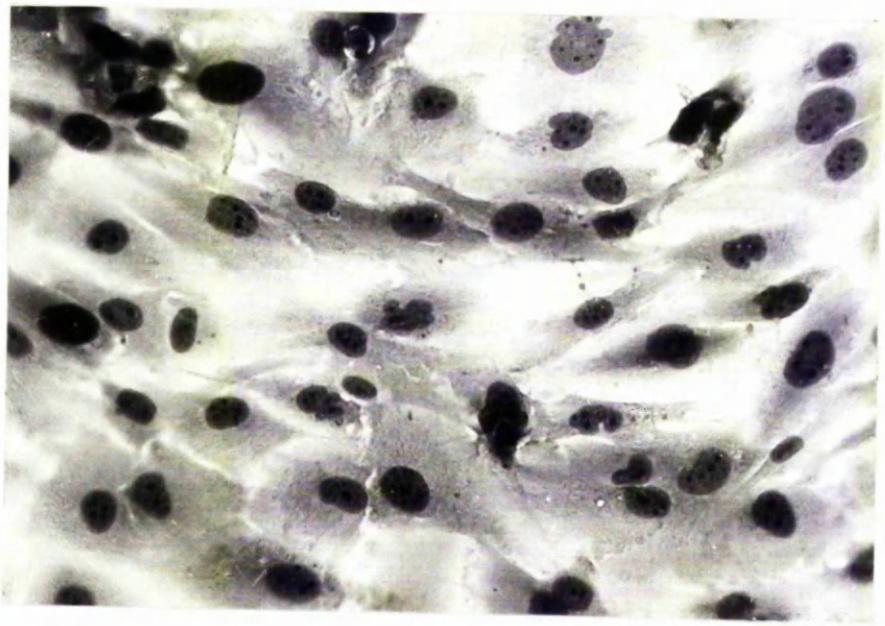
PLATE VIe

"Fibroblastic", G-UVW cells. Giemsa stained
(x 300)

VIc



VId



VIe

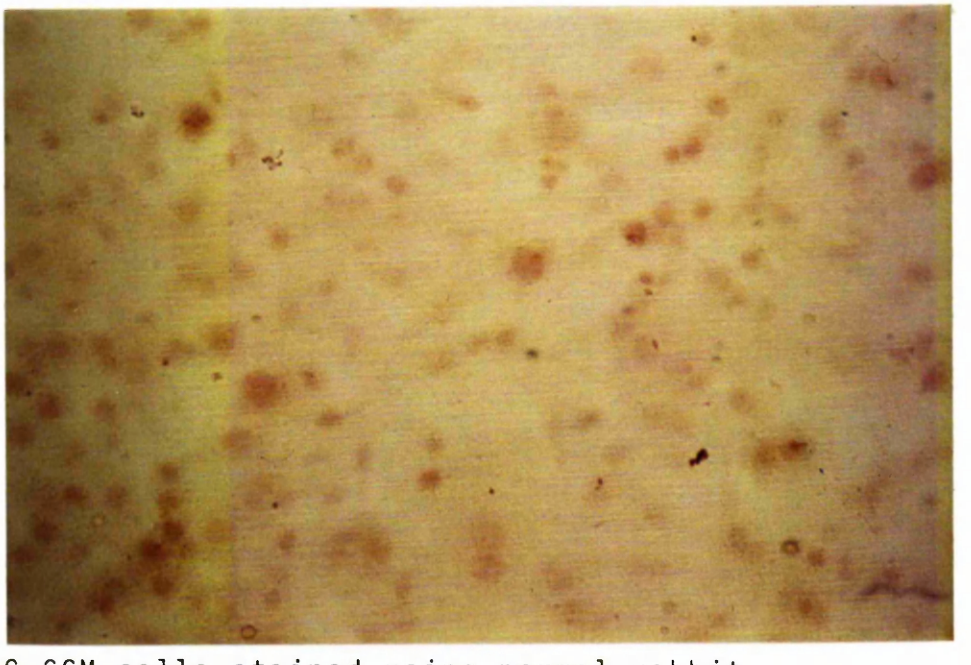
Table 2 Glioma Morphology

DESCRIPTION	CELL LINES
Large Astrocytic	G-ROG, G-ARY, G-JPT
Small Astrocytic	G-IJK, C_6
Flattened Polygonal	G-ATA, G-RAT
Elongated Polygonal	G-CCM, G-EME, G-MCN
Fibroblastic	G-UVW

PLATE VIIa

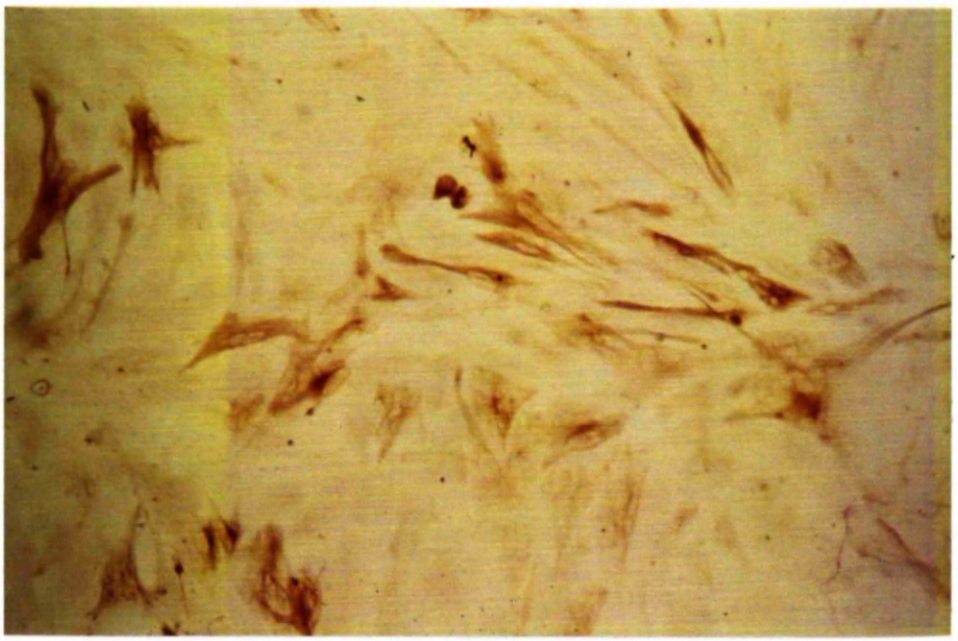
G-CCM cells positvely stained for GFAP
by immunoperoxidase (x 250)

Control



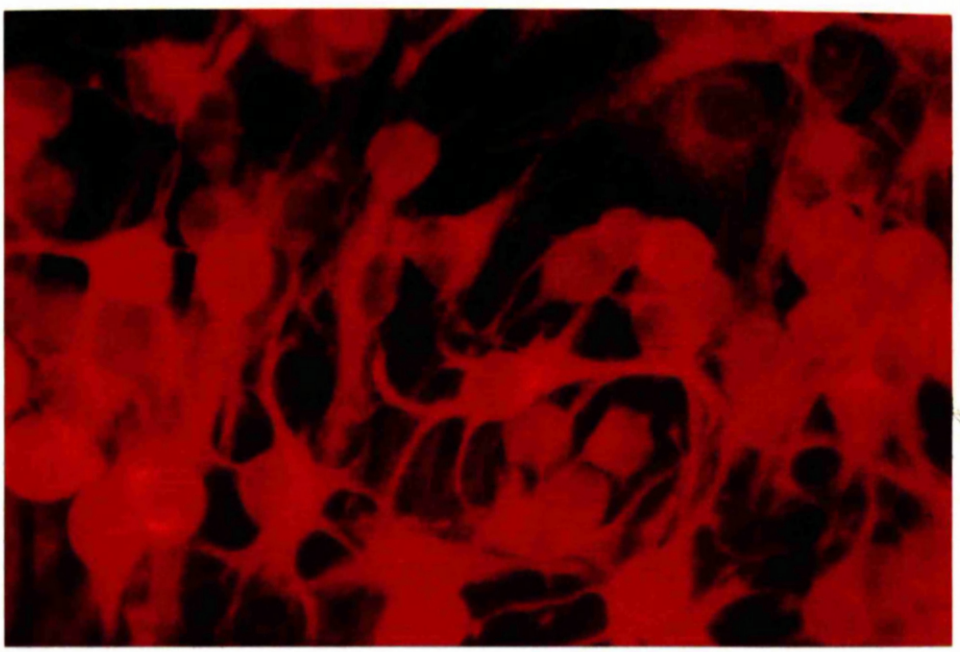
G-CCM cells stained using normal rabbit
serum as primary antibody (x 250)

PLATE VIIb



NFM culture stained for GFAP by immunoperoxidase (x 250)

PLATE VIIc



NFQ cells stained for GFAP by immunofluorescence using rhodamine conjugated antibody (x 320)

High Affinity Amino Acid Transport

1. Glutamic Acid

The uptake kinetics of glutamic acid was studied for a variety of cell lines. The velocity of glutamic acid uptake was determined for a range of substrate concentrations using radiolabelled glutamic acid, as described in the Materials and Methods section. Confluent cells were used for the uptake studies. The results were analysed by double reciprocal (Lineweaver - Burk) plots as shown for the rat C_6 glioma in Figure 4a and for endothelial GMS cells in Figure 4b. The biphasic nature of the plot for C_6 implied the existence of a dual affinity mechanism of glutamic acid uptake, one low affinity (Km 0.4mM) and the other relatively high affinity (Km 0.03mM). The high affinity mechanism could be competed out by 2mM aspartate (Figure 5). The double reciprocal plot for GMS was monophasic with only the low affinity (Km 0.25mM) uptake process evident. These Km values are comparable with those obtained by Logan and Snyder (124) for the low affinity (Km 0.15mM) and high affinity (Km 0.04mM) glutamate uptake into homogenates and slices of rat cerebral cortex.

The specificity of high affinity glutamic acid uptake with respect to cell type is shown in Table 3. Normal brain dervied, glioma and melanoma cell lines were able to take up glutamic acid by both a low and high affinity process, wherease factor VIII positive GMS endothelial cells, human and mouse fibroblasts and foetal human intestine cells possessed only the low affinity mechanism of uptake. These observations clearly showed that high affinity glutamic acid uptake was not restricted to cells of glial origin. Melanoma

FIGURE 4a and b

Lineweaver-Burk plots of $\frac{1}{Vi}$ against $\frac{1}{[\text{Glutamic Acid}]}$ for the uptake of glutamic acid into post-confluent and C_6 GMS cells respectively. Initial velocities (Vi) were determined as described in Materials and Methods section over a range of substrate concentrations.
Duplicate values are shown.

FIGURE 4a

DOUBLE RECIPROCAL PLOT FOR GLUTAMIC ACID UPTAKE IN C_6 CELLS

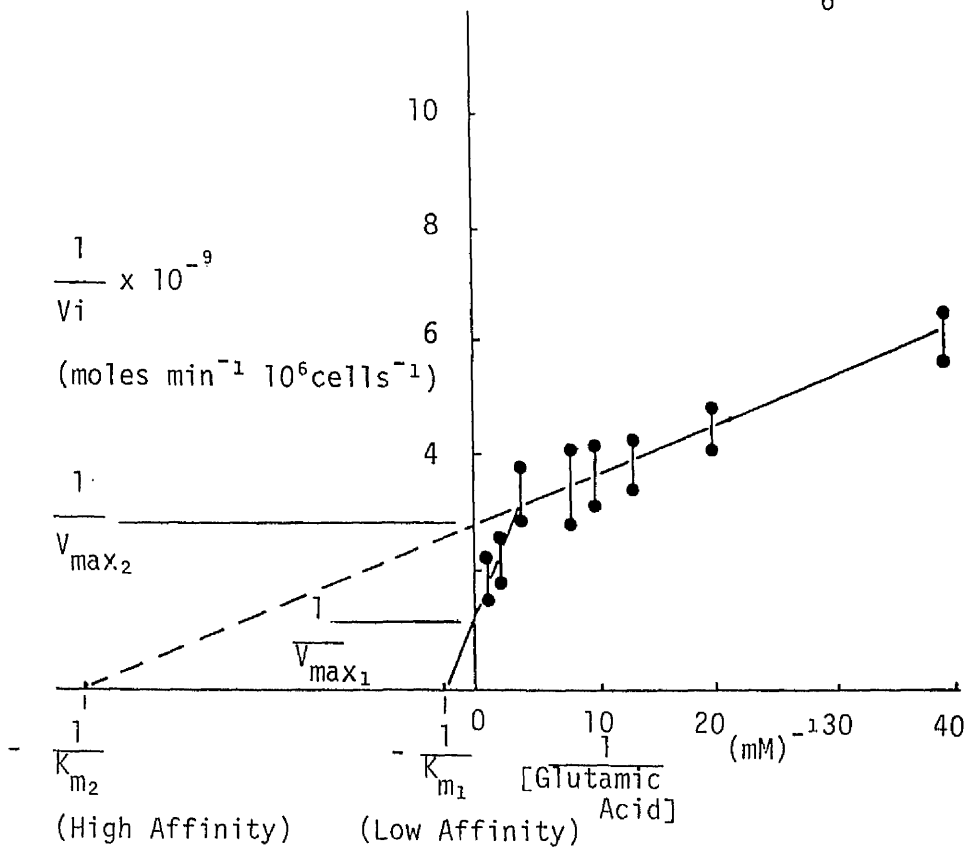


FIGURE 4b

DOUBLE RECIPROCAL PLOT FOR GLUTAMIC ACID UPTAKE IN GMS CELLS

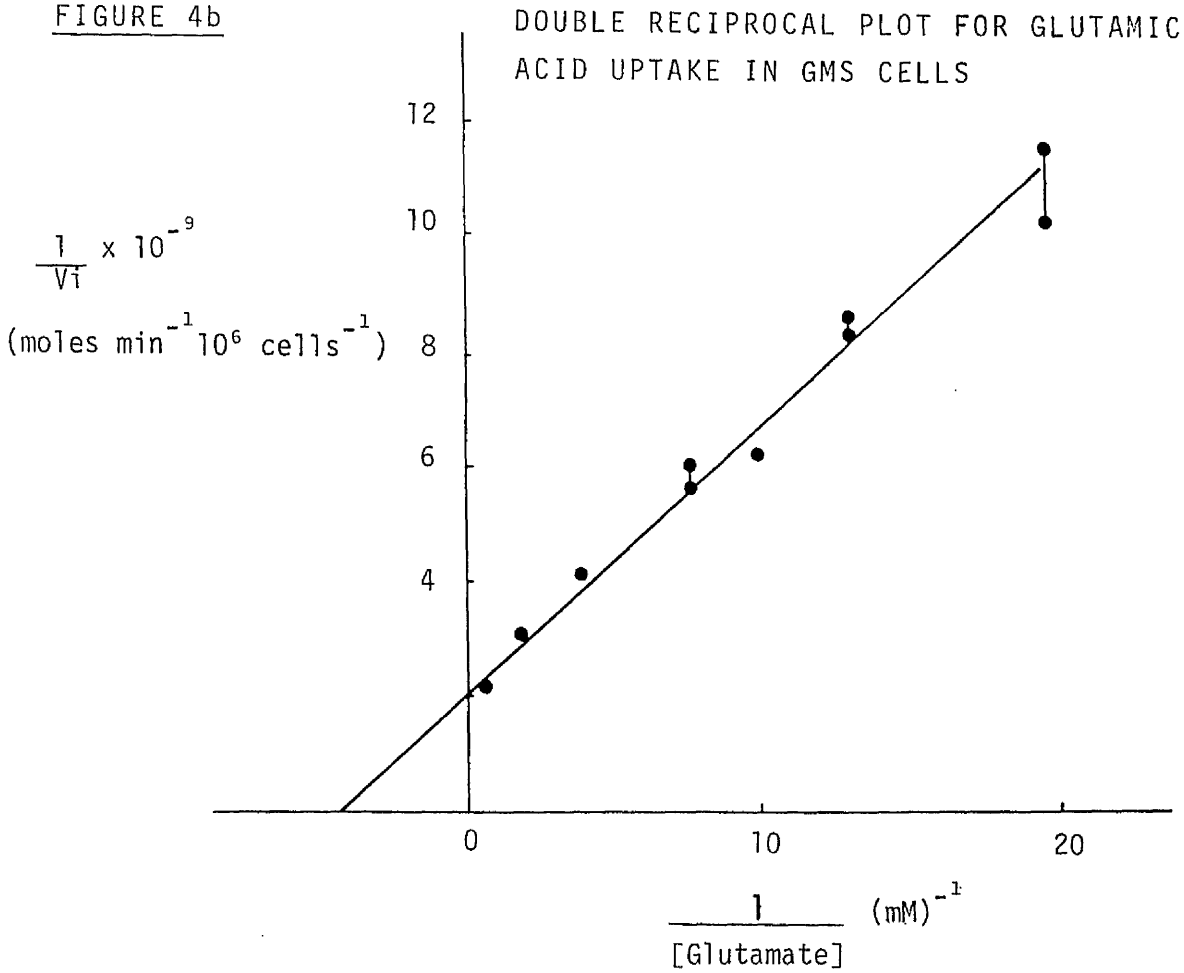


FIGURE 5

Lineweaver-Burk plots for the uptake of glutamic acid into C_6 cells in the presence and absence of 2mM aspartate.

FIGURE 5

THE EFFECT OF 2mM ASPARTIC ACID ON GLUTAMIC ACID UPTAKE KINETICS IN C_6 CELLS

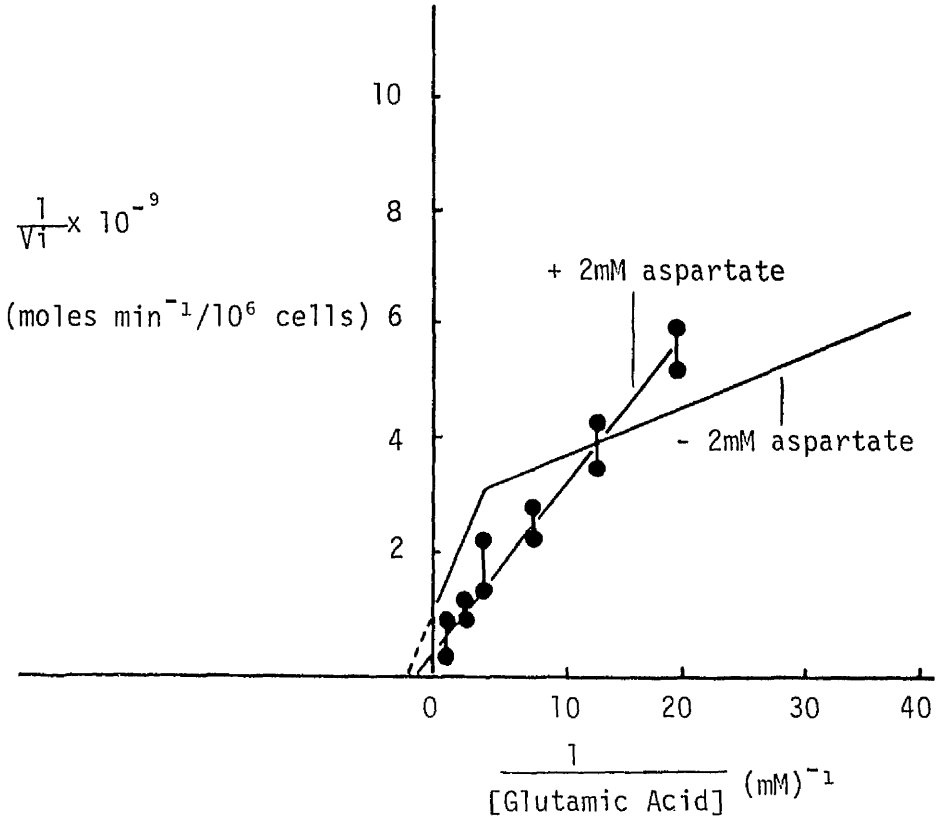


Table 3 SPECIFICITY OF DUAL AFFINITY GLUTAMATE UPTAKE

CELL LINE	CELL TYPE	DOUBLE RECIPROCAL PLOT	
		MONOPHASIC	BIPHASIC
NMB-C	Normal Human Brain		+
NAB-P	Normal Human Brain		+
G-ARY	Human Glioma		+
G-EME	Human Glioma		+
G-CCM	Human Glioma		+
G-IJK	Human Glioma		+
G-ATA	Human Glioma		+
G-RAT	Human Glioma		+
M-BRO	Human Melanoma		+
M-AVO	Human Melanoma		+
C_6	Rat Glioma		+
GMS	Human Glioma with probable large endothelial component	+	
MRC-5	Human Diploid Fibroblast	+	
3T3	Mouse Fibroblast	+	
FH1	Foetal Human Intestine	+	

were also capable of this process. This marker is possibly, therefore, a marker for cells of neuroectodermal origin, rather than being restricted to cells of glial origin.

2. GABA Uptake

The uptake of GABA was studied for a variety of cell lines in a manner similar to that described for glutamate. The results, however, were more complex. Many cell lines were either constitutive for high affinity GABA uptake or could be induced by steroid (β or dexamethasone) alone, by dbcAMP in the absence of serum, or by a combination of both steroid and dbcAMP, for about 7 days. The combination of dexamethasone ($10\mu gml^{-1}$) and dbcAMP (0.1mM) in the absence of serum, had been previously determined to be the maximum inducing conditions for GFAP in C_6 cells (Figure 2b). Some cell lines, however, could not be induced. An example of a biphasic double reciprocal plot for GABA uptake in the absence of inducers, by the GFAP positive cell line G-CCM, is shown in Figure 6a. The Km value of 0.038mM for the high affinity uptake of GABA by G-CCM was similar to the range of Kms. 0.013 - 0.03mM previously observed for high affinity uptake into various glial tumours maintained in tissue culture (131).

The specificity of high affinity GABA uptake and the induction requirements for a variety of cell lines are shown in Table 4. Of the normal brain derived lines only NOR-F was constitutive. NMB-C, GDU-T and NOR-T required induction. The two foetal lines NFF and NFQ were also constitutive, along with the two GFAP positive glioma lines G-CCM and rat C_6. The other gliomas, with the exception of G-ATA and G-ARY,

were induced for high affinity GABA transport by the steroid/dbcAMP combination. The factor VIII positive endothelial cell line GMS, the human melanoma line M-ERS and MRC-5 fibroblasts were not inducible. High affinity GABA uptake therefore appeared to be a more selective marker for cells of glial origin than high affinity glutamic acid uptake.

Results obtained in repeating some of the GABA uptake kinetic experiments implied that the batch of serum used in the culture medium for cell growth, could affect the ability of cells to express high affinity GABA uptake. In C_6 cells, in particular, the batch of serum determined whether or not the cells were constitutive or required induction. The importance of keeping the batch of serum constant throughout a series of experiments and of decreasing the serum concentration as much as possible, was therefore recognised.

The effect of β alanine, reported to compete specifically for the glial GABA high affinity uptake system, on the uptake kinetics of a few cell lines was investigated. Figure 6b shows that 2mM β alanine competes out the high affinity uptake process for NFF, resulting in a monophasic double reciprocal plot with only the low affinity uptake apparent. This selective inhibition by β alanine provided a useful means of quantitating the high affinity GABA uptake system. In further experiments, the sensitivity of uptake of 25 μM GABA to inhibition by 2mM β alanine was used to quantitate this process.

Treatment of cells with steroid (β or dexamethasone), often part of the induction procedure, had another significant effect on the uptake kinetics of GABA. Figure 6c shows the double reciprocal plot for G-EME using untreated cells and

FIGURE 6a

Lineweaver-Burk plots of $\frac{1}{Vi}$ against $\frac{1}{[GABA]}$ for the uptake of GABA into post-confluent G-CCM cells. Initial velocities (Vi) were determined as described in Materials and Methods section over a range of substrate concentrations.
Duplicate values are shown.

FIGURE 6b

Lineweaver-Burk plots for the uptake of GABA into NFF cells in the presence and absence of 2mM β alanine.
Duplicate values are shown.

FIGURE 6a

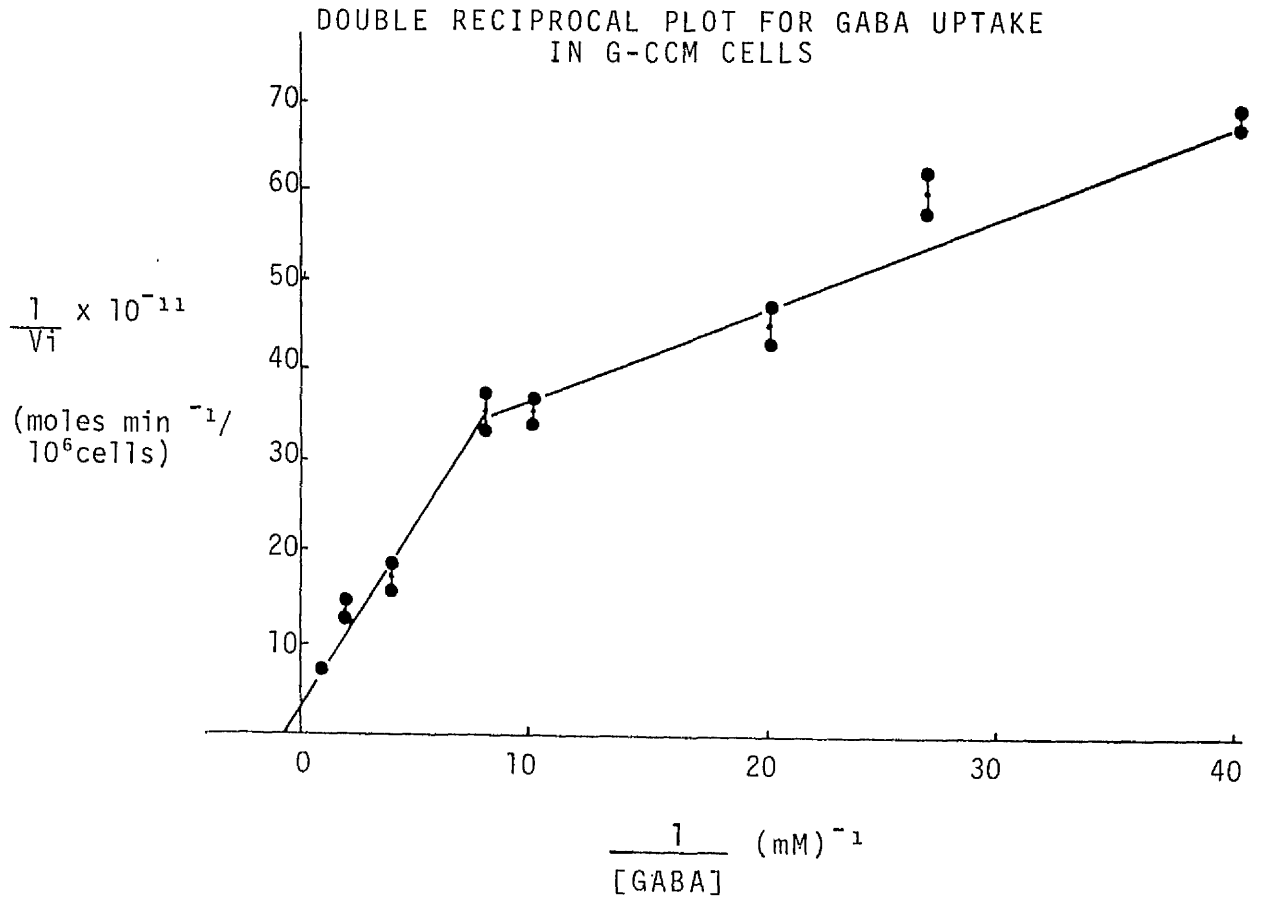


FIGURE 6b

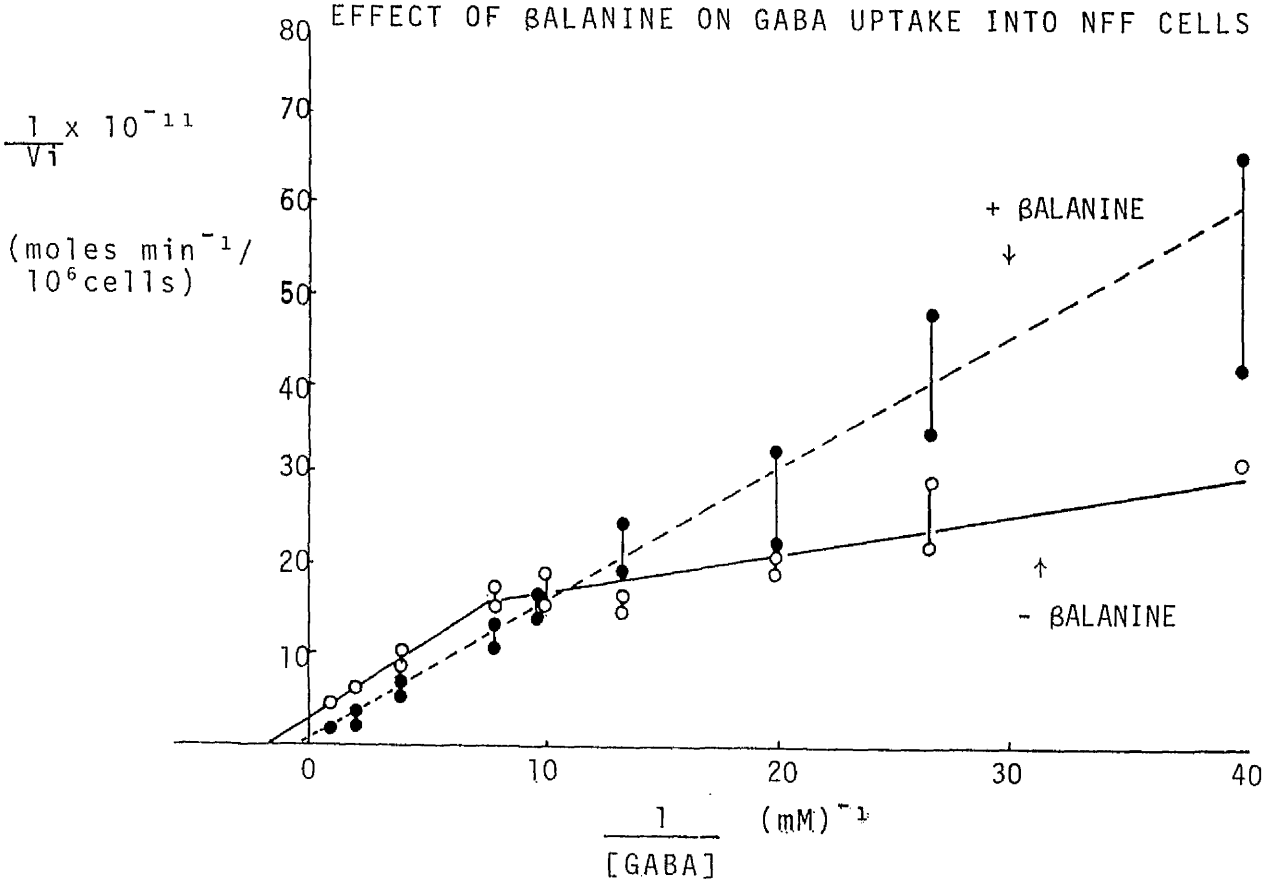


Table 4 SPECIFICITY OF HIGH AFFINITY GABA UPTAKE

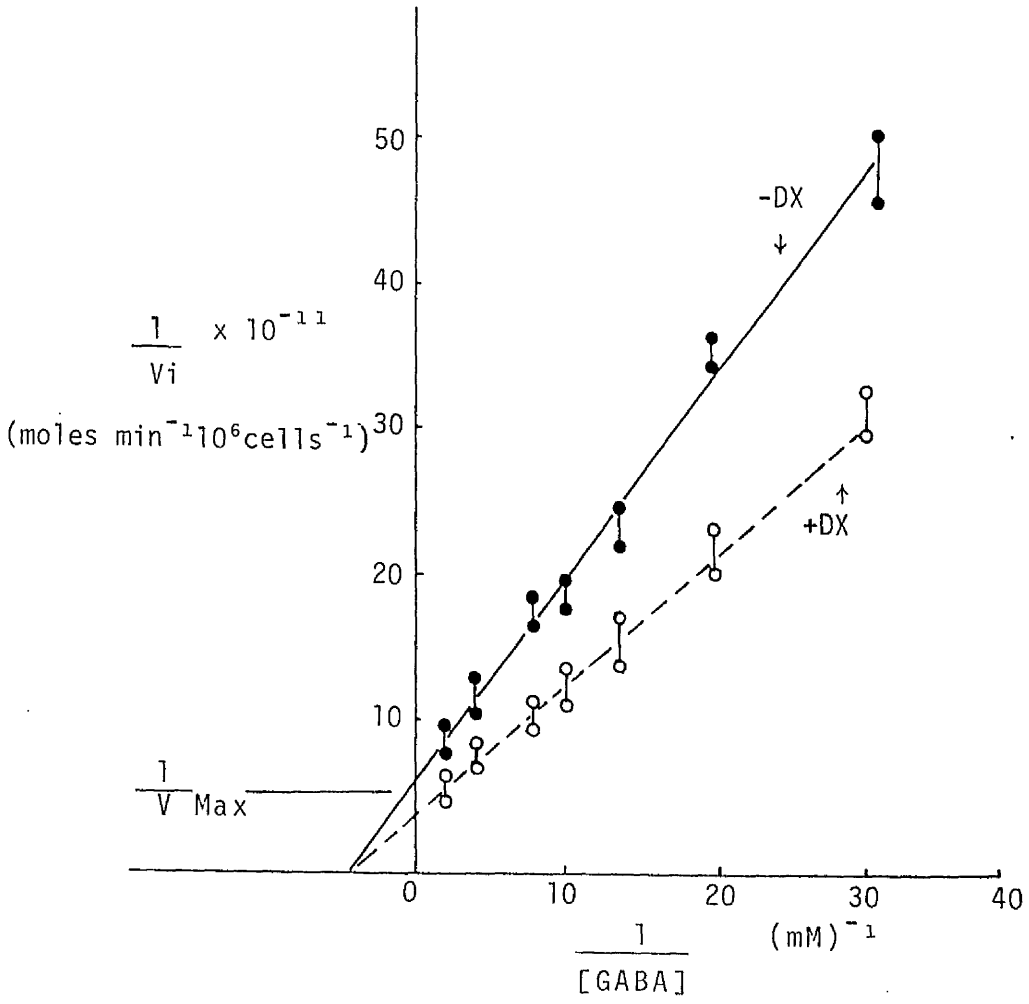
CELL LINE	CELL TYPE	INDUCER	TRANSPORT	
			Low Affinity	Low and High Affinity
NOR-F	Normal Human Brain	-		+
NMB-C	Normal Human Brain	-	+	
" "	" " "	β methasone		+
" "	" " "	dbcAMP (-serum)		+
GDUT	Normal Human Brain	-	+	
"	" " "	β methasone		+
"	" " "	dbcAMP (-serum)		+
NOR-T	Normal Human Brain	-	+	
" "	" " "	dbcAMP+βmethasone (-serum)		+
NFF	Foetal Brain	-		+
NFQ	" "	-		+
C_6	Rat Glioma	-		+
G-CCM	Human Glioma	-		+
G-EME	Human Glioma	-	+	
" "	" "	β methasone	+	
" "	" "	dbcAMP (-serum)	+	
" "	" "	dbcAMP+βmethasone (-serum)		+
G-ARY	Human Glioma	-	+	
" "	" "	dbcAMP+βmethasone (-serum)	+	
G-ATA	Human Glioma	-	+	
" "	" "	dbcAMP+βmethasone (-serum)	+	
G-RAT	Human Glioma	-	+	
" "	" "	dbcAMP+Dmethasone (-serum)		+
M-ERS	Human Melanoma	-	+	
" "	" "	dbcAMP+Dmethasone (-serum)	+	
MRC-5	Human Diploid Fibroblasts	-	+	
" "	" "	dbcAMP+Dmethasone (-serum)	+	
GMS	Endothelial (Fac. VIII +ve)	dbcAMP+Dmethasone	+	

FIGURE 6c

Lineweaver-Burk plots for the uptake of GABA into G-EME cells in the presence and absence of dexamethasone ($10\mu g ml^{-1}$). Duplicate values are shown.

FIGURE 6c

EFFECT OF STEROID ON MAXIMUM VELOCITY OF GABA UPTAKE INTO G-EME CELLS



cells treated with steroid for several days prior to kinetic analysis. Dexamethasone alone, in this case, does not induce high affinity transport but increases the maximum velocity of uptake (V max). The affinity (^{1}/km) is unaffected. These results suggest that dexamethasone increases the uptake of GABA by the unsaturable, diffusive, low affinity process, probably by a general effect on the cell membrane, increasing the permeability of cells to GABA and other small compounds. This complicates analysis of specific induction of the high affinity uptake by dexamethasone, as the steroid not only increases the β alanine sensitive GABA uptake, but also increases the low affinity uptake. The other compounds used for induction experiments did not show this effect.

Glutamine Synthetase

The glutamine synthetase (GS) activities of extracts prepared from various cell lines grown in F10 with 10% serum and 2mM glutamine are listed in Table 5. The normal brain derived cells had low levels of the enzyme. The foetal lines had about 2-3 times greater activity than the normal adult lines and the gliomas had variable amounts of GS activity with G-CCM having the highest level detected. Replacement of glutamine with equimolar amounts of glutamate in the culture medium, stimulated cellular GS activity in both normal adult and most glioma cell lines by about 2-5 fold. This implied that the glutamine/glutamate ratio in the environment was important in determining GS activity.

Malignancy Associated Properties

The development of marker assays for malignant phenotype was carried out using normal adult brain and glioma derived

Table 5 GLUTAMINE SYNTHETASE SPECIFIC ACTIVITIES

(nmoles product formed min^{-1} mg $protein^{-1}$)

CELL LINE	DERIVATION	GS ACTIVITY
GDUT	Normal Brain	16.8, 5.6
NOR-T	" "	10.0 ± 5.0
NBL	" "	6.0 ± 2.4
NMM	" "	8.6 ± 3.3
NFH	Foetal Brain	36.0 ± 27.0
NFJ-BG	" "	20.4 ± 15.1
NFJ-C	" "	29.9 ± 19.0
NFO-BG	" "	39.0, 32.0
NFO-C	" "	20.0, 39.0
NFP-BG	" "	19.0, 32.0
NFP-C	" "	29.0, 11.0
C_6	Rat Glioma	4.0 ± 0.5
G-IJK	Human Astrocytoma	24.0 ± 10.6
G-CCM	" "	45.0 ± 15.0
G-RAT	" "	22.0 ± 12.0
G-ATA	" "	10.0 ± 4.7

Results courtesy of Dr P.F.T. Vaughan and Mr A. Wilson

Department of Biochemistry, University of Glasgow.

cell lines.

Plasminogen Activator

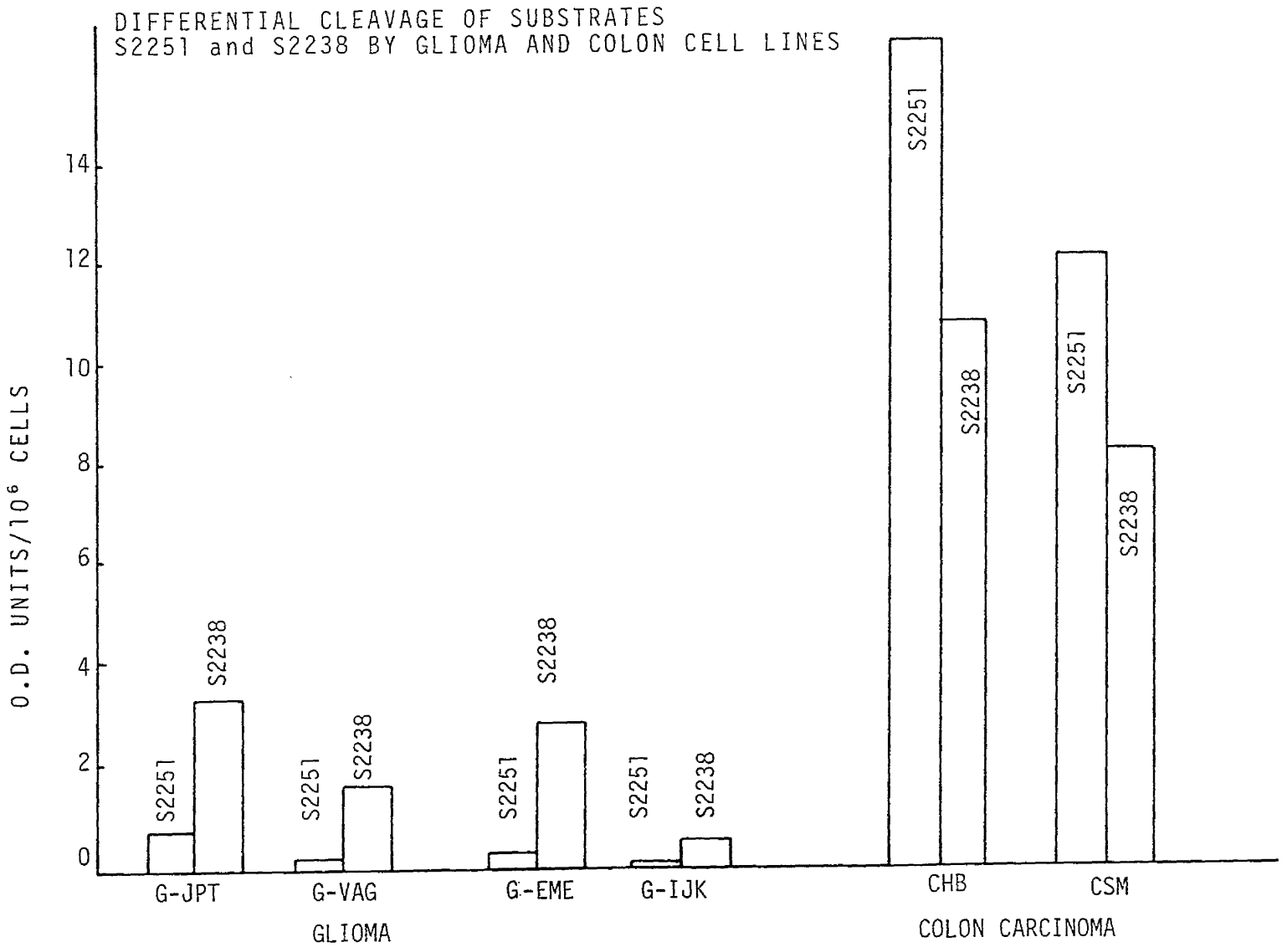
The chromogenic assay system of Whur et al (157), as described in Materials and Methods, was modified and used to detect PA produced by the normal and malignant brain cells. The addition of $0.1 mgml^{-1}$ poly-D-lysine increased the detectable PA levels 2-3 fold, probably by binding to both activator and plasminogen, altering their conformation in such a way as to increase efficiency of activation. The synthetic substrate used for detection of plasmin was S-2302, the preferred substrate for both normal and malignant brain cells. Experiments which tested the preference of a variety of glioma and colon carcinoma cell lines for chromogenic substrates, showed a significant difference between the two cell types. In particular, Figure 7 shows the PA activities of glioma and colon carcinoma cell lines detected using the chromogenic substrates S-2251 and S-2238. The two cell types differed in their relative activities with the two substrates implying that the molecular forms of PA produced by the two cell types differed. This in turn resulted in a variation in plasminogen cleavage patterns and hence molecular forms of plasmin which were not identical. This was consistent with the findings of Tucker et al, that human brain tumour PA is different from the PA of other human normal and neoplastic cell types (152).

In the initial experiments, confluent cells were washed three times with Ca^{++}, Mg^{++} - free PBS, incubated in a BSS/ glucose/vitamin solution for 1 hour and washed again in PBS

FIGURE 7

The cleavage of chromogenic substrates S-2238 and S-2251 (Kabivitrum) as a result of plasminogen activator (PA) produced by 4 glioma cell lines and 2 colon carcinoma cell lines at post-confluent densities. PA assays were carried out as described in Materials and Methods section. The OD 405nm is a measure of para-nitrophenol released by chromogenic substrate cleavage.

FIGURE 7


DIFFERENTIAL CLEAVAGE OF SUBSTRATES
S2251 and S2238 BY GLIOMA AND COLON CELL LINES
O.D. UNITS/10⁶ CELLS
0
2
4
6
8
10
12
14
S2251
S2238
G-JPT
G-VAG
G-EME
G-IJK
GLIOMA
CHB
CSM
COLON CARCINOMA

prior to the incubation with plasminogen and chromogenic substrate. Figure 8 shows the levels of PA activity for a number of cell lines as detected by optical density and related to urokinase standards. In general, the normal adult cell lines had lower levels of activity than the GFAP negative gliomas. The GFAP positive gliomas, however, G-CCM and rat C_6, had very low PA levels.

The effects of serum on the PA production of cells, as detected in the assay system described above, is shown in Figure 9. A considerable increase in the PA level of G-ATA was apparent when the cells were maintained in medium containing serum replacement (Ventrex Laboratories) as opposed to foetal calf serum itself. The inhibitory effect of serum is probably due to the presence of plasmin inhibitors in serum e.g. α 2-macroglobulin and α 2-antiplasmin, which remained bound to the cell surface despite the washing procedure described above. These results again emphasised the need to decrease the experimental serum concentration as much as possible. The PA levels shown in Figure 8 were, therefore, not absolute measures of the cellular production of PA since a proportion of that produced would be masked by serum inhibitors on the cell surface. The extent to which serum masked the detection of PA also varied from batch to batch (Figure 9). In most of the further experiments involving PA determinations, cells were grown to confluence and maintained in serum free medium for one week prior to PA assay. The effect of cell density on PA activity is presented later, in 3.2.

FIGURE 8

Plasminogen activator activities of a variety of GFAP negative astrocytomas, GFAP positive gliomas and cell lines derived from normal adult brain. PA assays were carried out on post confluent cultures as described in Materials and Methods section using the chromogenic substrate S2251. OD 405nm values were converted to equivalents of urokinase (Plough Units (PU)) from a standard curve. Duplicate PA values are shown.

FIGURE 8

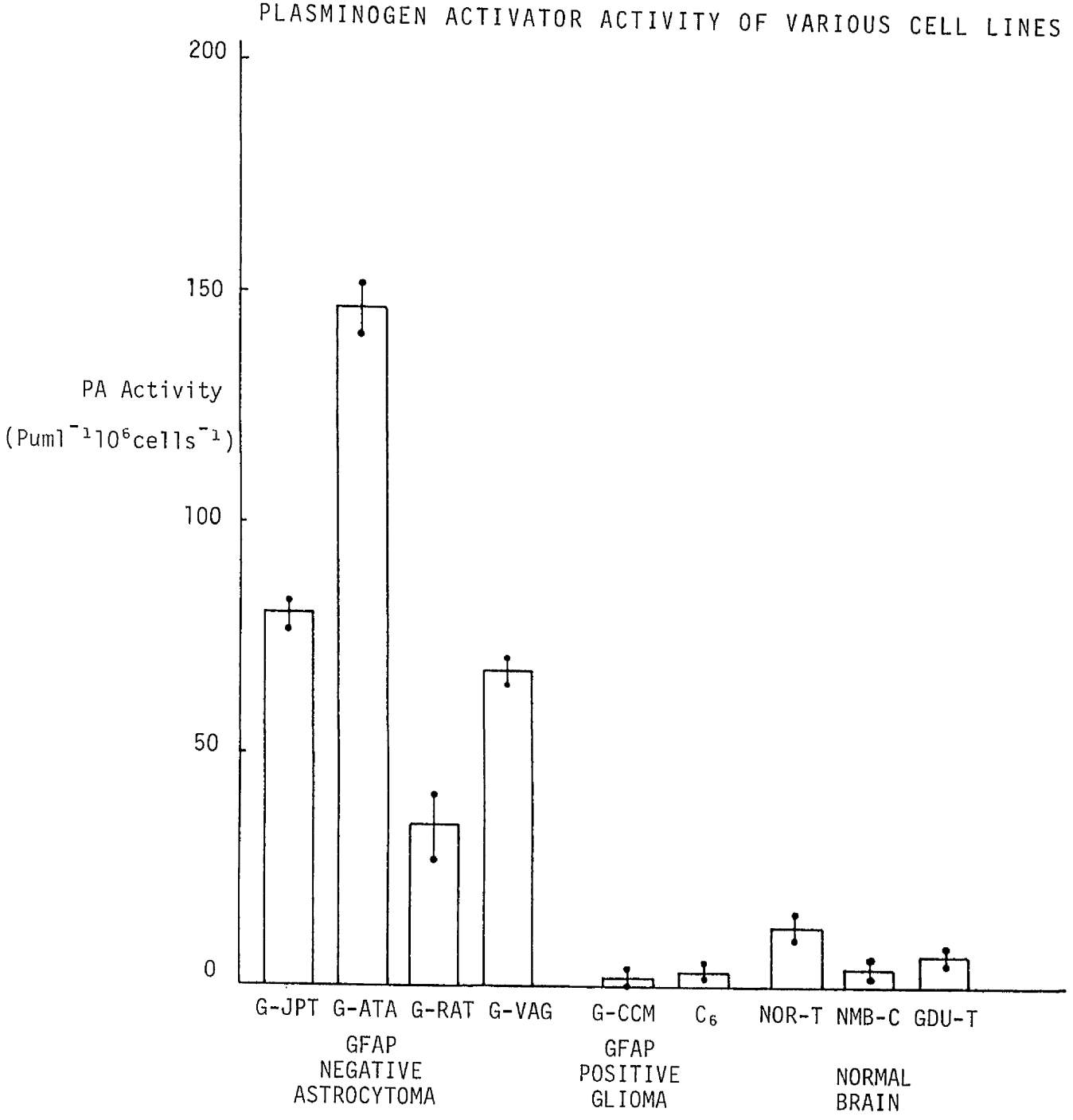
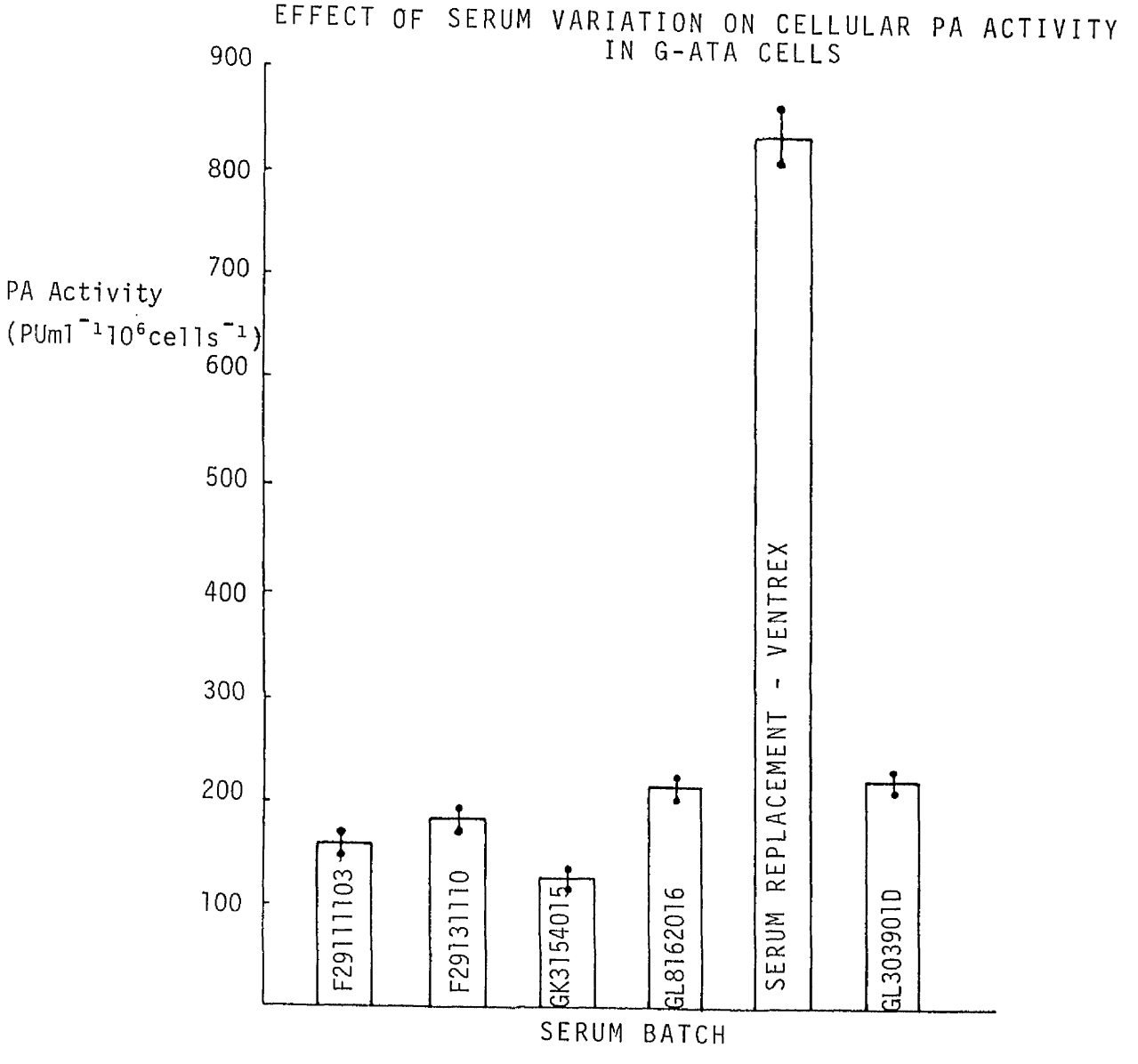

PLASMINOGEN ACTIVATOR ACTIVITY OF VARIOUS CELL LINES
200
150
100
50
0
PA Activity
(Puml⁻¹10⁶cells⁻¹)
G-JPT
G-ATA
G-RAT
G-VAG
G-CCM
C6
NOR-T
NMB-C
GDU-T
GFAP NEGATIVE ASTROCYTOMA
GFAP POSITIVE GLIOMA
NORMAL BRAIN

FIGURE 9

Plasminogen activator activities of G-ATA grown in the presence of various batches of foetal calf serum and serum replacement (Ventrex Laboratories) at 10%.
Duplicate values are shown.

FIGURE 9


EFFECT OF SERUM VARIATION ON CELLULAR PA ACTIVITY IN G-ATA CELLS
PA Activity
(PUml⁻¹10⁶cells⁻¹)
900
800
700
600
500
400
300
200
100
F29111103
F29131110
GK3154015
GL8162016
SERUM REPLACEMENT - VENTREX
GL303901D
SERUM BATCH

Tumour Angiogenesis Factor (TAF)

Chick chorioallantoic membrane (CAM)

Intracellular extracts of normal adult brain and glioma derived cell lines were prepared by repeated freezing and thawing of single cell suspensions followed by centrifugation at 48,000 x g for 30 mins. at 4°C. The supernatants were then tested for angiogenic activity on the CAM, as described in the Materials and Methods. Plates VIIIa, b and c show CAMs on which small pieces of Millipore filters soaked in 2mg ml^{-1} protein extracts of NOR-F, C_6 and G-RAT cells respectively, were implanted. These three samples showed a gradation in response from no obvious vasoproliferation for NOR-F to considerable activity for G-RAT. In this case, C_6 showed an intermediate response.

An attempt was made to grade angiogenic responses on the CAM. The negative response of Bovine Serum Albumin (BSA) protein control at 2mg ml^{-1} was designated as 0; the almost complete surrounding of Walker Rat Carcinoma - 256 extract soaked filters by radial blood vessels was designated as 4 and intermediate responses were designated as 1, 2 or 3. For example, the responses shown in Plates VIIIa, b and c would be designated 0, 2 and 4 respectively. Several eggs were used for each cell extract and responses on the 6th day after implantation were recorded. The means and standard deviations of the angiogenic responses are shown in Figure 10. Spent medium from WRC-256 showed no angiogenic activity on the CAM.

This technique clearly demonstrated that the glioma cell lines were able to induce angiogenesis although the responses were difficult to quantitate precisely. The large standard deviations were, in part, due to unavoidable variation in the site of implantation of the extract-soaked

PLATE VIIIa

Millipore filter soaked in NOR-F cell extract ($2mgml^{-1}$) placed on 9 day old chick CAM. After a 6 day incubation no obvious vasoproliferation was evident.

Designated 0 in response gradation.

PLATE VIIIb

Millipore filter soaked in C_6 glioma cell extract placed on chick CAM. After 6 days some radial vasoproliferation was seen towards the site of filter implantation.

Intermediate response designated 2 in gradation.

PLATE VIIIc

Millipore filter soaked in G-RAT glioma cell extract placed on chick CAM. After 6 days extensive radial vasoproliferation was evident.

Complete response desginated 4 in gradation.

VIIIa

VIIIb

VIIIc

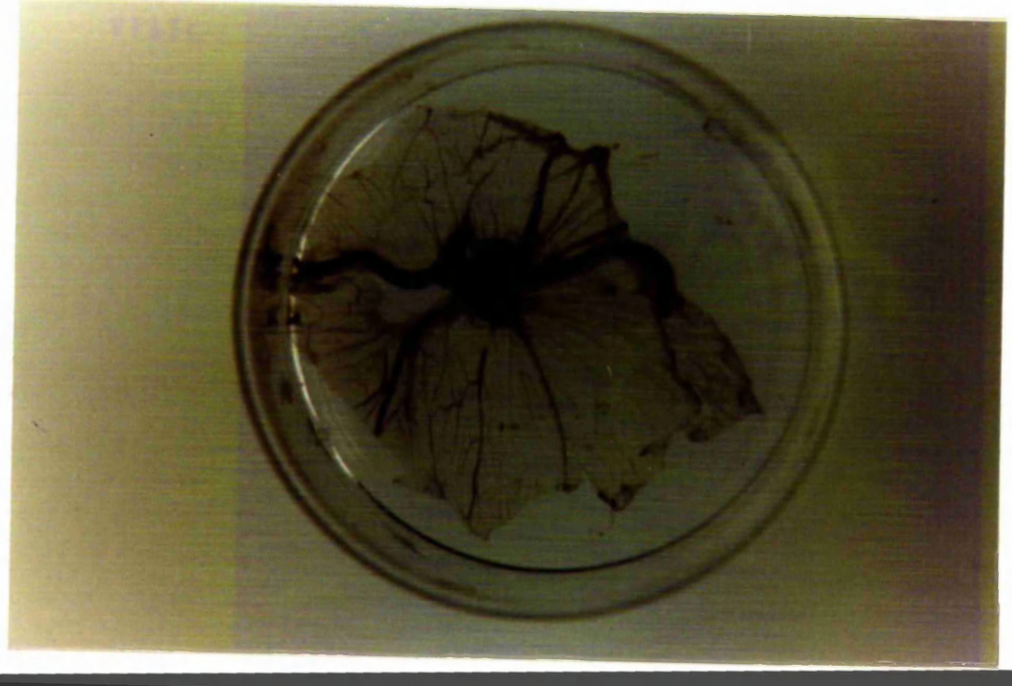
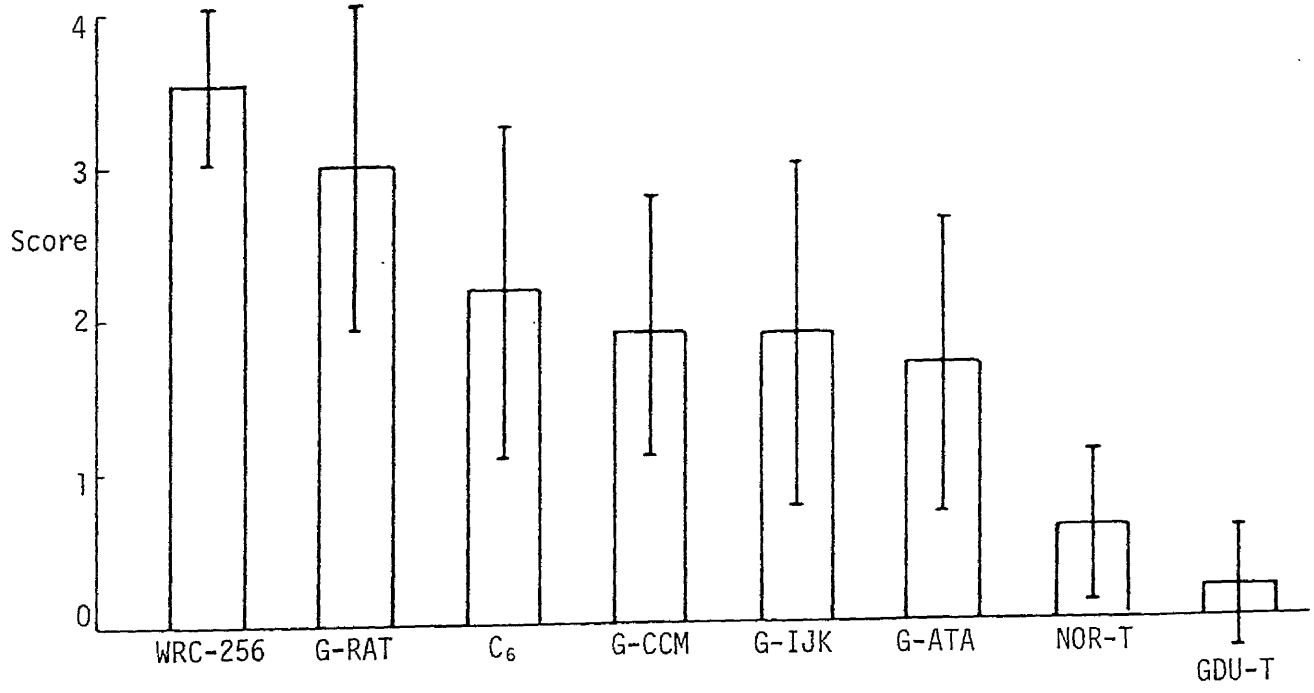


FIGURE 10

Semi-quantitative gradation of the angiogenic response of extracts prepared from various cell lines as measured by vasoproliferation on the chick chorioallantoic membrane. The preparation of cell extracts and the method of quantitation are described in the Text. The means and standard deviation of 7 to 10 replicates are shown.

FIGURE 10

CAM ANGIOGENESIS GRADATION FOR EXTRACTS OF VARIOUS CELL LINES



filter on the CAM. In particular, the proximity of the filter to major blood vessels often affected the magnitude of angiogenic response for a particular cell extract. The gradation of responses was also subjective and this introduced further variation to the results.

These findings prompted an attempt to find an *in vitro* assay for TAF which would allow more precise quantitation of the malignancy associated marker. The rationale behind the development of this assay was that a significant proportion of the cells *in vivo* which respond to the mitogenic effect of TAF are likely to be host vascular endothelial cells. The fact that tumour blood vessels often lack the normal smooth muscle component, forming tubes of undifferentiated endothelium (160), seems to confirm endothelial cells as the primary target for TAF. Attempts were therefore made to isolate and characterise endothelial cell cultures from a variety of sources.

1. GMS Human Glioma

A human glioma digested with collagenase (as described in the Material and Methods) and subsequently subcultured with trypsin, yielded a factor VIII positive cell line. Plate IXa shows immunoperoxidase stained factor VIII antigen in GMS cells with its characteristic granular staining. This observation emphasised the possibility that cultures derived from human glioma could be contaminated with endothelial cells.

2. Rat Brain White Matter

Rat Brain White Matter was cultured according to the method of Phillips *et al* (253). White matter from adult rats, killed by overanaesthetisation, was finely dissected and

trypsinised (0.5% trypsin) for 5 minutes. The monolayer cell clones which were seen after several days in culture were examined for morphology and factor VIII antigen. Plate IXb shows the morphology of factor VIII positive clones derived from the rat brain white matter. However, also present in these cultures were large factor VIII negative putative smooth muscle cells (Plate IXc). These observations differed from those of Phillips et al who obtained pure endothelial cultures by this technique.

3. Human Umbilical Vein

Collagenase digestion of the lumen of human umbilical vein (HUV) was carried out as described in the Materials and Methods. The primary disaggregates consisted of small strips of the endothelium lining the interior of the vein which attached to plastic or gelatin substrate and grew to form colonies of endothelial cells. Plate Xa shows the typical morphology of primary HUV cells in culture. The requirement of HUV cell proliferation for endothelial growth supplement (ECGS, Collaborative Research) is shown in Figure 11. ECGS was clearly an important medium constituent for the growth and maintenance of these cells in culture and was routinely used at 100 $\mu g\ ml^{-1}$.

HUV Cell Characterisation

HUV cultures were entirely factor VIII positive, by immunoperoxidase staining, when tested at the first, third and fifth passages. Plate Xb shows factor VIII positively stained HUV cells. Transmission electron microscopy of post confluent cultures revealed the presence of Weibel Palade Bodies, electron dense organelles consisting of a

PLATE IXa

GMS cells positively stained for factor VIII by immunoperoxidase.
(x 320)

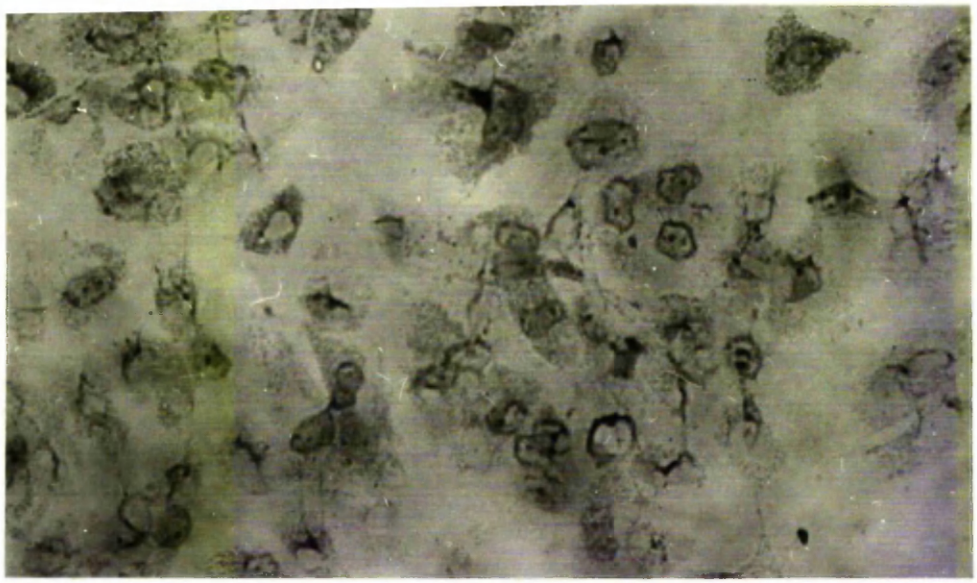
PLATE IXb

Endothelial clones cultured from rat brain white matter. Phase contrast.
(x 180)

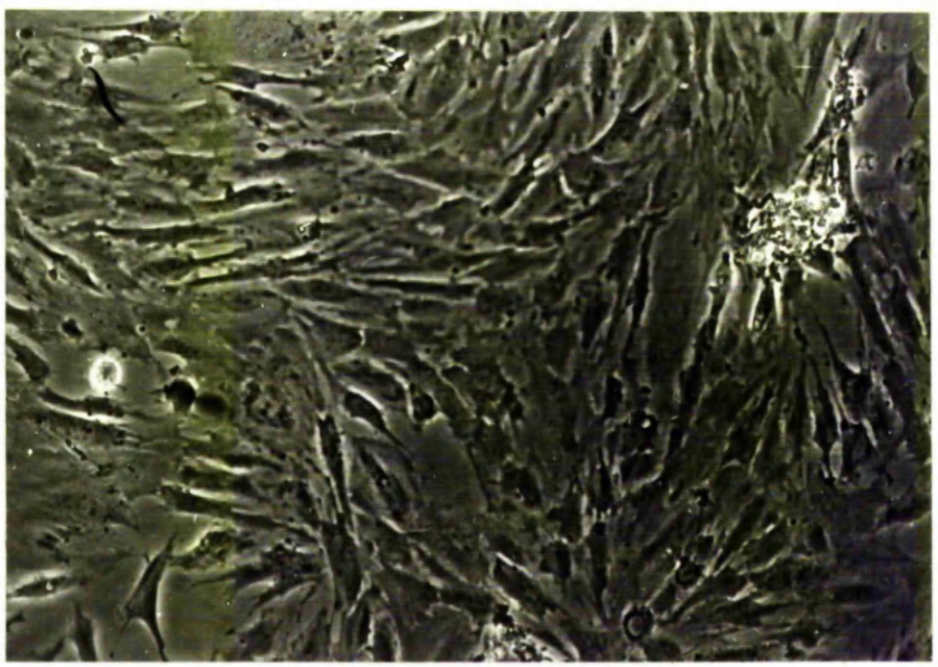
PLATE IXc

Putative smooth muscle cells present in primary cultures from rat brain white matter. Phase contrast.
(x 180)

IXa

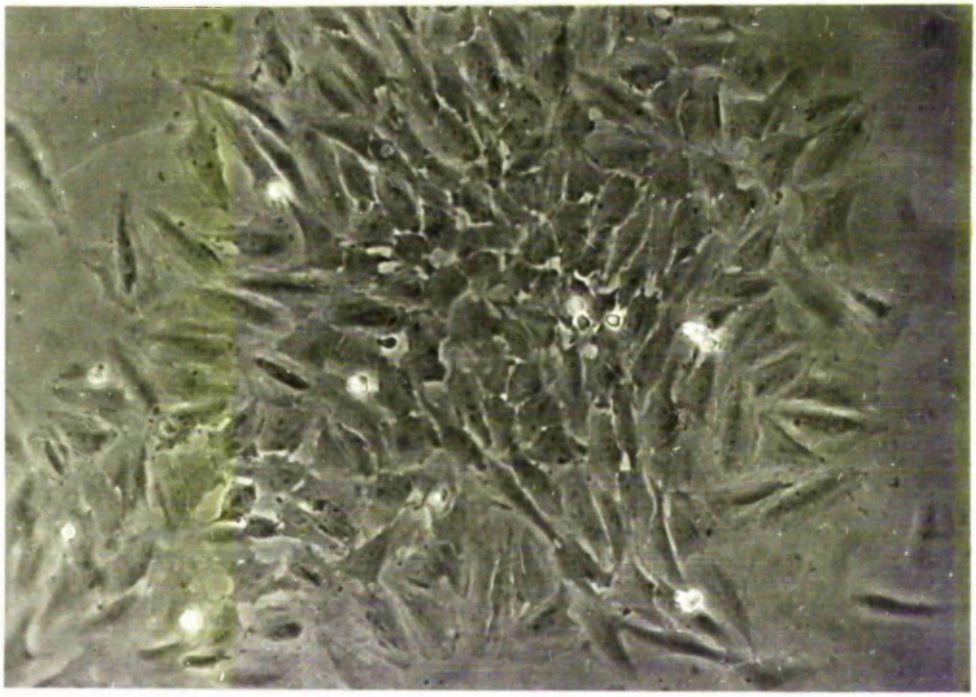


IXb



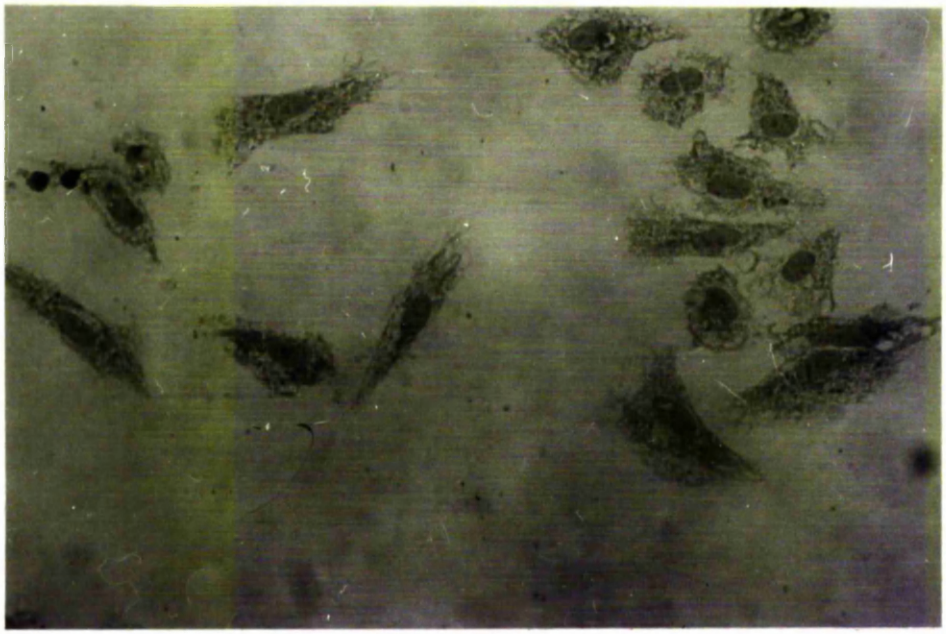
IXc

PLATE Xa



Primary clone of endothelial HUV cells derived from the lumen of human umbilical vein.
(x 180)

PLATE Xb

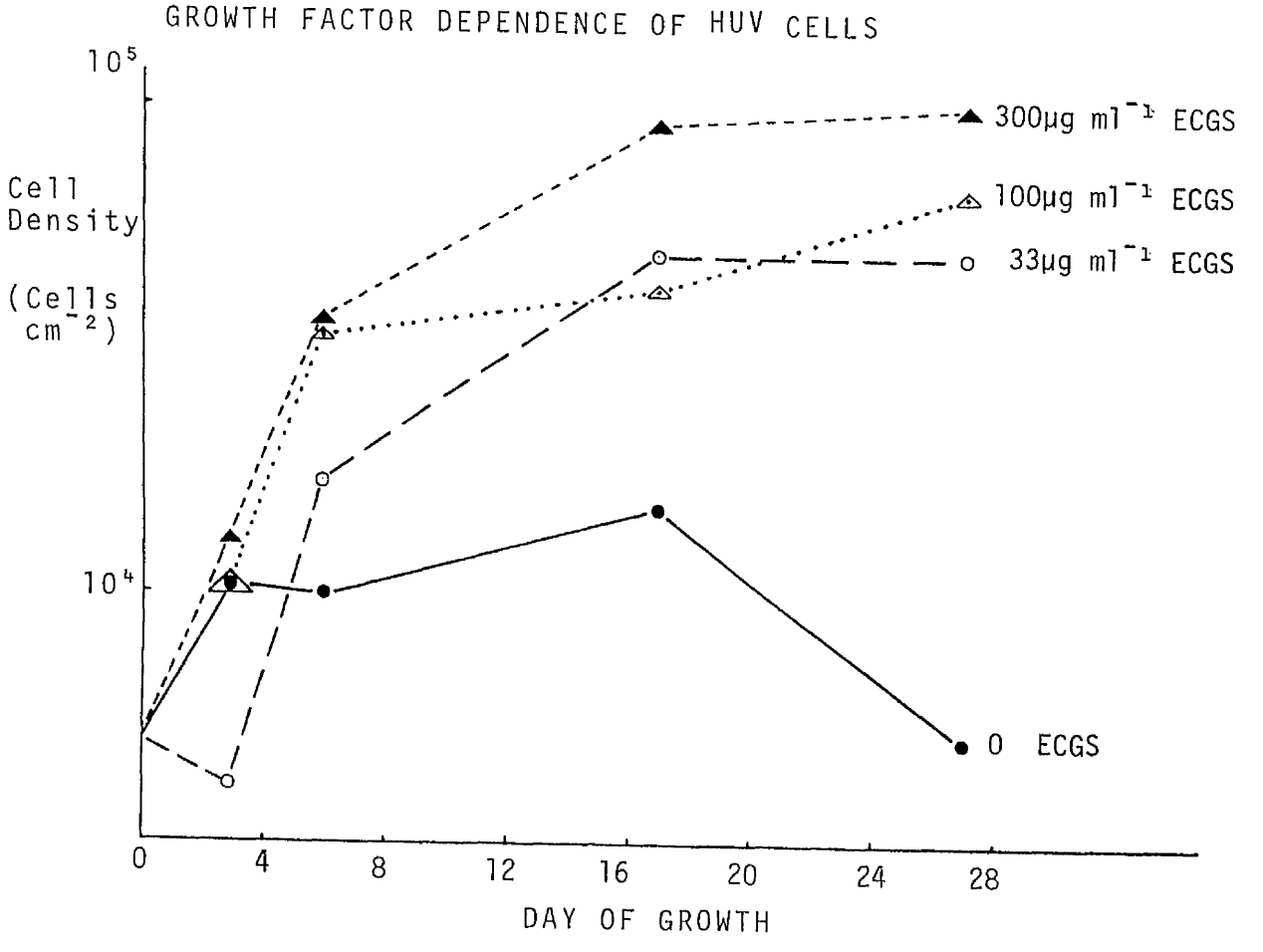


HUV cells positively stained for factor VIII by immunoperoxidase. Characteristic granular staining is evident.
(x 320)

FIGURE 11

Semi-logarithmic plot of cell density against time for HUV endothelial cells grown in culture medium containing various concentrations of Endothelial Cell Growth Supplement (ECGS). The cells were grown in 24-well plates and culture medium renewed every 2 to 3 days. Each time point represents the mean of cell counts from 4 replicate wells.

FIGURE 11


GROWTH FACTOR DEPENDENCE OF HUV CELLS
10⁵
10⁴
Cell Density (Cells cm⁻²)
300µg ml⁻¹ ECGS
100µg ml⁻¹ ECGS
33µg ml⁻¹ ECGS
0 ECGS
0
4
8
12
16
20
24
28
DAY OF GROWTH

membrane bound tubular matrix, as shown in Plate Xc and tight junctions between cells as shown in Plate Xd. Plate Xe shows the way in which the cells tightly pack at confluence forming the "pavement" pattern observed microscopically. When HUV cultures were maintained at high density for a few weeks, with continual medium renewal, a large proportion of the cells detached from the substrate and were released into the medium. Accompanying this were morphological alterations of the remaining cells, many of which fused together and formed a tubular network structure over the growth surface. Plates Xf and g show the initial and later stages of tube formation respectively.

Mitogenesis of HUV cells in Culture

In a preliminary experiment, the ability of the Walker Rat Carcinoma-256(WRC-256) cell extract, to stimulate endothelial cell mitogenesis, was investigated.

Cells were seeded at low density (2×10^4 cells cm^{-2}) into wells of plastic microtitration dishes, half of which had been coated in collagen and allowed to dry. The cells were fed every 2 - 3 days with 200µl fresh medium (F10/20% foetal calf serum/100µg ml^{-1}ECGS) and the cells counted by trypsinisation at the times indicated in Figure 12a. The cells grown on plastic reached a much higher density (2.5×10^5 cells cm^{-2}) than those grown on collagen (7.5×10^4 cells cm^{-2}). However, on reaching these densities, many of the cells grown on plastic began to shed into the medium and structure formation took place, while those grown on collagen could be maintained as a complete monolayer without any cell loss or morphological alteration.

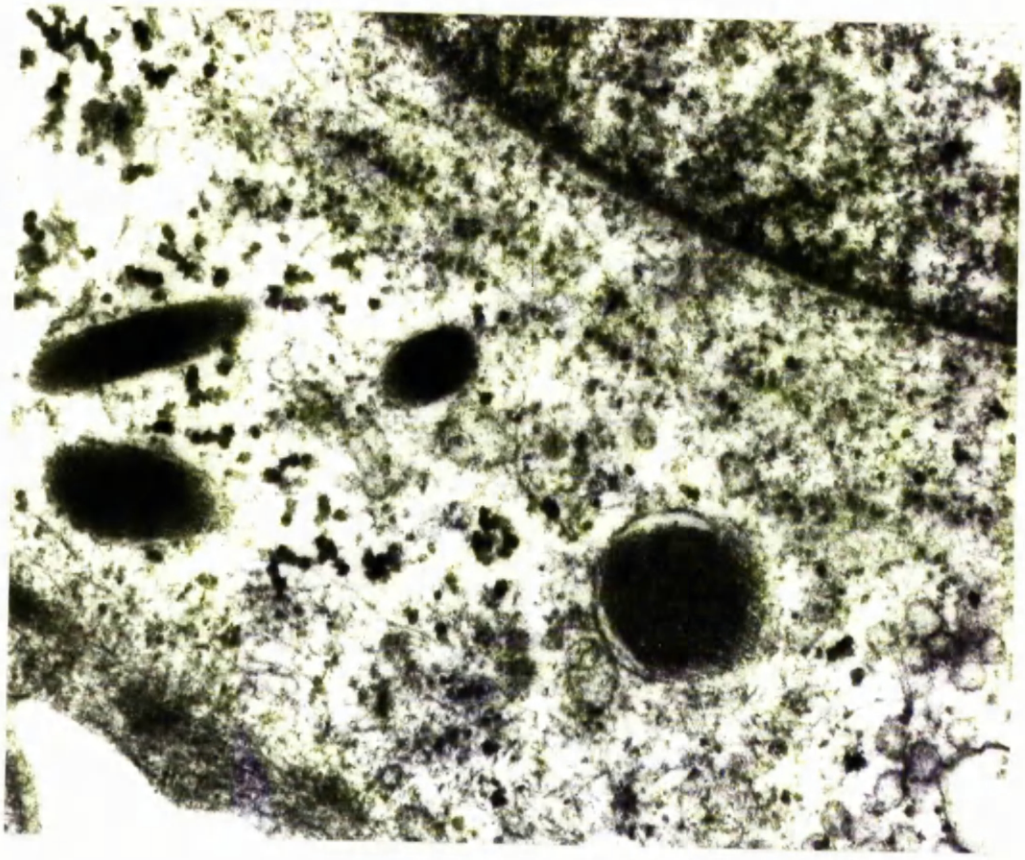
PLATE Xc

Electron micrograph revealing the presence of Weibel-Palade bodies in the cytoplasm of HUV cells.
Magnification 75,000

PLATE Xd

Membrane contacts between HUV cells.
Magnification 33,333

Xc



Xd

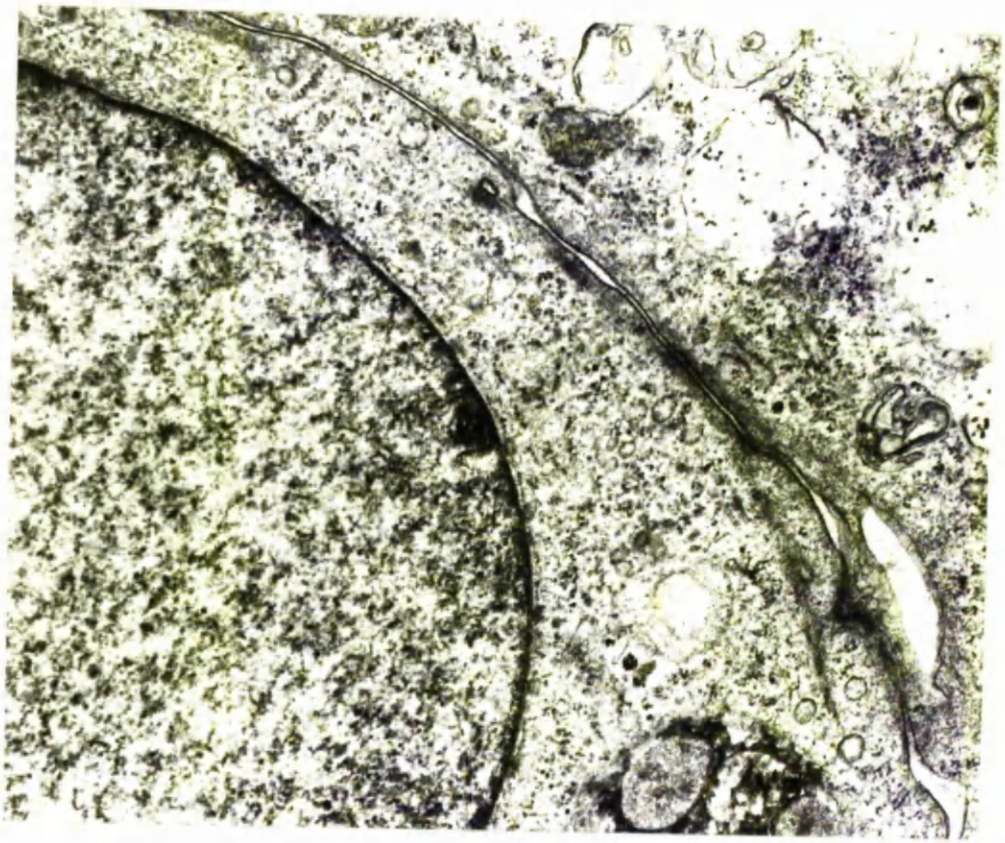
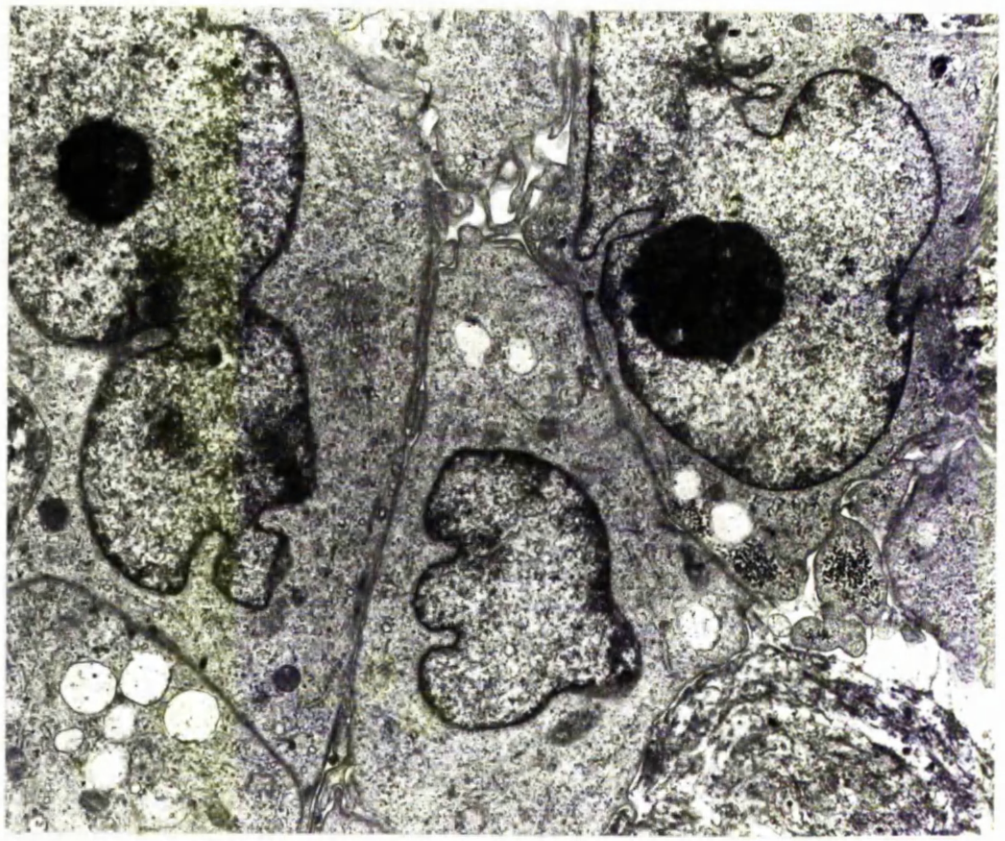
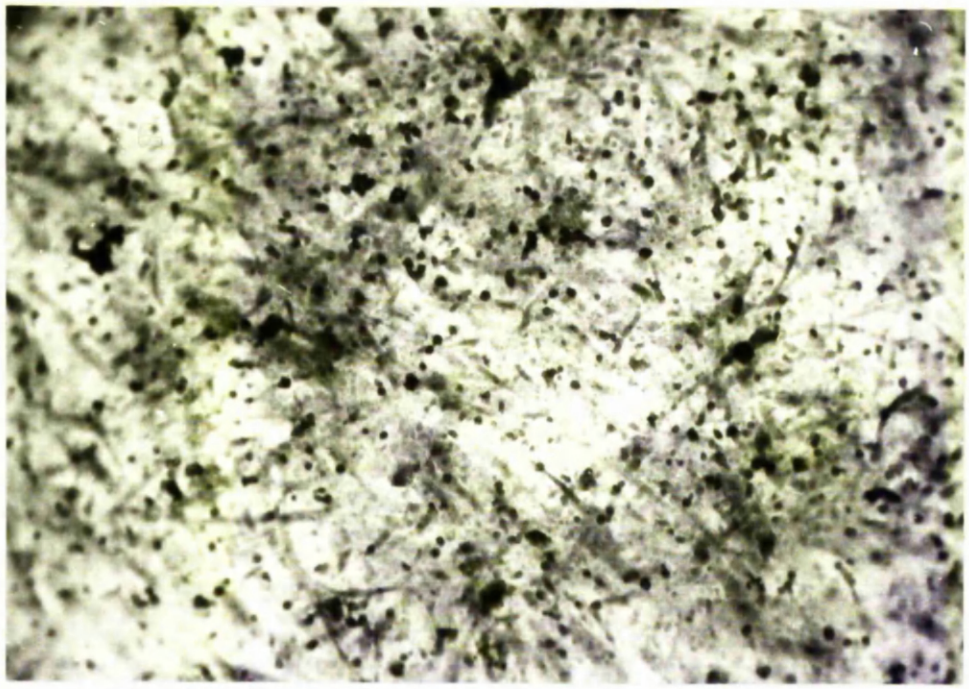


PLATE Xe



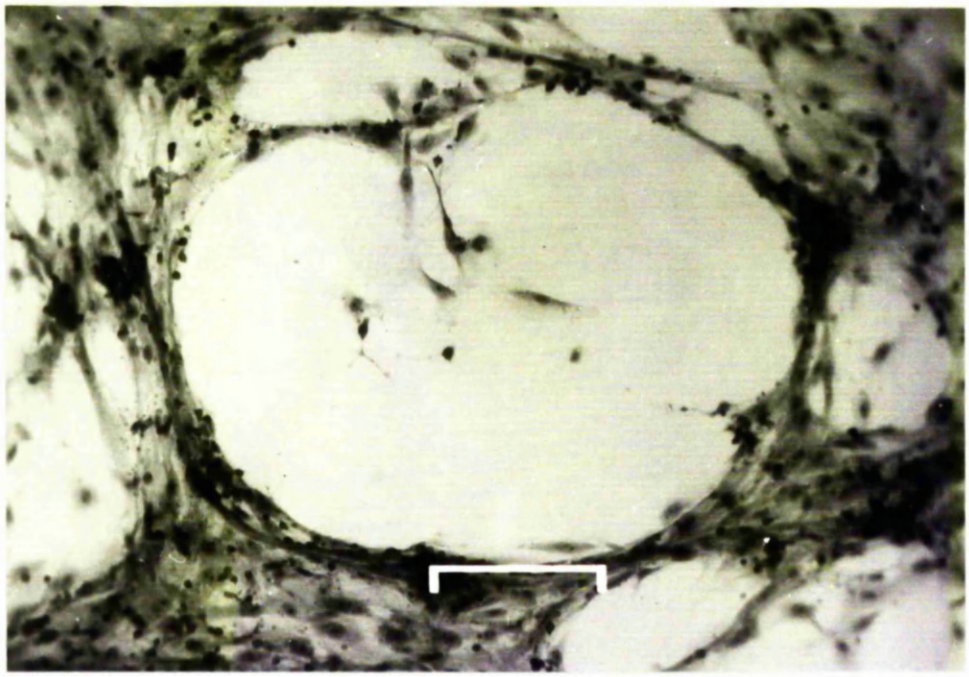
Electron micrograph showing the tightly packed "pavement" pattern of confluent HUV cells.
Magnification 11,666

PLATE Xf



HUV cells after 21 days at high density. The initial stages of reorganisation and structure formation are evident. Giemsa stained.
(x 200)

PLATE Xg



Later stages of structure formation. HUV cell shedding has taken place and the formation of "tubes" or <u>in vitro</u> "capillaries", is evident.
(x 220)

The addition of WRC-256 extract to HUV cells grown on collagen, at the plateau phase of the growth curve, stimulated further proliferation as seen in Figure 12b. This indicated that the WRC-256 extract contained a factor or factors capable of stimulating resting endothelial cultures to proliferate *in vitro*. Whether this effect of WRC-256 extract was due to the presence of active TAF in the extract or to some less specific mitogenic factor(s) required further experimentation. Extracts from a variety of normal brain and malginant glioma derived cell lines, some of whose angiogenic activity on the CAM were known, were therefore tested for their ability to stimulate endothelial mitogenesis. The results, presented in Figure 12c, showed that extracts from most cell lines, both normal and malignant, were able to stimulate HUV cell division to various extents. However, the relative abilities of cell line extracts to induce angiogenesis on the CAM (Figure 10) was not the same as their relative activities in stimulating HUV mitogenesis. A particular example of this was seen with G-RAT and G-CCM whose relative activities were reversed in the two assay systems. The normal brain derived cell line GDU-T, which showed very little angiogenic activity on the CAM, was able to stimulate mitogenesis considerably. These results implied that endothelial cell mitogenesis *in vitro* did not relate directly to angiogenic activity, as determined by vasoproliferation on the CAM. However, there were some similarities. With the exception of GDU-T, all the extracts of the malignant gliomas and WRC-256 cells, previously shown to induce angiogenesis on the CAM, stimulated HUV to a greater

FIGURE 12a

Semi-logarithmic plot of HUV cell growth to saturation density on plastic and collagen-coated plastic in microtitration plates. Culture medium was renewed every 2 to 3 days. Points represent the means and standard deviations of cell counts from 6 replicate wells.

FIGURE 12b

Induction of mitogenesis in density limited cultures of HUV cells grown in collagen coated microtitration plates. Replicate samples from plateau phase (Figure 12a) were treated with Bovine Serum Albumin (BSA) or WRC-256 cell extract as indicated. Further growth was measured by cell counts. Time points represent the means and standard deviation of 6 replicate wells.

FIGURE 12a

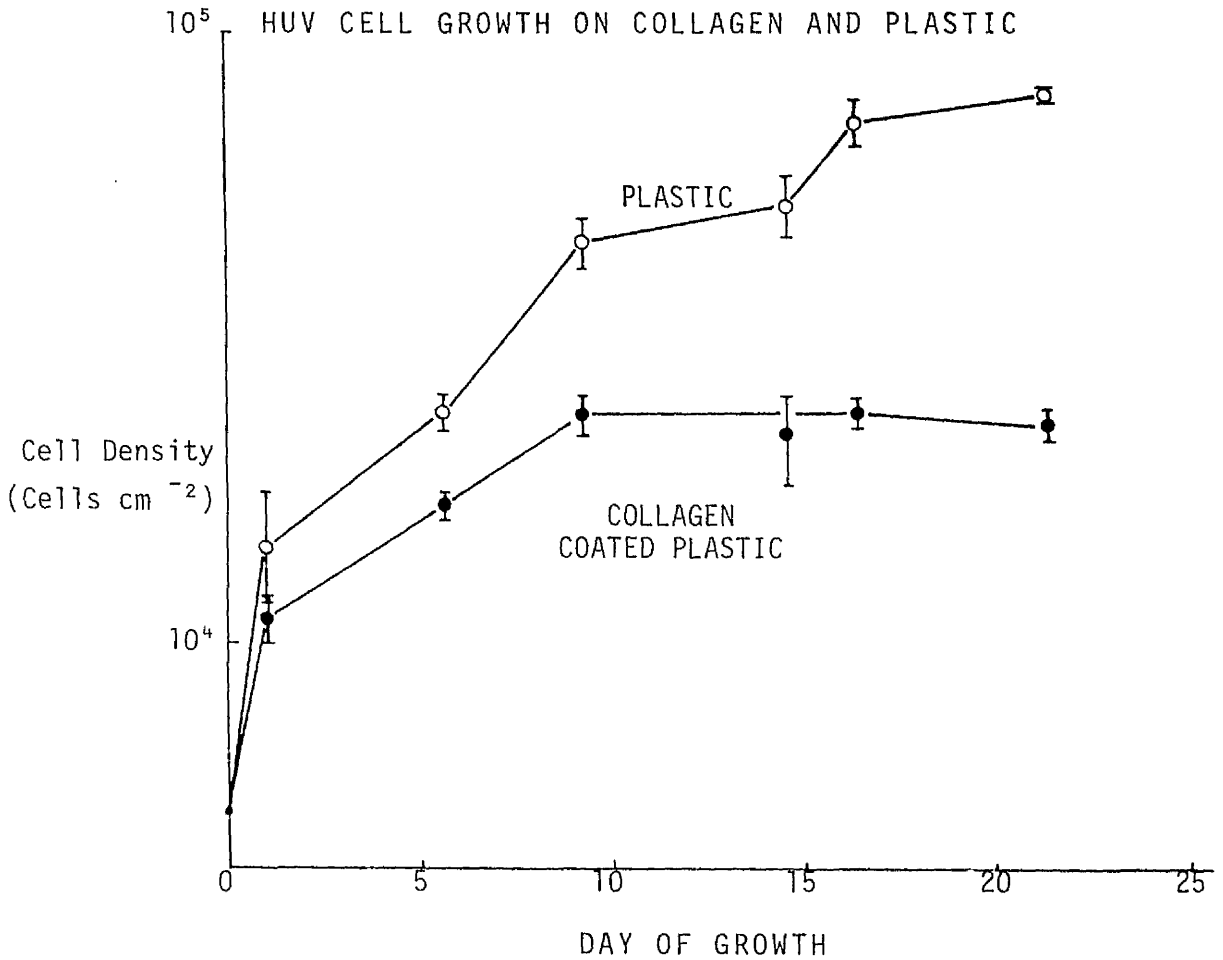


FIGURE 12b

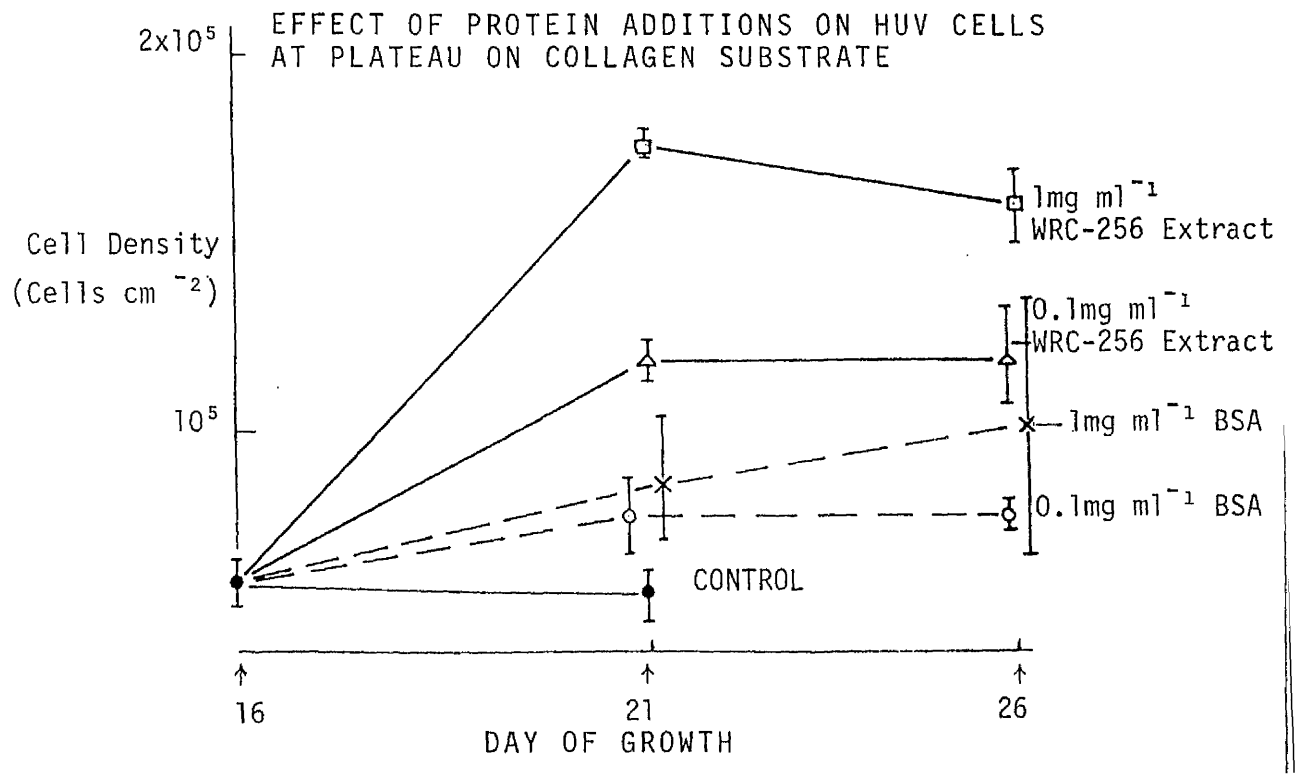
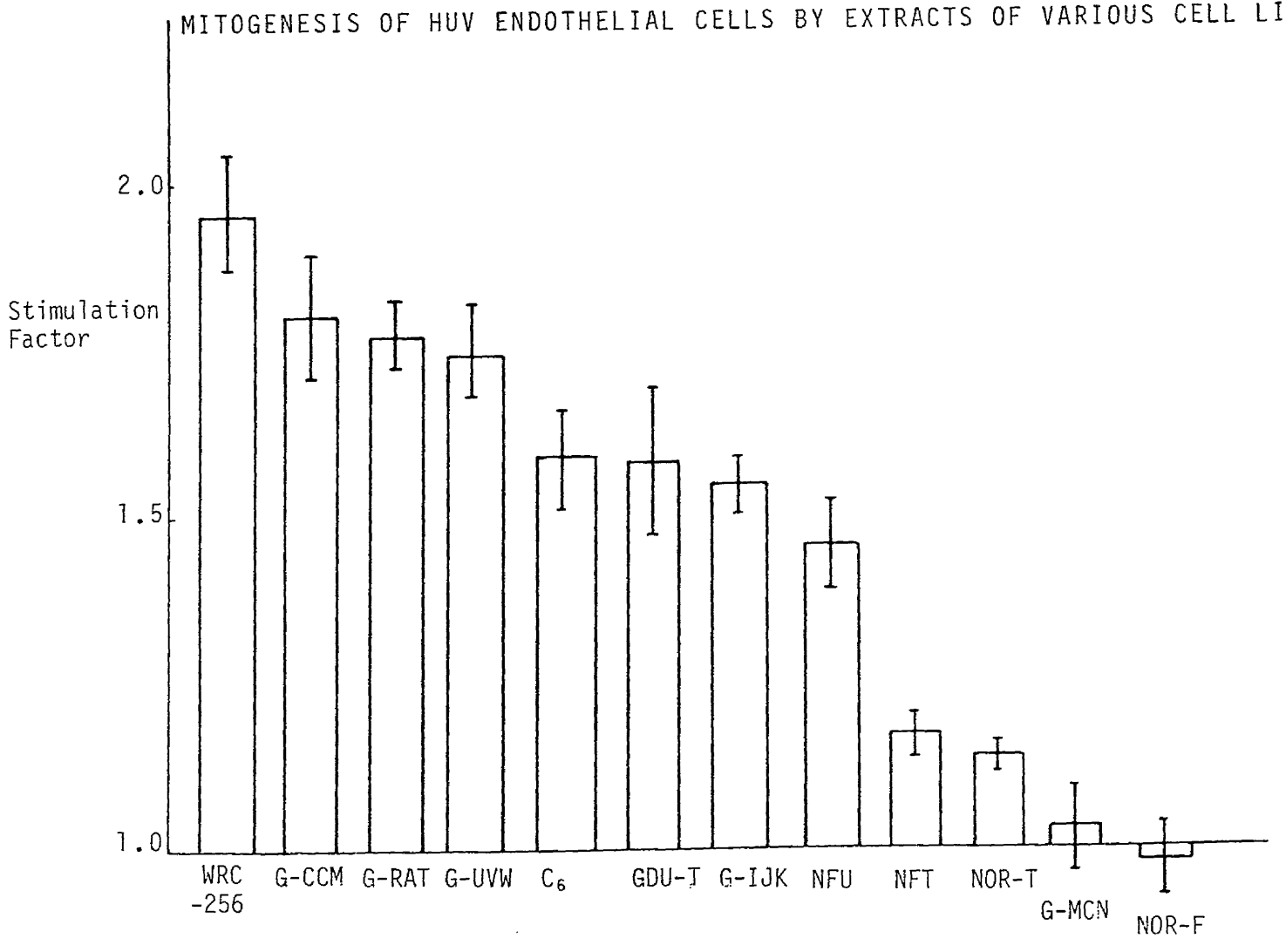


FIGURE 12c

Stimulation of HUV cell growth at high density by extracts of various cell lines. The stimulation factor assigned to a cell line was the plateau density after exposure to its extract at $1 mg ml^{-1}$ divided by the HUV plateau density under standard culture conditions. The mean and standard deviations of 6 replicate microtitration wells are shown.

FIGURE 12c


MITOGENESIS OF HUV ENDOTHELIAL CELLS BY EXTRACTS OF VARIOUS CELL LINES
Stimulation Factor
2.0
1.5
1.0
WRC -256
G-CCM
G-RAT
G-UVW
C6
GDU-T
G-IJK
NFU
NFT
NOR-T
G-MCN
NOR-F

extent than the foetal and normal adult brain derived cells. G-MCN, a glioma cell line not previously tested on the CAM, had no stimulatory effect on HUV.

3.2 Cell Density

Monolayer Culture

The effect of increasing cell density on the expression of GFAP in rat C_6 cultures is shown in Figure 2a. As the cells reached confluence and cell-cell contacts were formed, the proportion of GFAP positive cells increased dramatically from less than 50% to around 75%. C_6 was the only cell line which showed density induction of GFAP. It was particularly suitable since,in control populations about half the cells were GFAP positive. The human glioma cell lines studied were either 100% GFAP positive in control cultures (G-CCM) or were predominantly or entirely GFAP negative. In the latter case there was no visually detected increase in the number of GFAP positive cells with increasing cell density.

Experiments which studied the effect of monolayer cell density on the expression of β-alanine sensitive GABA (BASG) uptake and plasminogen activator (PA) production were also carried out. The results for BASG uptake by NOR-F and C_6 presented in Figure 13a and b, showed a similar, but less dramatic effect to that seen for GFAP in C_6 cultures. As the NOR-F cells reached confluence BASG uptake increased implying enhanced activity of the high affinity GABA uptake system. Similar results were obtained for NOR-T and G-IJK cultures.

Increasing monolayer cell density also decreased cellular PA production. This was true for all the cell lines

FIGURES 13a and 13b

Semi-logarithmic plots of NOR-F and C_6 (respectively) mean cell densities against day of growth in 24-well plates. Culture medium was renewed every 2 to 3 days. β Alanine sensitive GABA uptake (BASG) measurements were made at various time points as described in Materials and Methods section. BASG points are the means and standard deviation of 4 replicate wells.

FIGURE 13a

EFFECT OF CELL DENSITY ON βALANINE SENSITIVE GABA UPTAKE

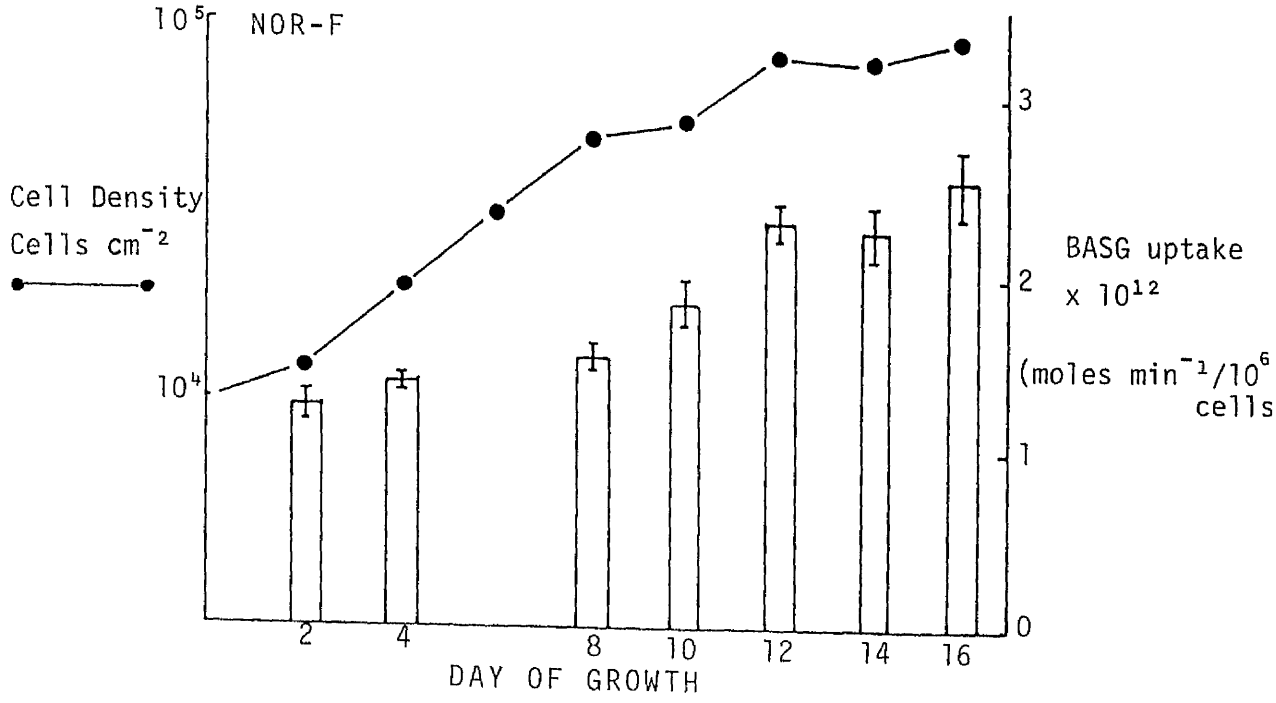
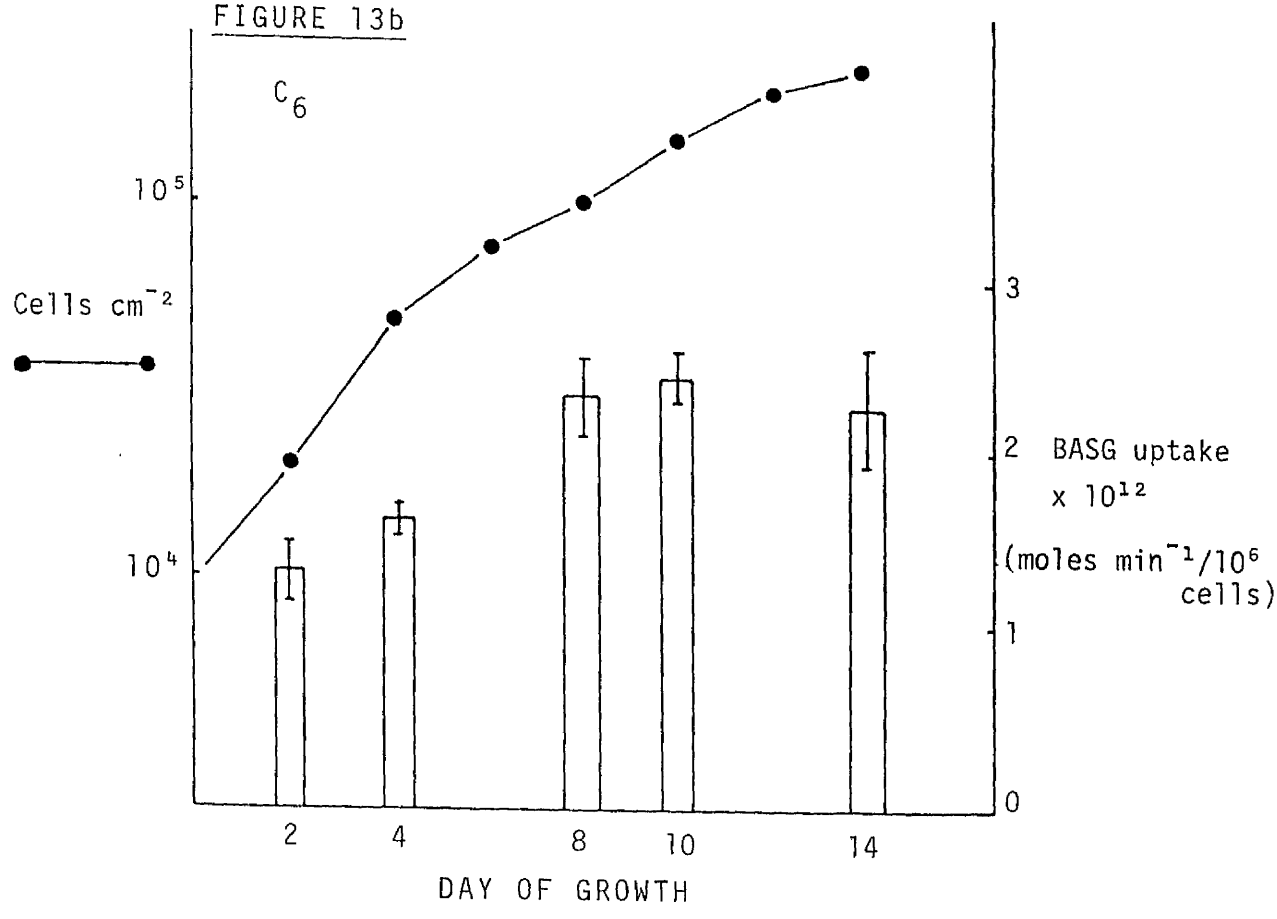


FIGURE 13b



investigated to different extents, although the effect was most clearly demonstrated by cell lines which exhibited high PA levels. Figure 14 shows the decline in PA production by G-ATA with increasing cell density. These results clearly showed the importance of carrying out chemical phenotypic manipulation experiments on post confluent cultures, when the levels of expression of at least three of the parameters used to represent the differentiated and malignancy-associated phenotypes were stable.

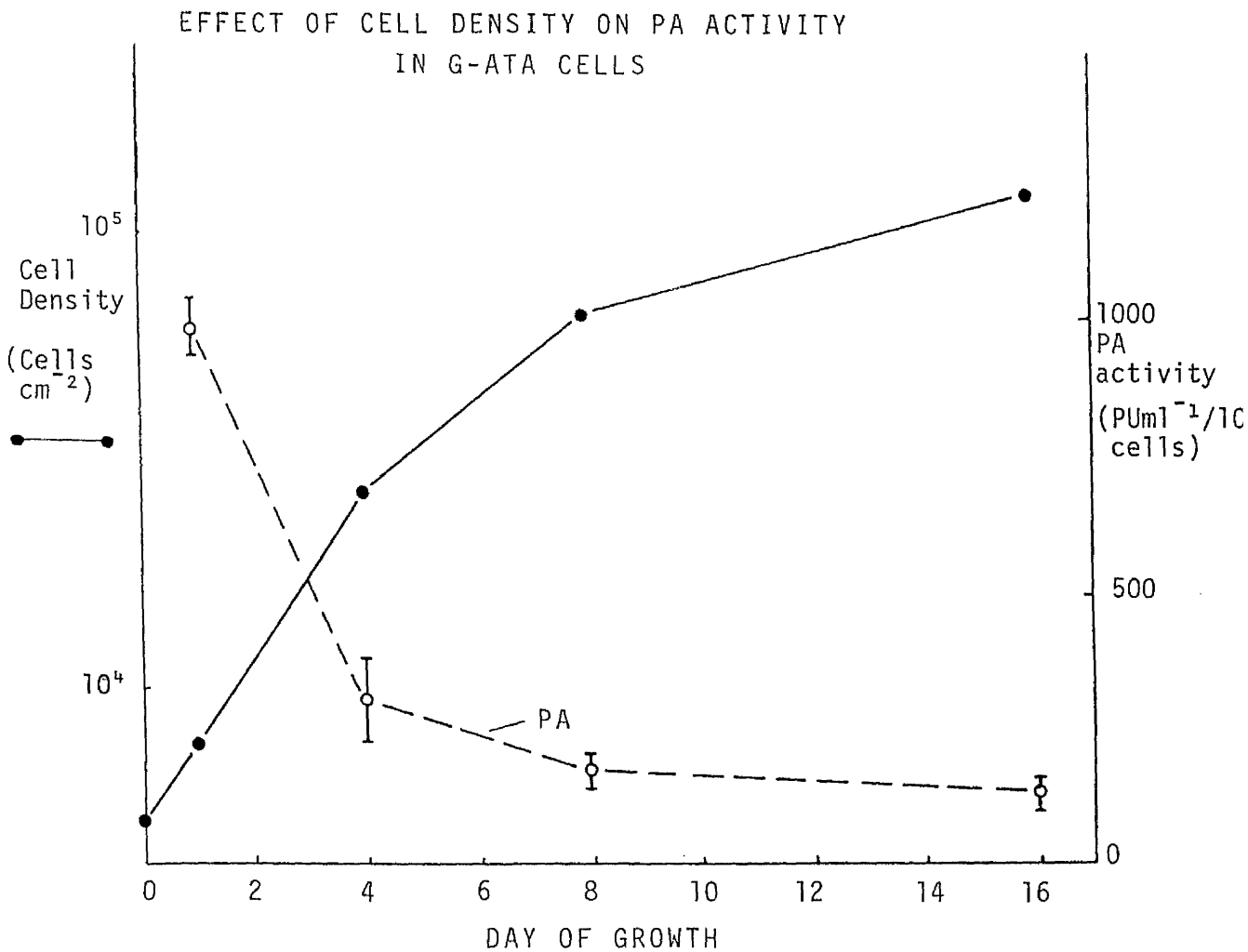
The alterations in phenotypic expression as a result of cell cultures reaching a high density, could be due to one or both of the consequences of dense monolayer culture i.e. reduction in cell proliferation and the formation of cell-cell contacts. In order to distinguish between these, serum was withdrawn from exponentially growing cultures and BASG uptake assayed prior to, and a few days after, serum withdrawal. NOR-F cells, at a density of 5.2×10^4 cells cm^{-2} when the cells were rapidly dividing, took up 1.30×10^{-12} moles min^{-1} per million cells by the β alanine sensitive pathway. At a density of 5.9×10^4 cells cm^{-2}, when the cultures had stopped dividing as a result of serum withdrawal, the β alanine sensitive uptake of GABA was 1.33×10^{-12} moles min^{-1} per million cells. This suggested that the increase in BASG uptake as cells reached confluence was not due to reduction in proliferation and the onset of cytostasis. In both these cases, cultures were pre-confluent and few cell contacts formed.

A different approach was necessary to study the alteration in cellular PA production with increasing cell density, due to the complicating effect of serum. As discussed previously, serum withdrawal for several days, automatically increases the detected levels of PA by removal of cell surface bound inhibitors. A

FIGURE 14

Semi-logarithmic plot of G-ATA mean cell density against day of growth in 24-well plates. At each density point cells were assayed for plasminogen activation (PA) by the chromogenic assay described in Materials and Methods section. The substrate was S-2302. PA measurements are the means and standard deviation of 4 replicate wells.

FIGURE 14


EFFECT OF CELL DENSITY ON PA ACTIVITY
IN G-ATA CELLS
10^5
Cell
Density
(Cells
cm^{-2})
10^4
1000
PA
activity
($PUml^{-1}$/1C
cells)
500
0
PA
0
2
4
6
8
10
12
14
16
DAY OF GROWTH

comparison of cells at high and low densities, under proliferating and non-proliferating conditions was made in the following way:-

1. Cells seeded at low density and allowed to proliferate to high density in medium containing 10% serum.

11. Cells seeded at low density in serum free medium and cells seeded at post confluent density in serum free medium.

PA assays were carried out on low and high density cells under conditions 1 and 11 above. The results for G-ATA are shown in Figure 15. In 1 the absolute difference in PA production per million cells between low and high density cultures was 322 PU ml^{-1} and could have been due to reduced cell proliferation and/or contact formation. In 11, however, although detected PA levels were greater, as expected, the absolute difference between low and high density cultures was reduced to 110 PU ml^{-1}. As cells were not dividing at either low density or high density in 11, due to the absence of serum, the reduction in detected PA at high density was probably the result of formation of cell-cell contacts. The values for the differences in PA activities for low and high density cultures in 1 and 11, implied that although cell-cell contacts played a part in the considerable reduction in cellular PA production at high density, the decrease in the rate of cell proliferation was also important.

Three Demensional Perfusion Culture

The Vitafiber culture system, as described in 1.6.1 and illustrated in Plate III was used to grow normal brain (NOR-F) and glioma (G-RAT) cells in high density, three dimensional clusters. The capillary units were assembled in a Laminar Flow cabinet after steam sterilisation and the polycarbonate fibre bundles coated in foetal calf serum

FIGURE 15

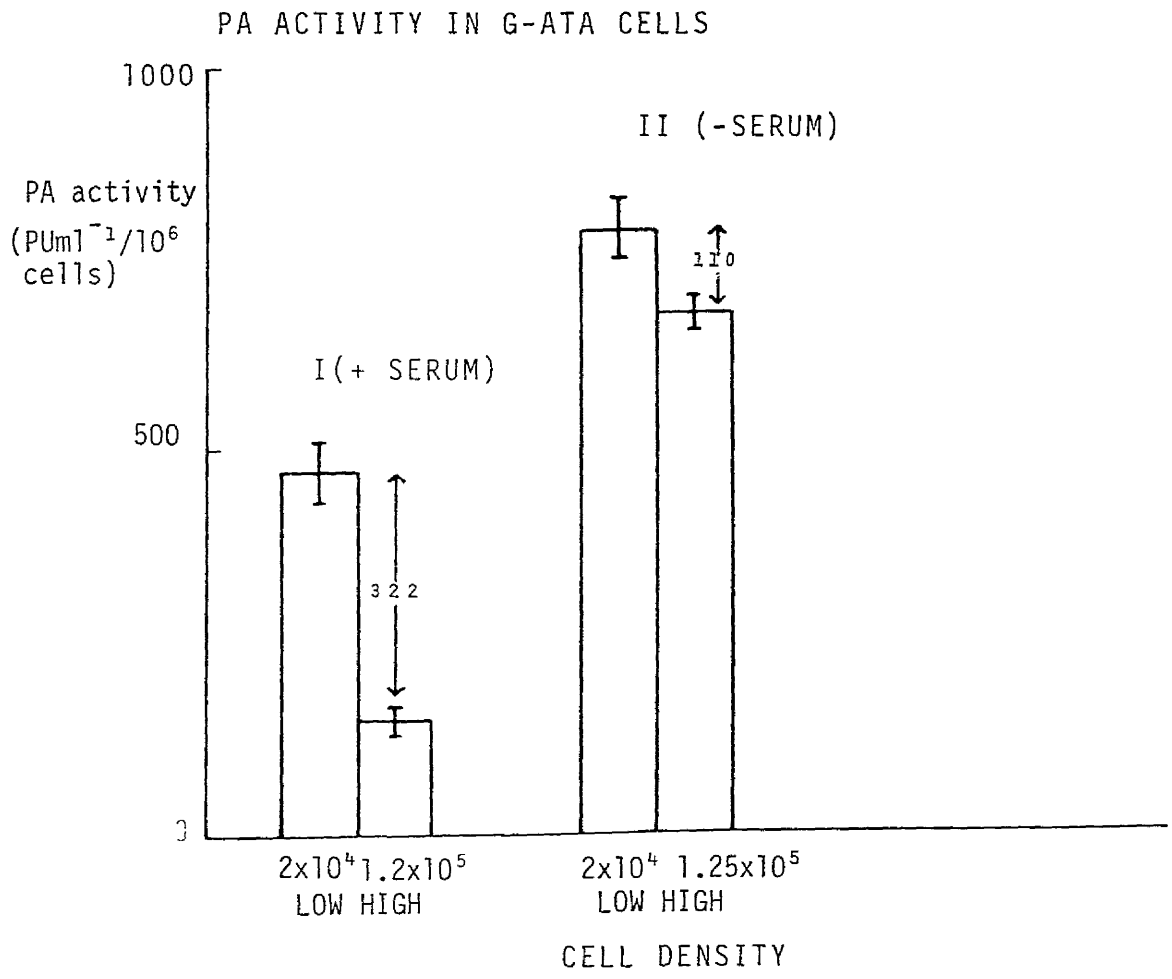
Plasminogen activator (PA) activities of G-ATA cultures under low and high density conditions and

I in the presence of serum, and

II in the absence of serum.

PA assays as described in Materials and Methods section using the chromogenic substrate S-2302. Points are the mean and standard deviation of 4 replicate wells of 24-well plates.

FIGURE 15


PA ACTIVITY IN G-ATA CELLS
1000
500
0
PA activity
(PUml^{-1}/10^6 cells)
I(+ SERUM)
II (-SERUM)
322
110
2x10^4 1.2x10^5
LOW HIGH
2x10^4 1.25x10^5
LOW HIGH
CELL DENSITY

to enhance cell attachment. Cells were inoculated by injecting a single cell suspension containing 5 x 10^6 cells in 1ml of medium (F10/10% serum) through the top ports into the extracapillary space. The suspension was flushed back and forwards by two syringes for a few seconds and the cells allowed to settle for two hours. The chambers were then rotated through 180° to allow any unattached cells to settle in the opposite direction. The rotation was carried out every four hours for 24 hours at which time the peristaltic pump was switched on and the medium allowed to circulate continuously. These conditions were found to be optimum for "loading" cells on to the polycarbonate fibre bundles to achieve maximum attachment.

After an initial lag period of about 2 - 3 days, the cells began to divide and eventually formed dense masses of cells at particular areas on the capillary bundles, notably directly below the top ports. Medium in the perfusate reservoir (250 ml) was replaced with fresh medium every 4 - 5 days. Cell growth was monitored by the rate of glucose utilisation which reached a steady state level after about 21 days of growth. The cells were maintained in the high density clusters for a few days after the rate of medium glucose utilisation had become constant, before removal from the capillary bundles by digestion with trypsin and collagenase. The resulting cell suspension, containing both single cells and cells which remained clumped, was then seeded into a 24 well plate, allowed to settle and attach, and examined for BASG uptake and PA production. After the cells had attached to the wells of the 24 well plate, further trypsinisation yielded single cell suspensions

which were used to obtain cell counts.

The initial inoculum for both NOR-F and G-RAT was 5×10^6 cells. The final yield of cells from the culture chambers obtained after two enzymatic digestion steps, was approximately 2.9×10^7 and 3.5×10^7 for NOR-F and G-RAT respectively. The results for BASG uptake and PA production, after the high density cell masses were removed from the perfusion culture chambers and seeded into the 24 well dishes, are shown in Table 6. The values were compared with those observed in high density monolayer culture. BASG uptake of cells grown in three dimensional cell masses was greater by a factor of about 1.4 in the case of NOR-F and a factor of 2 in the case of G-RAT, than equivalent cells in high density monolayer culture. This implied that rises in cell density over the monolayer terminal cell density as achieved in the three dimensional perfusion culture system, further increased high affinity GABA uptake in these cells. There was no detectable difference, however, between PA production by cells grown to high density in a monolayer or in three dimensional clusters for either of the cell lines looked at.

3.3 Heterologous Co-culture

Two cell lines, one normal brain derived (NOR-F) and one glioma (G-UVW) were grown in two distinct co-culture systems with the human neuroblastoma cell lines TRK-14 and IMR-32. In the first of these the glial and the neuroblastoma cells were grown on separate plastic coverslips, placed in the same petri dish containing 20ml medium (F10/10% serum). Although no heterologous contacts were formed the two cell types shared the same culture medium. In the second co-culture system glial cells were grown on the same substrate as the

Table 6 Effect of three dimensional high density perfusion culture on BASG uptake and PA

		MONOLAYER CULTURE CELLS (24 well plate)	VITAFIBER PERFUSION CULTURE CELLS (24 well plate after removal from fibres)
NOR-F	CELL DENSITY	1.9×10^{5} cm^{-2}	6.0×10^{5} cm^{-2}
	BASG UPTAKE	2.3×10^{-12} moles min^{-1} /10^{6} cells	3.26×10^{-12} moles min^{-1} /10^{6} cells
	PA	20.6 ± 3.2 PUml^{-1} / 10^{6} cells	22.3 ± 4.6 PUml^{-1} / 10^{6} cells
G-RAT	CELL DENSITY	2.3×10^{5} cm^{-2}	7.2×10^{5} cm^{-2}
	BASG UPTAKE	0.098×10^{-12} moles min^{-1} /10^{6} cells	1.87×10^{-12} moles min^{-1} /10^{6} cells
	PA	85.3 ± 9.2 PUml^{-1} / 10^{6} cells	83.7 ± 12.1 PUml^{-1} / 10^{6} cells

neuroblastoma cells,in 75 cm^2 plastic culture flasks, thus allowing both homologous and heterologous cell-cell contacts to be formed.

The normal and malignant glial cells were maintained in co-culture with neuroblastoma for between 7 - 10 days. After this time the morphologies of the cells were observed and BASG uptake and PA activities of NOR-F and G-UVW determined. In the case of the heterologous contact co-culture, prior separation of the cells on Percoll gradients was carried out. BASG uptake and PA activities were compared with these for NOR-F and G-UVW grown in homologous culture.

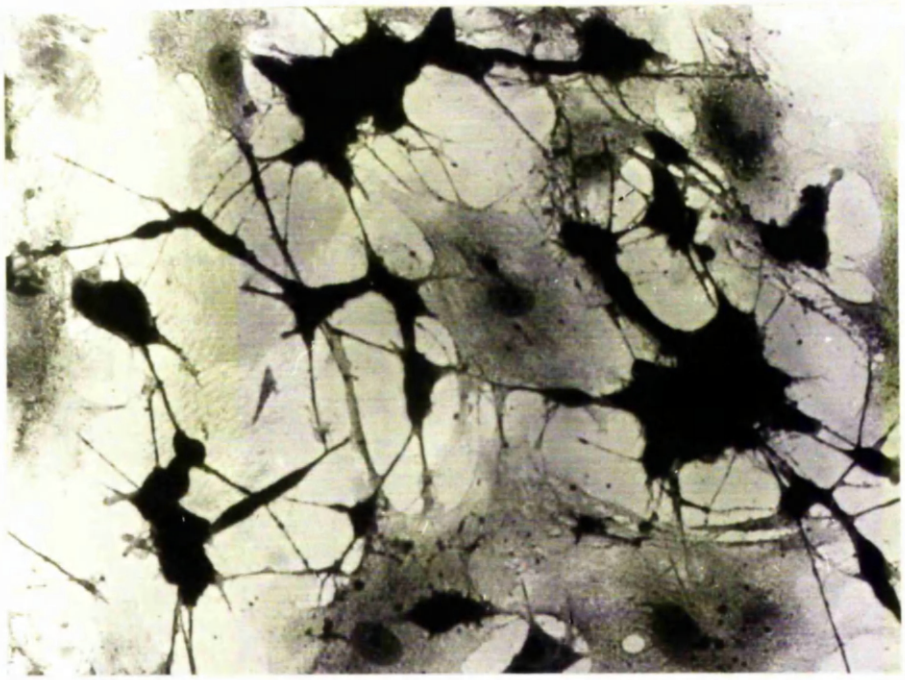
Morphology

When TRK-14 and IMR-32 cells were co-cultured with NOR-F, active extension of neuroblastoma processes were seen giving rise to very long thin cell extensions (Plate XIa) not evident in the neuroblastoma cells cultured alone or with G-UVW (Plate XIb). This change in morphology was not reciprocal i.e. the presence of neuroblastoma caused no morphological alteration of NOR-F cells, and was not seen when the cells were cultured separately in the same medium. These observations implied that NOR-F cells, when in contact with TRK-14 and IMR-32 induced morphological differentiation in the neuroblastoma cells which was not seen when in contact with G-UVW.

BASG Uptake

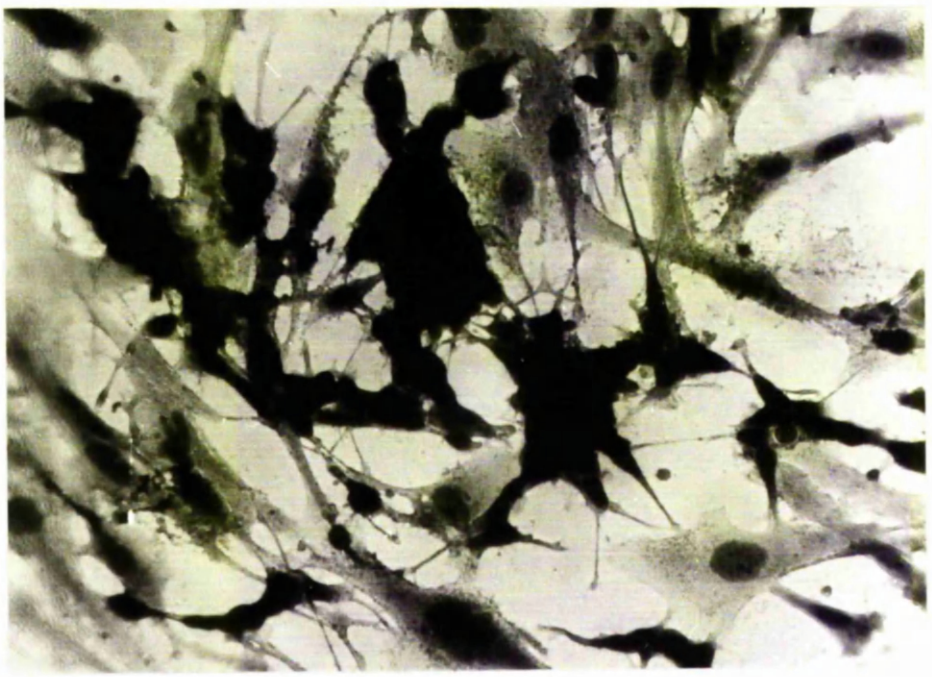
There was no detectable difference in BASG uptake by NOR-F or G-UVW between cells grown in homologous culture and those grown separately in the same culture vessel, sharing the same medium as the neuroblastomas. When the cells

PLATE X1a

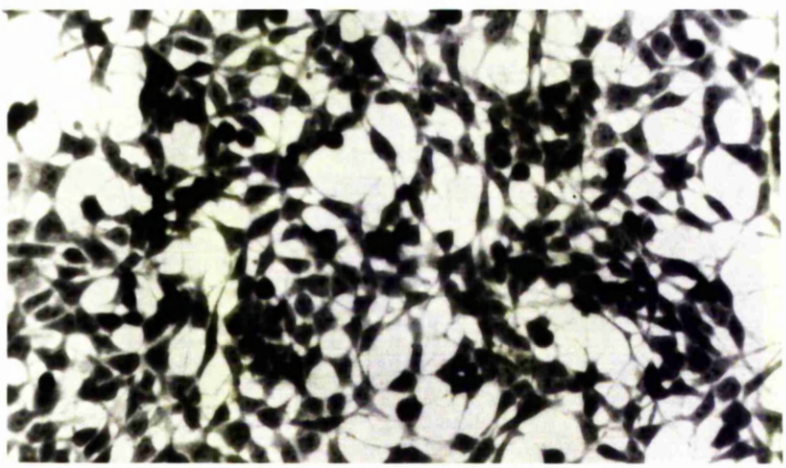


IMR-32 neuroblastoma (densely stained) co-cultured with NOR-F cells (lightly stained). The neuroblastoma cells formed dense aggregates when seeded with cells of a different type. IMR-32 - NOR-F co-cultures led to the extension of long thin processes by the neuroblastoma cells. Giemsa stained.
(x 360)

PLATE X1b



IMR-32 neuroblastoma co-cultured with G-UVW cells. Giemsa stained.
(x 360)



IMR-32 neuroblastoma homologous culture. Cells attached and growing as single cells. The formation of dense aggregates by neuroblastoma, as seen in co-cultures, is not evident. Giemsa stained.
(x 360)

were grown in contact with TRK-14 or IMR-32 the BASG uptake by NOR-F cells increased from 2.4×10^{-12} units to 3.17×10^{-12} units (1 unit ≡ 1 mole/min per 10^6 cells). There was no increase in the BASG uptake by G-UVW cells co-cultured with neuroblastoma.

Plasminogen Activator (PA)

The PA activities of both NOR-F and G-UVW cells were not significantly altered by growth in either of the co-culture systems described. Activities of 47 PU $ml^{-1}/10^6$ cells and 115 PU $ml^{-1}/10^6$ cells respectively, were very close to the values obtained for NOR-F and G-UVW grown in homologous culture.

3.4 Chemical Agents : Effect on phenotypic expression

A number of cell lines (normal adult, foetal and malignant brain derived) were grown to confluence in monolayer culture systems i.e. microtitration plates, 24 well dishes or plastic flasks, and treated for 6 - 7 days with medium containing 10% serum, 1% serum, and 1% serum plus a variety of chemical agents. Lists of the cell lines and the culture conditions to which these were subjected are presented in Tables 7 and 8 respectively. The cellular levels of differentiation markers i.e. GFAP, high affinity GABA uptake, glutamine synthetase, and malignancy associated markers i.e. plasminogen activator and endothelial cell mitogenic activity, were then determined. Owing to the large number of cell lines and chemical conditions investigated for the five marker properties, it was not possible to use large sample numbers in each assay. Statistical analyses were therefore only carried out for each cell line under control conditions i.e. F10 medium,low serum. This series

Table 7 Cell Lines used in Chemical Induction Studies

ADULT BRAIN (NORMAL)

NOR-T, NOR-F, GDU-T

FOETAL BRAIN

NFQ, NFR, NFS, NFT, NFU

ASTROCYTOMA

G-CCM, G-UVW, G-MCN, G-RAT, G-JPT, G-IJK, C_6

CONTROLS

WRC-256, HUV

Table 8

CHEMICAL AGENTS/DRUGS

1. F10/10% fcs (foetal calf serum)
2. F10/1% fcs
3. F10/1 + DEXAMETHASONE (DX)
4. F10/1 + ISOPROTERENOL (IP)
5. F10/1 + DX + IP
6. F10/1 + RETINOIC ACID (RET.Ac.)
7. F10/1 + INTERFERON (IFN β)
8. F10/1 + MITOMYCIN C(MIT.C)
9. F10/1 + PIG BRAIN EXTRACT (PBE)
10. F10/1 + N-METHYL ACETAMIDE (NMA)
11. F10/1 + PHORBOL MYRISTATE ACETATE (TPA)
12. F10/1 + METHYL NITROSOUREA (MNU)
13. F10/1 + TPA + MNU

of experiments was thus regarded as a screening system, looking for chemically induced changes in phenotypic expression, consistent for a number of cell lines and leading to identification of agents of particular interest for further study.

GFAP

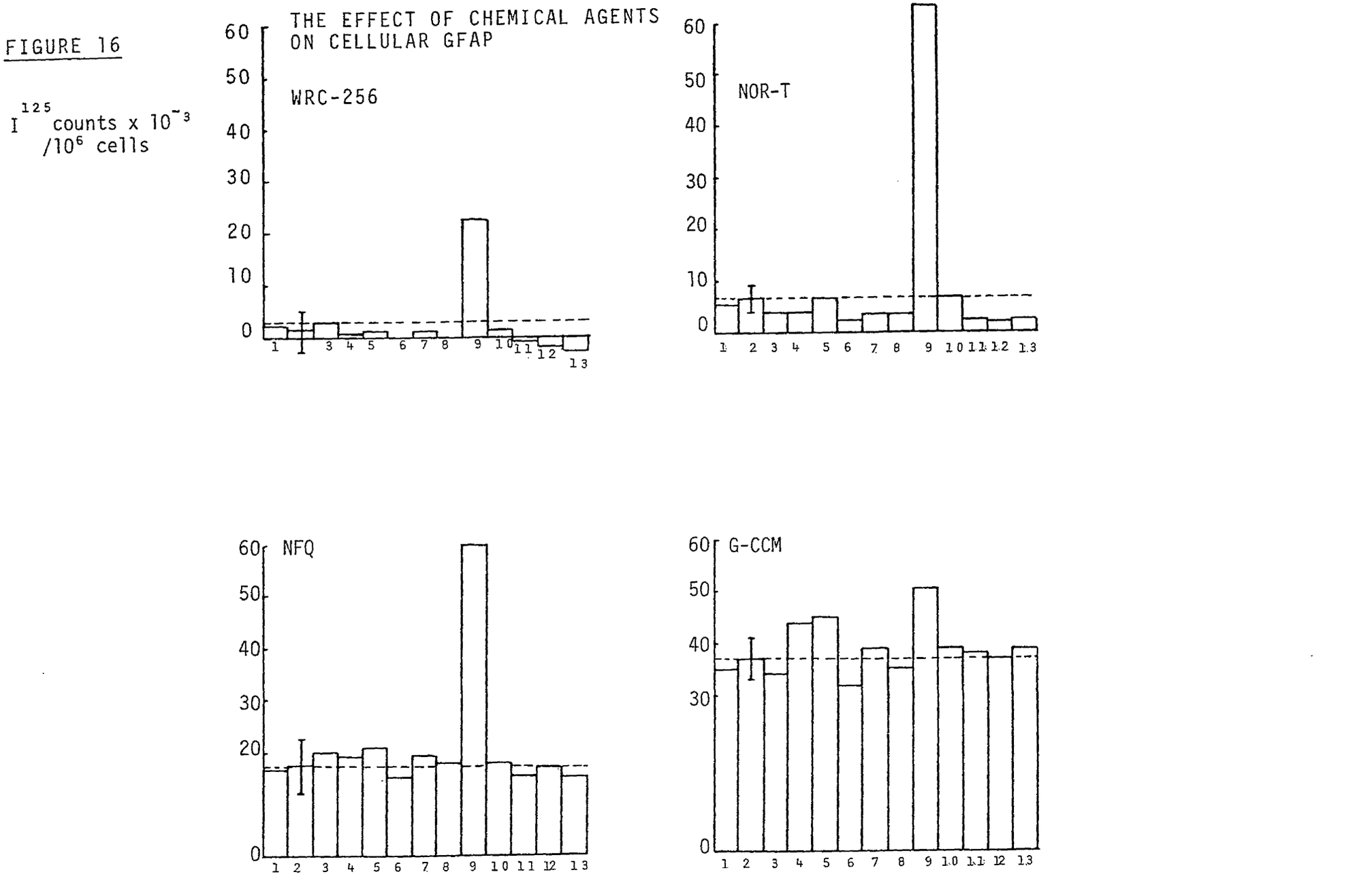
GFAP was quantitatively assessed after the cells had been grown and treated with drugs in microtitration plates, by fixing the cells in methanol and treating with firstly a rabbit anti-human GFAP antiserum followed by a goat anti-rabbit IgG, previously labelled with I^{125}. The unbound antisera were washed off with several PBS washes and the labelled cells dissolved in 0.3N/NaOH/1%SDS. The iodine counts were determined by gamma counting and corrected for cell number. The results for the GFAP content of the WRC-256 negative control, NOR-T, NFQ and G-CCM under various conditions of drug treatment are shown in Figure 16. The three normal lines NOR-F, NOR-T and GDU-T all had control GFAP levels which were only slightly higher than those detected for the WRC-256 negative control. This was consistent with the lack of visual detection of GFAP in these cells by immunoperoxidase or immunofluorescence. The foetal cell line, NFQ, previously found to be GFAP positive by immunofluoresence, had a control GFAP level which was intermediate between the level detected in normal adult cells and that in G-CCM. G-CCM had the highest detected level of any cell line studied and this was again consistent with visual observations.

With the exception of Pig Brain Extract (PBE), treatment of cell lines with the various drugs had no significant effect on the GFAP content of any of the cell lines studied. The

FIGURE 16

Histograms showing cellular GFAP content of WRC-256, NOR-T, NFQ and G-CCM cells. Conditions 1 to 13 refer to the culture conditions to which the cells were exposed for 6 to 7 days prior to assay, as described in Table 8. Each value is the mean of duplicates.

FIGURE 16


THE EFFECT OF CHEMICAL AGENTS ON CELLULAR GFAP
I^{125} counts x 10^{-3} /10^6 cells
WRC-256
NOR-T
NFQ
G-CCM
0
10
20
30
40
50
60
1 2 3 4 5 6 7 8 9 10 11 12 13

level of GFAP remained relatively constant over the range of treatment conditions. The apparent stimulation of cellular GFAP by PBE treatment was probably a consequence of pig GFAP being present in the crude PBE preparation, which cross reacted with rabbit anit-human GFAP. The cross reactivity of the crude PBE with the anti-serum was tested using a laser nephelometer. This measures the amount of light scatter produced as a result of an incident beam of light striking antigen-antibody complexes, while selectively minimising light scatter produced by interfering substances. With the nephelometer set to zero with a 1:100 dilution of anti-serum in polymeric buffer solution, the sample blank of 1:20 PBE in polymeric buffer gave a mean reading of 37 arbitrary units. The combination of anti-serum and antigen containing extract, at 1:100 and 1:20 respectively gave a mean reading of 178 arbitrary units, showing that an antibody-antigen complex had been formed. PBE thus contained GFAP, indicating that the protein is not species specific. The actual effect of PBE on the level of expression of cellular GFAP therefore remained undetermined. Further analysis would require the GFAP in the PBE to be absorbed out or further purification of the component in PBE responsible for the morphological differentiation of precursor glial cells.

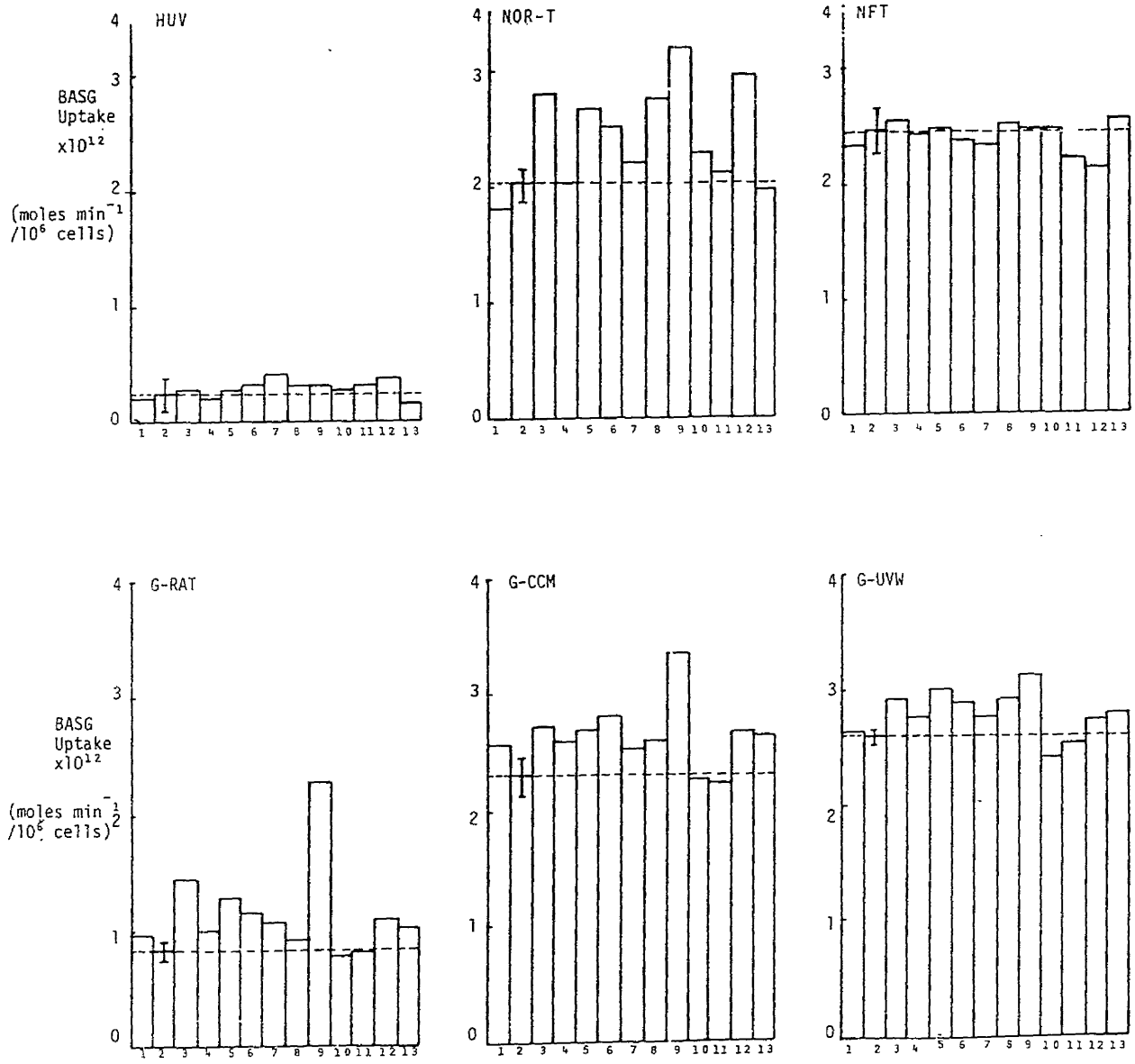
β Alanine Sensitive GABA (BASG) Uptake

Histograms representing the uptake of GABA (25μM), which was sensitive to 2mM β alanine for various cell lines (one endothelial, one foetal, one normal adult, and three gliomas) under the drug conditions described, are shown in Figure 17. In this case, the non-glial control was the endothelial cell line HUV, which had BASG uptake of less

FIGURE 17

Histograms showing high affinity GABA uptake of HUV, NOR-T, NFT, G-RAT, G-CCM and G-UVW cells, after 6 - 7 days exposure to conditions 1 to 13 (Table 8). Each value is the mean of duplicates. Variation between duplicate samples was always less than 4.4×10^{-13} moles $min^{-1}/10^6$ cells.

FIGURE 17 EFFECTS OF DRUGS ON HIGH AFFINITY GABA UPTAKE IN VARIOUS CELL LINES



than 0.5×10^{-12} unit (1 unit ≡ 1 mole $min^{-1}/10^6$ cells) and was unaffected by the various drug treatments.

The foetal cell lines NFT and NFR both had constitutive BASG uptake of 2.4×10^{-12} and 2.5×10^{-12} units respectively, reduced slightly by treatment with the phrobol ester (TPA) alone and in the case of NFT by N-methyl nitrosourea (MNU) also. The other drugs and chemical agents had no significant effect. The normal adult cell line NOR-T, under control conditions, had BASG uptake of about 2×10^{-12}units,which was increased by treatment with pig brain extract (PBE), methyl nitrosourea (MNU), dexamethasone (DX), mitomycin C (Mit.C), retinoic acid (Ret.Ac.),N-methyl acetamide (NMA), and interferon (IF), in order of magnitude of stimulation. Similar results were obtained for NOR-F, with the exception that NMA caused no significant increase for this cell line.

Within the group of glioma cell lines tested the results were more variable. Certain trends in response to drugs, however, were evident. In almost all cases DX, isoproterenol (IP), IFN, Ret.Ac., PBE, Mit.C and MNU increased BASG uptake with the greatest stimulation by PBE. Actinomycin D inhibited the PBE induced increase in BASG uptake from 2.28×10^{-12} units to less than 1×10^{-12} units, in G-RAT cells. Interferon (IFN) in each case, gave a small increase in BASG uptake. G-UVW, however, differed in drug response from most of the other gliomas. This cell line had a high constitutive BASG uptake of about 2.6×10^{-12} units which was only slightly increased by IP, Ret.Ac., IFN, PBE,MNU and DX.

Glutamine Synthetase (GS)

Determination of cellular GS levels was carried out using homogenates of post confluent cultures, treated with drugs as for GFAP and BASG uptake studies, except that glutamine in the culture medium was replaced with an equimolar amount of glutamate. This was to allow determination of GS levels in the absence of any repression by glutamine as previous work by De Mars (254) and Fottrell and Paul (255) has shown that the growth of some cells in the presence of glutamate rather than glutamine, resulted in an increase in GS activity.

In all cases DX treatment stimulated the levels of GS detected in cell homogenates by between 2-10 fold in gliomas, about 1.4 fold in foetal lines and only very slightly, 1.05-1.19 fold, in normal adult lines. The DX stimulations are shown in Table 9. Actinomycin D completely abolished the DX stimulation of GS in G-UVW and NOR-F cells. The combination of DX and IP stimulated GS activity to about the same level as DX alone; IP alone had no effect on GS. For the rest of the drugs used in this series of experiments, there was no obvious general pattern for the effect on cellular GS activity. In some cell lines a few of the compounds, notably IFN,and Mit.C. and MNU caused small increases in GS levels. In all the cell lines investigated PBE caused between 1.2-1.8 fold stimulation of GS activity.

Plasminogen Activator (PA)

Cells grown in 24 well dishes for PA analysis were treated with drugs in serum free medium as opposed to the medium containing 1% serum used for GFAP, BASG and GS analysis.

Table 9

DX Stimulation of Glutamine Synthetase Activity

(nmoles product formed min^{-1} mg $protein^{-1}$)

Cell Line	GS Activity (F10/1% fcs)	GS Activity (F10/1% fcs + DX)	DX Stimulation
G-MCN	23.3	51.9	2.2
G-UVW	68.8	156.7	2.3
G-CCM	20.9	89.5	4.3
G.IJK	92.6	843.3	9.1
C_6	13.8	56.9	4.1
NOR-F	47.4	50.0	1.05
GDU-T	45.0	53.5	1.19
NFU	57.2	79.1	1.38
NFT	25.5	36.0	1.41

The complete absence of serum was required, to avoid the reduced detection of PA activity caused by serum inhibitors.

The results for PA levels in post confluent, drug treated cells are shown for four cell lines (one endothelial, one foetal, one normal adult and one glioma) in Figure 18. HUV endothelial cells had very low PA activity which did not alter in response to any of the drugs. The foetal cell line, NFT, had a control PA level above that of the normal adult lines. DX alone and in combination with IP decreased activity by about 50%. With the exception of small increases in PA by Mit.C and MNU in foetal cell lines, the other drugs had no effect.

The PA activity of normal brain derived cell lines, of which NOR-T was typical, was significantly decreased by DX, IFN and PBE and increased by IP, Ret.Ac., Mit.C, MNU and MNU in combination with TPA. TPA itself had no effect. The observations on drug responses of glioma cell lines were similar to those for the normal adult lines. PA activity was almost abolished by DX and DX and IP in combination, slightly by IFN and quite considerably by PBE. IP, Mit.C, TPA, MNU and MNU and TPA in combination all significantly increased activity.

Endothelial Cell Mitogenic Activity.

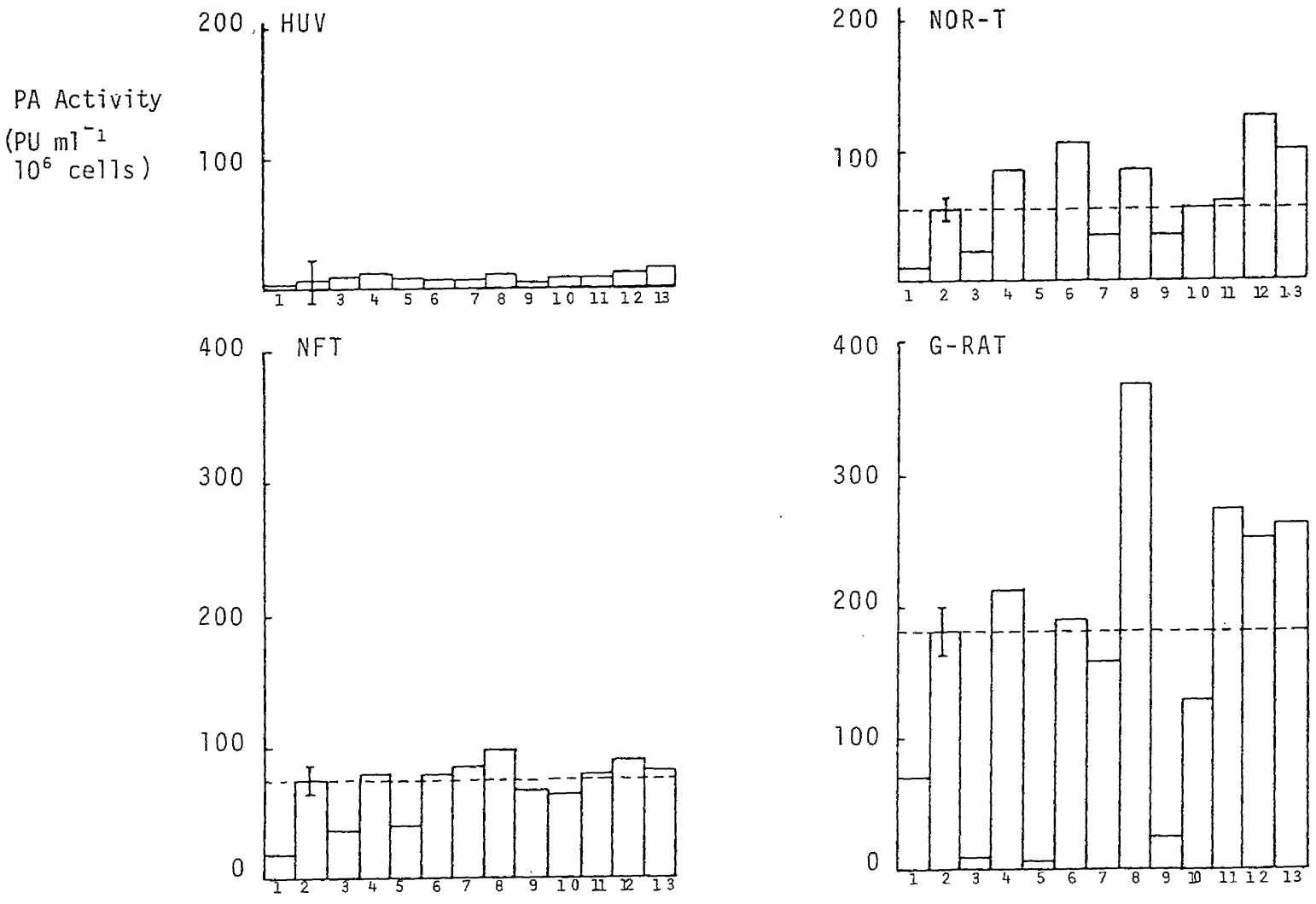
The effect of drugs on the mitogenic activity of malignant glioma cell extracts on HUV endothelial cells was investigated. Two cell lines, G-CCM and G-RAT, were grown to confluence in 75cm² flasks, treated with drugs in F10/1% serum for 6-7 days, harvested and cell extracts prepared by repeated freezing and thawing followed by centrifugation for 30 minutes at 48,000 x g.

FIGURE 18

Histograms showing cellular plasminogen activator production by HUV, NOR-T, NFT and G-RAT cells after 6 to 7 days exposure to conditions 1 to 13 (Table 8). Each value is the mean of duplicates. Variation between duplicate samples was always less than 46 $Puml^{-1}/10^6$ cells.

FIGURE 18

THE EFFECT OF CHEMICAL AGENTS ON CELLULAR PA ACTIVITY



The supernatants were diluted to $1mgml^{-1}$ in F10/10% serum and added to plateau phase HUV cells grown on collagen. The cells were counted after five days and the extent to which the extracts induced cell proliferation recorded. The stimulatory activity of cell extracts was designated as the final HUV cell density in the presence of $1mg\ ml^{-1}$ extract, divided by that achieved in the absence of extract. The results are shown in Figure 19. In both cases DX and PBE resulted in a small reduction in the ability of treated glioma extracts to induce mitogenesis of HUV cultures. Mit.C., TPA, MNU and TPA in combination with MNU caused small increases. The other drugs used in this series had no detectable effect. The drug induced fluctuations in mitogenesis, as detected by the increase in HUV cell number at terminal cell density, were small, and their significance therefore open to doubt. In comparison with the almost complete abolition of PA activity in glioma cells, observed as a result of DX and PBE treatment, the decrease in the endothelial mitogenic activity of treated glioma extracts, was very slight. However, these small drug fluctuations were consistent for both the glioma lines studied in detail.

Time courses of Dexamethasone and Pig Brain Extract effects.

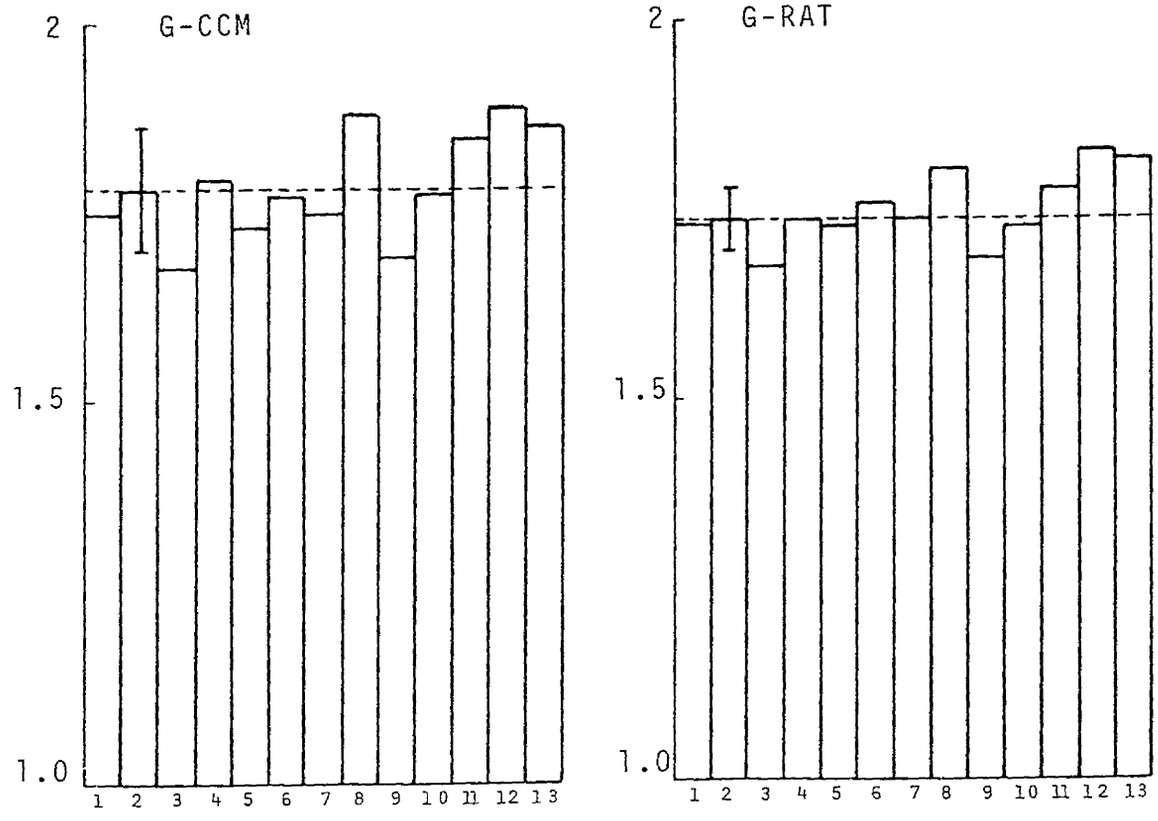
Dexamethasone (DX) and pig brain extract (PBE) had qualitatively similar effects on the phenotypic expression of malignant glial cells *in vitro* and provided potentially useful conditions for modulating tumour cell phenotype. The time courses for the effects of these agents, alone and in combination, on high affinity GABA uptake, glutamine synthetase and plasminogen activator, were investigated for the cell lines G-UVW and NOR-F. The results are presented in figures 20, 21 and 22 respectively.

FIGURE 19

Histograms showing the mitogenic effect of G-CCM and G-RAT cell extracts on HUV endothelial cells at high density. Cell extracts were prepated from cells after 6 - 7 days treatment with conditions 1 to 13 (Table 8). Each value is the mean of duplicates. Variation between duplicate stimulation factor values was always less than 0.22.

MITOGENESIS IN HUV ENDOTHELIAL CELLS

FIGURE 19



FIGURES 20a and 20b

Post-confluent cultures of G-UVW and NOR-F respectively were exposed to dexamethasone ($10\mu g ml^{-1}$) ●—●, pig brain extract (1:32) ▲—▲ and a combination of dexamethasone and pig brain extract ■—■ in F10 containing 1% foetal calf serum. BASG uptake was assayed at various times after drug exposure and the increased BASG uptake over that observed for control cultures plotted against time. Points represent means and standard deviation of 4 replicate wells.

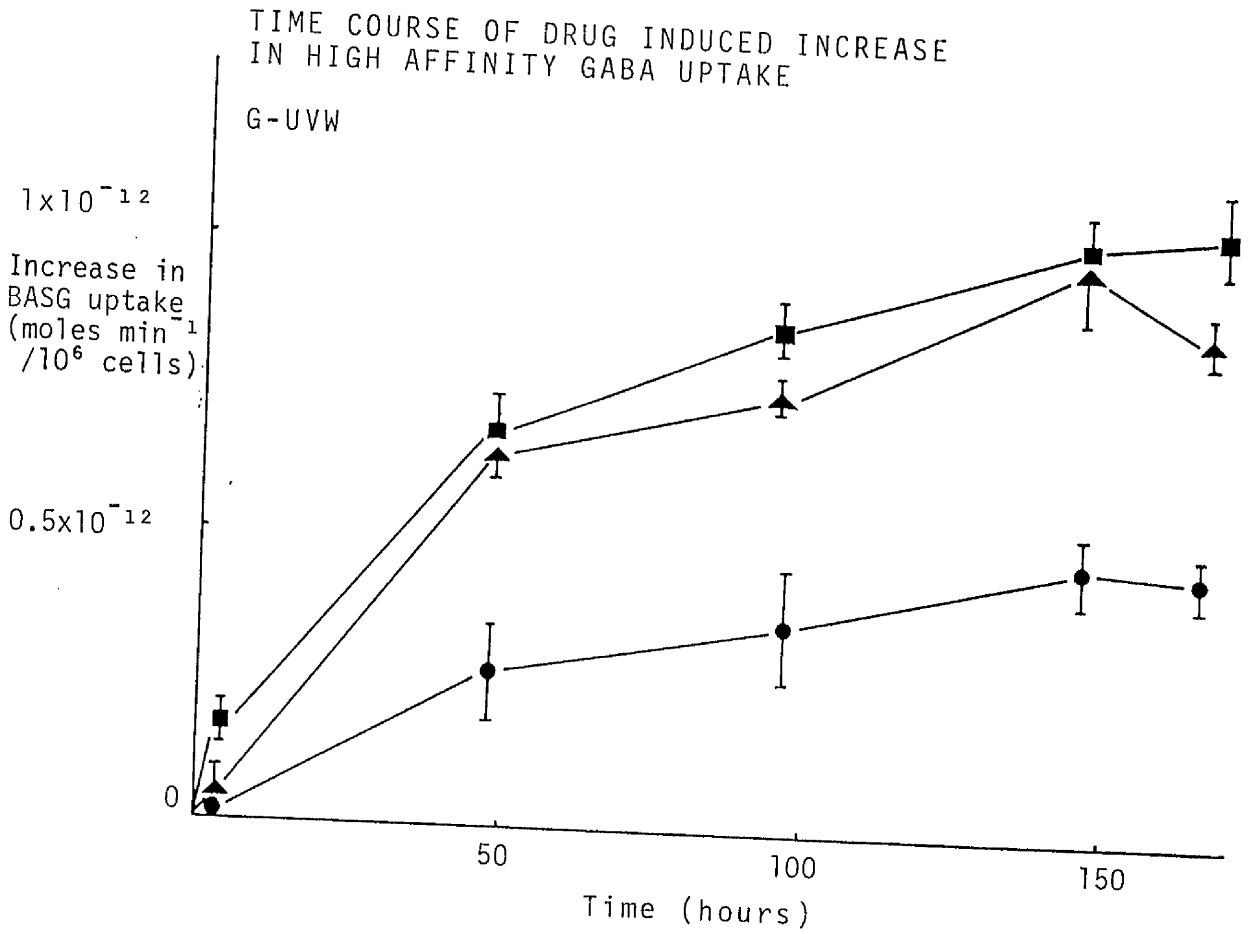
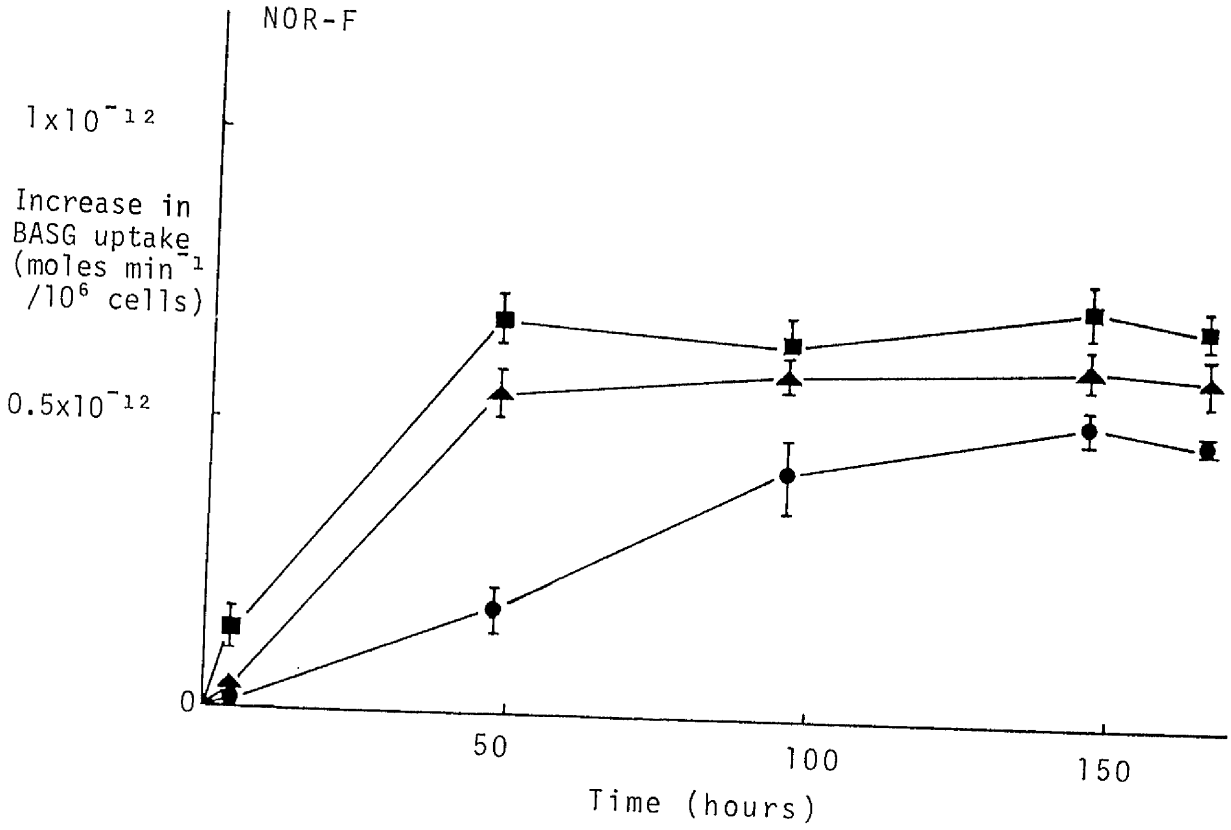

TIME COURSE OF DRUG INDUCED INCREASE
IN HIGH AFFINITY GABA UPTAKE
G-UVW
1x10⁻¹²
Increase in
BASG uptake
(moles min⁻¹
/10⁶ cells)
0.5x10⁻¹²
0
50
100
150
Time (hours)

FIGURE 20b


NOR-F
1x10⁻¹²
Increase in
BASG uptake
(moles min⁻¹
/10⁶ cells)
0.5x10⁻¹²
0
50
100
150
Time (hours)

FIGURES 21a and 21b

Post-confluent cultures of G-UVW and NOR-F respectively were exposed to dexamethasone ($10\mu g ml^{-1}$) ●——●, pig brain extract (1:32) ▲——▲ and a combination of dexamethasone and pig brain extract ■——■ , in F10 containing no serum. At various times after drug exposure cells were harvested and assayed for glutamine synthetase (GS) activity. GS was also measured for control cultures in F10 (no serum) with no drug O——O. Points represent individual measurements.

FIGURE 21a

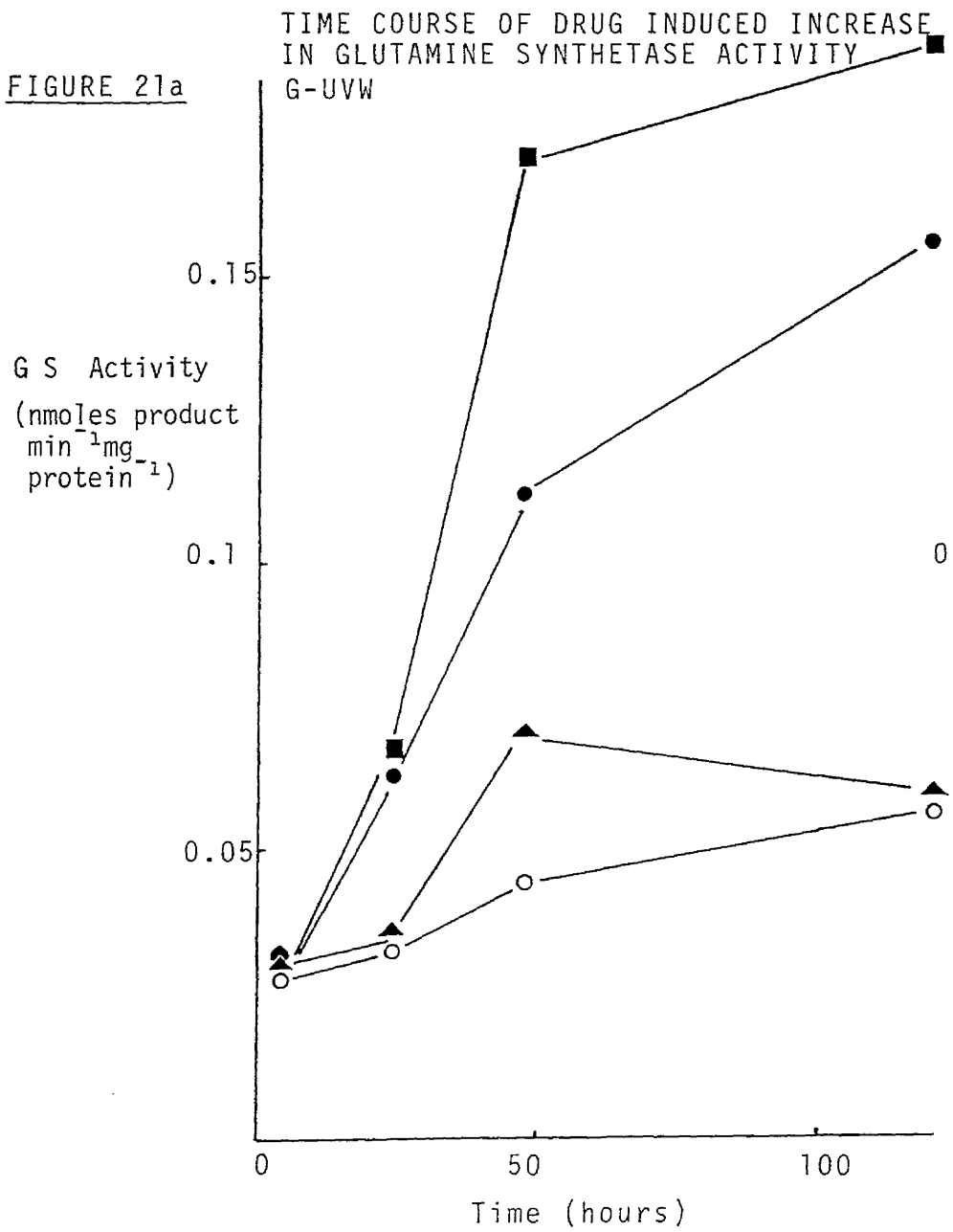
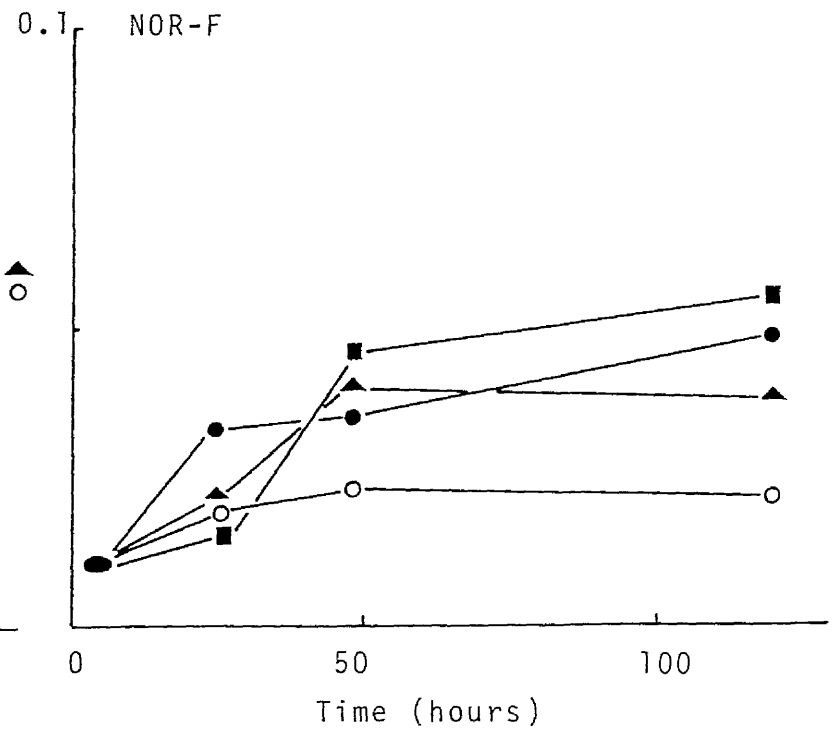


FIGURE 21b



FIGURES 22a and 22b

Post-confluent cultures of G-UVW and NOR-F respectively were exposed to dexamethasone ($10\mu g ml^{-1}$) ●——●, pig brain extract (1:32) ▲——▲ and a combination of dexamethasone and pig brain extract ■——■ in F10 containing no foetal calf serum. Plasminogen activator was assayed at various times after drug exposure and the decrease in cellular PA production over that observed for control cultures plotted against time. Points represent means and standard deviation of 4 replicate wells.

FIGURE 22a

TIME COURSE OF DRUG INDUCED REDUCTION IN PA ACTIVITY

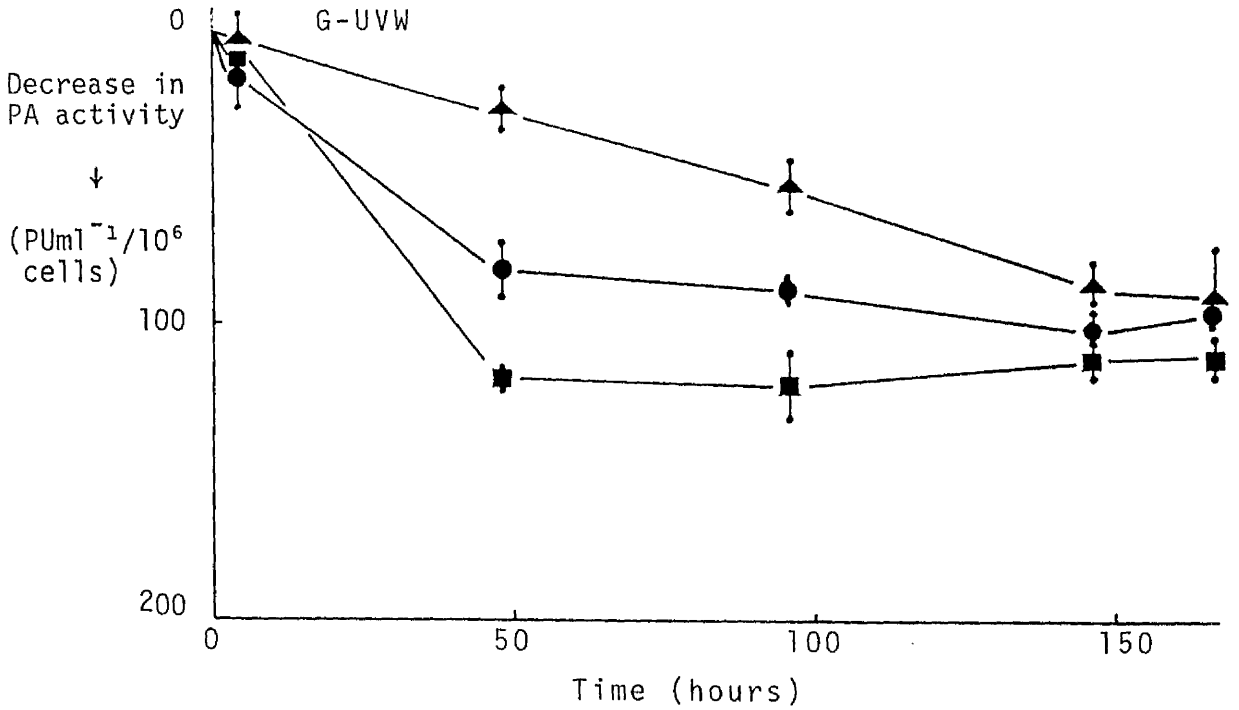
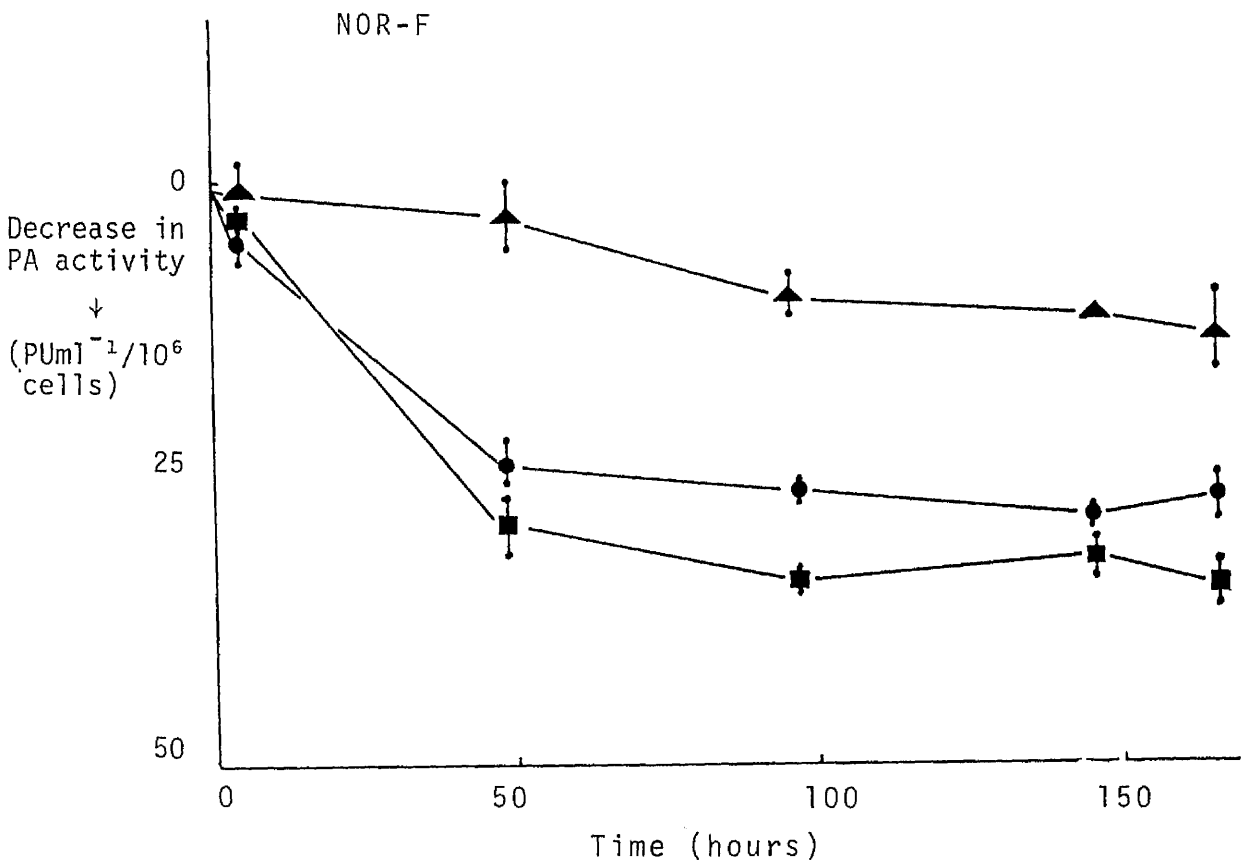


FIGURE 22b



For both cell lines the induction of high affinity GABA uptake by PBE was greater and more rapid than the DX induction. The time course for the combination of the two agents paralled that for PBE alone, although the effect of PBE was enhanced by the presence of DX. There appeared to be a degree of synergism between the two agents, although the effects were not additive.

In the case of glutamine synthetase, DX produced the greatest stimulation, particularly in the glioma line G-UVW. The combination of DX and PBE resulted in considerable enhancement of the glutamine synthetase stimulation caused by either agent alone.

The reduction in plasminogen activator activity of both G-UVW and NOR-F by DX was greater and more rapid than the PBE reduction, the opposite situation to that observed for the induction of high affinity GABA uptake by these agents. The time course for the two agents in combination paralleled that for DX alone, with the presence of PBE enhancing the reduction in cellular plasminogen activator activity by DX. In the case of DX alone and DX in combination with PBE, there was little further reduction in plasminogen activator activity after 48 hours. The PBE reduction, however, continued for more than 150 hours.

From the time course and syngergism studies using the three marker properties described above, it was concluded that DX and PBE were acting in a dissimilar manner, at least with respect to the timing of the observed inductions and repressions. The combination of the two agents enhanced the effect of either agent alone in modulating phenotypic expression.

D I S C U S S I O N

Part I Characterisation and Development of Marker Properties

4.1 Normal Adult Brain Cultures : 1. Morphology
: 2. Biochemistry

4.2 Foetal Brain Cultures

4.3 Glioma Cultures : 1. Characterisation

4.4 Postulation of an Astroglial Maturation Pathway

4.5 Marker Properties Associated with Malignant Behaviour

Part II Environmental Manipulation of Phenotypic Expression

4.6 Serum Effects

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: 3. Three Dimensional Perfusion Culture

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4.10 Concluding Remarks

PART I

1. Normal Adult Brain Cultures

1. Morphology.

The typical flat polygonal shape of cells from normal adult post-mortem brain material was similar to that described by Lim et al (202) for glioblasts cultured from 17 day foetal rats. The glioblasts observed in this study responded morphologically to Glia Maturation Factor (GMF), a protein of apparent molecular weight 350,000 purified from pig brain, and to dibutyrilcyclic AMP (dbcAMP), by forming multipolar, branched processes. The differences between GMF and dbcAMP in eliciting process formation in glioblasts (dbcAMP caused mainly retraction of cell bodies at selected points whereas GMF resulted in active extension of processes as well as retraction of cell bodies) was similar to the differences in the effects of the two agents on the normal adult brain derived cultures. Lim et al also observed a time difference between the two effects. Treatment with dbcAMP resulted in morphological alteration of the cells within a few minutes whereas, with GMF, there was a lag period of about 12 hours.

One important difference between the normal adult brain cultures and the rat glioblasts described above, was the presence of GFAP in the latter cells. The normal adult cultures, despite a morphology which closely resembled that of rat glioblasts and a similar response to treatment with dbcAMP and PBE, lacked GFAP. GFAP-containing cell cultures, derived from human adult brain, have been described by Gilden et al (109). They reported that by the 3rd to 5th subculture, cultures derived from cerebral white matter had a relatively homogenous appearance with one or two cell types predominating. In these

cultures 30-50% of the cells expressed GFAP, which remained throughout their *in vitro* lifespan. These observations implied that it was possible to grow GFAP positive cells from human adult brain tissue. However, despite many attempts,using different culture techniques, to derive cell lines from normal brain, no GFAP-expressing cells were obtained for study in this investigation. The procedures used, including collagenase digestion, cold trypsinisation and primary explantation all gave rise to cultures with the characteristic flat polygonal morphology and no detectable GFAP.

The development of a series of serum-free media for the culture of various cell types from newborn rat brain by Lang and Brunner (256), led to the observation that culture conditions could select not only for distinct cell types, but also for cells at various stages of differentiation. In particular, they identified four cells of the astroglial cell lineage in different hormonally-supplemented, serum-free media. The four cell types i.e. primitive astroglia, intermediate astroglia, astrocytes and astroblasts existed as homogeneous populations in the different media. On addition of serum to their growth medium, the astrocytes, which had long branched processes, morphologically altered to a flat form reminiscent of astroblasts and the normal adult brain cultures used in this project. These observations, together with the observations on alterations in morphology after dbcAMP and PBE treatment, indicated that the state of glial differentiation was at least partly determined by the microenvironment of the cells. The morphological observations support the hypothesis that the normal adult brain derived cultures consisted of astroglial precursor cells. However, morphological criteria alone, are never sufficient to determine the identity of cells in culture.

2. Biochemistry

As discussed above, the normal adult brain cultures lacked GFAP, present in the rat astroblasts and astrocytes described by Lang and Brunner (256) and in the foetal rat glioblasts described by Lim et al (202). This observation implied one of several possibilities:

1. the cells were not glial in origin,
2. the cells were non-GFAP-expressing glial cells e.g. oligodendroglia or,
3. the cells were glial precursors.

The immunological and biochemical characteristics of these cultures, while not providing unequivocal proof, threw further light on their identity.

The first possibility, that the normal adult brain cells were not glial, was excluded for several reasons. Of the possible cellular contaminants neurones do not divide in culture and the absence of factor VIII and merocyanin (257) antigens respectively, indicated that no endothelial or cells with excitable membranes(e.g. smooth muscle) were present. This together with the ability of these cultures to take up the putative neurotransmitters glutamate and GABA by saturable, high affinity processes, implied their probable glial nature.

The second possibility, that the cells were oligodendroglia, was excluded for two main reasons. Successful bovine oligodendroglial cultures have been obtained by Poduslo et al (258) and shown to produce whorls of membrane lamellae adjacent to the cell body. They also had high levels of the enzyme 2', 3' - cyclic nucleotide 3'- phosphodiesterase (CNPase), which is associated with myelin in the membrane lamellae. The normal adult brain cultures used in this project showed no unusual membrane production and contained low levels of CNPase (259).

The third possibility, that the cells were undifferentiated or precursor astroglia was the most favourable explanation of their identity. The time taken for monolayer cultures to grow from human adult brain tissue was 3-4 weeks after dissection and enzymatic digestion. This compared with the 24-72 hours taken to obtain monolayer cultures from foetal and malignant brain tissue, and implied that the flat, polygonal cells which grew from the normal adult tissue constituted a small proportion of the original cell mass. These cells were selected by being able to grow in culture, perhaps as a result of their precursor or undifferentiated status.

The uptake of glutamic acid and GABA by saturable, high affinity processes was probably the action of specific membrane permeases, acting in addition to the non-saturable, diffusive uptake of these amino acids. This reflected one of the suggested functional roles of glia in the central nervous system to remove neurotransmitters from the synaptic cleft, thus reducing their concentration at the post synaptic receptor sites. High affinity glutamic acid uptake was a less specific event than the high affinity GABA uptake. It was constitutively active in normal adult brain cultures, malignant astrocytoma and melanoma cultures, which like glial cells are neuroectodermal in origin. This provided one of the first pieces of evidence that the normal adult cells were neuroectodermally derived. The specificity of high affinity GABA uptake was more complex. One of the normal adult lines, NOR-F, was constitutive; the others, NOR-T and GDU-T, required induction by steroid and/or dbcAMP in the absence of serum. Melanoma cells, fibroblasts and endothelial cells could not be induced to take up GABA by the high affinity process and Lineweaver-Burk kinetics for those cells indicated operation of only the low affinity diffusive

mechanism of uptake. The induction requirement for expression of high affinity GABA uptake by some of the normal adult cell lines supported the hypothesis that these were undifferentiated, able to express specific astroglial properties only in the presence of appropriate inducers. Differences in the induction requirement for various normal adult lines might reflect their different states of differentiation i.e. the cells selected for growth in culture might be at different stages in an astroglial differentiation pathway.

The normal adult cultures also possessed much lower levels of glutamine synthetase activity, under standard culture conditions, than the foetal and some of the glioma lines. This could also be a reflection of their undifferentiated status. Precursor or undifferentiated cells in culture could have arisen, either by propagation of a pool of immature precursor cells or by selection for mature, fully differentiated cells which underwent spontaneous de-differentiation in culture. It was impossible to distinguish between these two possibilities and seems feasible that either could have occurred. A possible differentiation pathway,incorporating the chosen markers of astroglial differentiation will be described later, by considering the phenotypic expression of many cell lines, including those from normal adult, foetal and malignant brain tissue.

2. Foetal Brain Cultures

The absence of GFAP from normal adult cultures, and the consequent doubt about their identity, led to consideration of cells derived from foetal brain as more appropriate controls for malignant astroglia. Previous work has shown that although monolayer cultures derived from foetal mouse brain were originally

small, flat polygonal cells which did not express GFAP, they acquired GFAP after about 10-13 days in culture (116). At this time there was also a morphological change which resulted in the appearance of multiple branched processes. The spontaneous differentiation *in vitro* occurred at the same time as the peak GFAP content and expression of astrocytic differentiation *in vivo*, in 12 day neonatal mouse brain (260). These cells senesced after 50-70 days in culture.

Cultures derived from human foetal brain (12-16 weeks post-conception), contained cells which expressed GFAP. The proportion of GFAP positive cells varied between foetal lines, from around 30% to 100%, although it remained constant in any one culture throughout the finite lifespan of the cells of 8 to 12 generations. Two distinct morphological cell types were identified in foetal cultures; astrocytic cells, which were generally GFAP positive and elongated polygonal cells, which contained no GFAP. In the initial stages of culture the foetal cells grew very rapidly. They slowed down and senesced at around the 8th - 12th generations. The short life-span of the foetal cells in culture and their poor recovery after storage in liquid nitrogen, necessitated a continual supply of fresh foetal material from which fresh cultures could be obtained.

The foetal cell lines all expressed high affinity GABA uptake constitutively and expressed high levels of glutamine synthetase activity when grown under standard culture conditions. These properties, together with the expression of GFAP, indicated that highly differentiated astroglial cells, capable of rapid proliferation *in vitro*, could be derived from foetal brain tissue. The identity of the cells not expressing GFAP in the foetal cultures remained uncertain. They did not contain factor VIII.

The successful growth of differentiated astroglia from foetal brain tissue, using an identical culture procedure to that used for the normal adult tissue, showed an obvious difference between the foetal and adult systems, the latter yielding less differentiated cells. An interesting and perhaps relevant observation was made by Lim et al (202) on the GMF content of both foetal and adult rat brain tissue. They found that this factor was present at a high level in adult brain but at a low level in foetal brain, and postulated that GMF had a role in the maintenance of glial cell differentiation in the adult. Rat foetal glial cells did not appear to require GMF for maintainance of the differentiated phenotype in vivo. These observations, if also true for the human system, could explain the maturity of foetal glial cell cultures in the absence of GMF, while the morphological and biochemical differentiation of cells derived from normal adult tissue required this, or other differentiating agents. The phenotypic expression of the foetal cultures was insensitive to treatment with Pig Brain Extract.

3. Glioma Cultures

1. Characterisation

The primary cultures derived from anaplastic astrocytomas contained a heterogeneous mixture of cell morphologies. In most cases a proportion of the cells expressed GFAP. By the second or third subculture one of the cell types generally, predominated, and with the exception of G-CCM and G-ROG, the selection procedure favoured cells which did not express GFAP. Although GFAP can be detected in most astrocytic gliomas, only the most morphologically differentiated cells express it; the

more primitive and anaplastic cells do not (112, 113, 114). Thus the GFAP negative cell lines were composed of tumour cells which grew out from the more primitive or anaplastic cells in the tumour cell population. The different morphologies exhibited by a range of glioma lines was consistent with the heterogeneous morphological phenotypes of a number of established glioma lines previously reported (97). This probably reflected the heterogeneous nature of the cells in malignant astrocytomas and a selection procedure which favoured the cells most suited to in vitro growth. The result was astrocytoma derived cultures which exhibited morphological homogeneity within a culture but considerable heterogeneity between cultures.

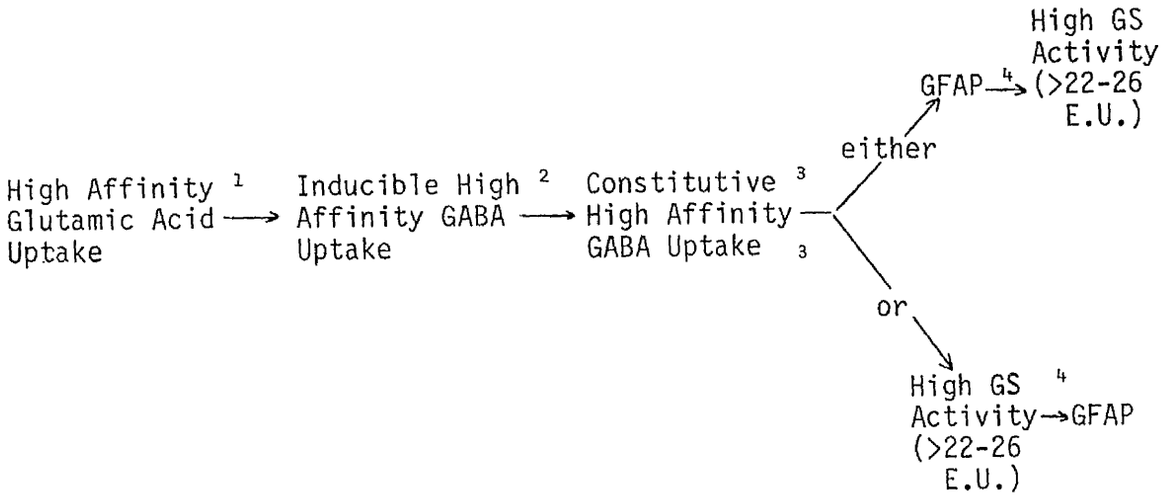
The absence of GFAP from most of the astrocytoma derived cell lines, as was the case with the normal adult brain derived lines, necessitated further immunological and biochemical characterisation. With the exception of GMS, an astrocytoma derived line found to be predominantly endothelial by virtue of the detectable presence of factor VIII, the glioma cell lines did not express the endothelial marker. They were able to take up glutamic acid by the high affinity process implying their neuroectodermal origin. The two GFAP positive glioma lines G-CCM and rat C_6 also expressed high affinity GABA uptake constitutively, implying that the cells possessed an active form of the high affinity astroglial GABA carrier (261), as a component of their membranes. This was consistent with the differentiated nature of their phenotypic expression. Of the non GFAP- expressing glioma lines investigated by Lineweaver-Burk analysis, G-RAT and G-EME were induced for high affinity GABA uptake by the combination of steroid (β-or dexamethasone)

and dbcAMP (in the absence of serum). In the case of G-EME, steroid or dbcAMP alone was not sufficient for induction. Two cell lines, G-ARY and G-ATA were not induced for GABA uptake by the combination.

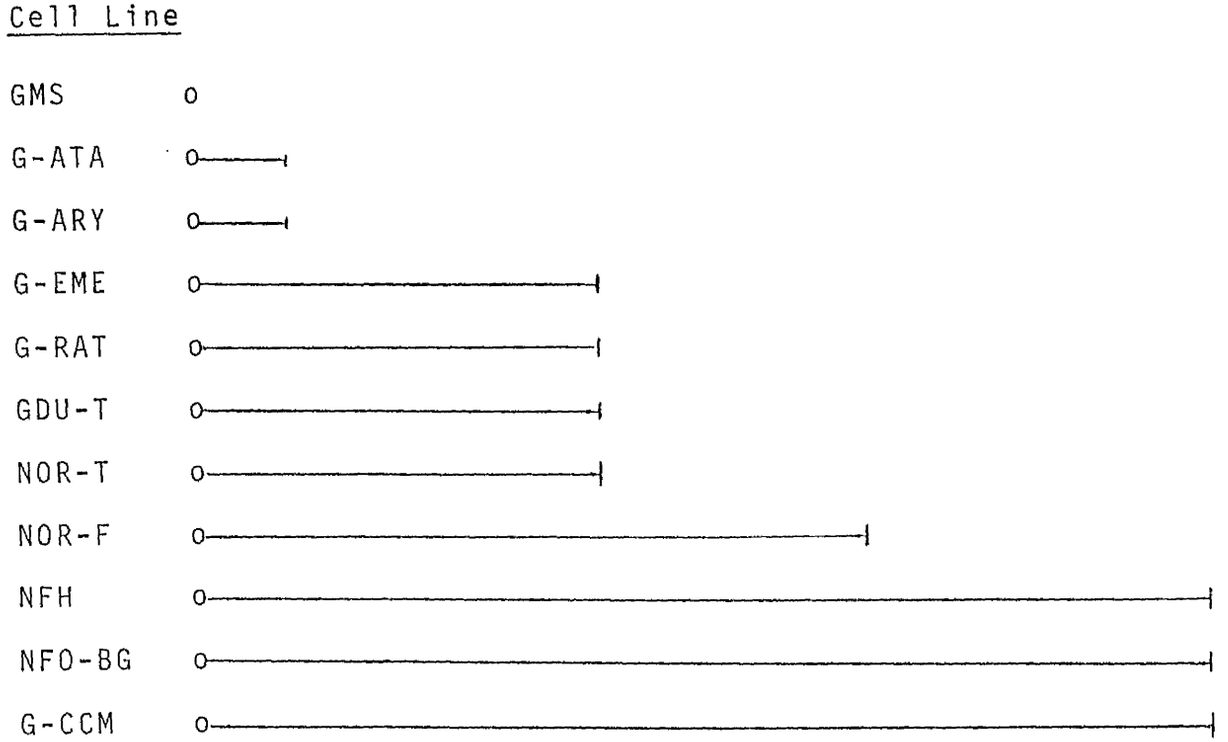
Under standard culture conditions, the glioma cell lines exhibited a wide range of glutamine synthetase activities, ranging from a value higher than the foetal cultures for G-CCM to a value equivalent to that for the normal adult cultures for G-ATA. This again emphasised the heterogeneity of phenotypic expression between glioma cultures. The human astrocytoma derived cell lines used in this project exhibited a range of differentiated states with respect to the chosen markers of astroglial differentiation. In particular, G-CCM showed a highly differentiated phenotypic expression, G-RAT showed intermediate and G-ATA relatively undifferentiated phenotypic properties. There was no obvious correlation between the morphological characteristics of cell lines and their levels of expression of differentiated properties.

4. Astroglial Maturation Pathway

The expression of differentiated astroglial properties by the normal adult, foetal and astrocytoma derived cultures allowed a maturation pathway to be postulated. This is presented below with the properties expressed by various cell lines.



E.U. = Enzyme Units
(nmoles product min^{-1} mg $protein^{-1}$)



This possible maturation pathway considers only a few of the differentiated astroglial features. If such a pathway exists then cells would acquire these properties in the sequence shown. There was no evidence to suggest whether GFAP was acquired before, after or at the same time in the sequence as high glutamine synthetase activity. These two properties were always co-expressed

in the more differentiated cultures. Out of a large number of cell lines investigated there were none whose phenotypic expression did not conform to the above pathway e.g. there were no GFAP positive cell lines which did not express the properties preceeding GFAP in the above pathway.

Normal adult cell lines appeared to be blocked at stage 2 or 3 in the hypothetical differentiation pathway. Less differentiated glioma lines were blocked at 1 or 2. G-CCM and the foetal brain derived cultures were not blocked at any point in the pathway and were apparently highly differentiated while continuing to proliferate rapidly in culture.

The biochemical differentiation pathway hypothesised above, might be considered analogous to that described for the haemopoetic system (69). In this case compounds such as dimethylsulphoxide and N-methylacetamide induce erythrocyte precursor cells to differentiate along the pathway resulting in the generation of mature erythrocytes. Potential inducers of astroglial differentiation, such as dbcAMP, dexamethasone and Pig Brain Extract, however, did not stimulate cells blocked at any stage in the astroglial pathway, to differentiate terminally. GFAP was not detected in such cells treated with inducers, although they showed enhanced expression of other differentiated characteristics.

5. Marker Properties associated with Malignant Behaviour

Plasminogen Activator (PA)

Increased PA activity has been correlated with expression of the malignant phenotype in a number of experimental systems (148, 149, 150), and is widely believed to have a role in the growth and spread of tumours. Pearlstein et al (262) however, have reported that high levels of fibrinolytic activity can

be demonstrated in some tumour cells, notably sarcomas, but not in all. They observed high levels in some normal cells, in particular lung and bladder. This was consistent with reports by other investigators (263, 264, 265).

The apparent inconsistencies in deciding whether or not PA was a useful malignancy associated marker, prompted comparisons between normal cell lines and those derived from malignant tissue. The results for colon carcinoma and cells derived from pathologically normal colon showed no obvious correlation. In some cases normal colon lines produced high PA and carcinoma lines only low amounts of PA, implying that for the colon cultures investigated PA was not a useful malignancy associated marker. However, there remained some doubt about the nature of the normal colon cultures. These were derived from ressected colon, at regions distant to the site of the tumour and might not represent truly normal tissue.

The results for cells derived from normal adult brain and anaplastic astrocytomas were more encouraging. In this system PA was inversely correlated with the degree of cellular differentiation. The GFAP-expressing glioma lines, G-CCM and C_6, had low PA activity. The non-GFAP-expressing gliomas had PA levels which were higher than those for the normal adult lines, which in turn were higher than G-CCM and C_6. Within the group of GFAP negative glioma lines, the highest PA activities were attributable to the least differentiated lines, notably G-ATA. G-RAT, a cell line of intermediate differentiation status, also had intermediate PA activity.

The *in vivo* tumourigenicity of the astrocytoma derived cell lines was not determined. Thus the relationship between differentiation and tumourigenicity was unknown. However the

degree of differentiation of a particular cell line was inversely related to its PA expression, a property often associated with malignant behaviour (152, 155). The PA activity of the normal adult lines, which was greater than the GFAP-expressing glioma lines, was consistent with the view that these cultures were less well differentiated than the GFAP positive gliomas.

The chromogenic substrate preferences of a variety of colon and brain derived cell lines showed an obvious difference between the two cell types. The implication was that the molecular forms of PA produced by brain and colon differed, resulting in different plasminogen cleavage patterns and hence molecular forms of plasmin which were also different. Tucker et al had previously reported that human brain tumour PA was different from the PA of other normal and neoplastic cell types (152).

Tumour Angiogenesis Factor (TAF)

The neovascularisation requirement for the survival and growth of all solid tumours implies that tumour cells express the potential to induce angiogenesis i.e. the growth of new capillary blood vessels from the host vascular bed, in response to tumour produced stimulus. The angiogenic substance, TAF, has been partially purified by several groups of workers (166,167), and has been shown to stimulate endothelial cell growth in vitro (163, 167).

The chick chorioallantoic membrane (CAM) has provided a useful biological system for demonstrating angiogenesis. In the present study tumour cell extracts containing active TAF resulted in visually obvious vasoproliferation when placed on

the CAM. Normal adult brain cell extracts showed no angiogenic response. This technique proved difficult to quantitate and an attempt to grade tumour cell extract responses, relative to the positive response of a WRC-256 cell extract and the negative response of BSA, resulted in large standard deviations between samples. The subjective nature of the gradation and the uncontrolled variation in the assay due to environmental factors e.g. the site of implantation of the TAF fraction on the CAM, were probable explanations for the observed variations in response.

Despite the difficulties in quantitating the angiogenic response on the CAM, TAF production by the astrocytoma derived cell lines appeared to be a good tumour marker. Regardless of their degree of morphological or biochemical differentiation the glioma lines all produced TAF. There was no inverse correlation between differentiation and TAF production, as was the case with PA. This might reflect the absolute requirement of a tumour for TAF and suggested that perhaps all the cells of a heterogenous tumour cell population produce an angiogenic factor.

Endothelial Cell Growth

The poor quantitation of the TAF mediated response on the CAM and the consequent large numbers of chick embryos required, prompted the development of an assay which might be more precise and practically feasible for large numbers of samples. The primary assumption was that endothelial cells are the *in vitro* targets for TAF produced by tumour cells, which induces their proliferation resulting in the formation of blood capillaries. Attempts were therefore made to culture

endothelial cells, and their possible use in an *in vitro* assay for TAF was investigated.

As discussed previously, endothelial cells were cultured from an astrocytoma by collagenase digestion. However, the factor VIII positive cell line, GMS, could not be successfully propagated for more than a few generations. Attempts to culture endothelial cells from rat brain white matter, by the method of Phillips *et al* (253), yielded clones of factor VIII positive cells surrounded by large cells, probably smooth muscle. The endothelial cells remained in small discrete colonies and could not be propagated. These observations differed from those of Phillips *et al*, who obtained pure endothelial cultures from rat brain.

The most successful method for culturing endothelial cells was found to be collagenase digestion of the internal surface of human umbilical vein (HUV). The endothelial colonies grew up after a few days in culture to form complete monolayers. After the first few subcultures the integral "pavement" structure of the monolayer was lost and the individual cell morphology altered. These cells, which were factor VIII positive throughout their *in vitro* lifespan, required Endothelial Cell Growth Supplement (ECGS, Collaborative Research) to proliferate. Further indication of the endothelial nature of the HUV cultures came from scanning electron micrographs, in which Weibel-Palade bodies and tight junctions between cells were identified.

The morphological transition observed in HUV cultures, which had been maintained at high density for a few weeks, was similar to the transition observed by another group of workers. In particular, the cell fusions and formation of a tubular network structure over the growth surface was reminiscent of the

capillary tubes formed spontaneously by bovine capillary endothelial cells *in vitro* (170). The structure formation probably represents a pre-programmed cellular event which results in the cell orientations and interactions required to form differentiated endothelium. The stimulus for this i.e. the trigger at high cell density, remained uncertain. One possibility was the deprivation of an essential medium nutrient after continued maintenance at high density. An analogous situation exists with the cellular slime mould Dictyostelium discoideum. In this case, free living amoebae grow and divide by binary fission until a high density is reached and the local environment is depleted of essential nutrients. This provides the signal for cell aggregation, the first step in the differentiation process, which finally culminates in the formation of mature fruiting bodies. As with this eukaryotic amoeba, the information for the formation of differentiated endothelial structures is entirely contained within the endothelial cells; the stimulus is environmental.

Endothelial Cell Mitogenesis

When HUV cultures were grown to high density in micro-titration dishes, cells were shed into the medium and structure formation occurred within a few days. When the cells were grown on collagen, however, a lower plateau density was attained and the cells could be maintained as a confluent monolayer, without structure formation, for a few weeks. HUV growth on collagen therefore provided a more suitabe system for investigating mitogenesis. Schor *et al* previously reported that mitogenesis of endothelial cells in culture by a purified component from WRC-256 cells required growth of the target

cells on collagen (166).

A preliminary experiment demonstrated that addition of a crude WRC-256 extract to HUV growth medium significantly increased the plateau density attained by these cells. The tumour cell extract clearly contained a factor (or factors) which stimulated endothelial cell growth at high density. The question of whether or not this effect was partly or wholly due to TAF in the crude extract was considered by investigating the stimulation of HUV cells by extracts from normal adult, foetal and malignant brain derived cell lines. The results demonstrated that many cell lines had the ability to stimulate endothelial cell growth, and that this ability was not restricted to tumour cells. Extracts of the normal adult line, GDU-T, and the foetal line, NFU, also stimulated HUV growth. Two other normal adult lines, NOR-T and NOR-F, the foetal line NFT and the glioma line, G-MCN (whose angiogenic response on the CAM was not known), did not stimulate growth appreciably.

A comparison of the relative activities of cell extracts in inducing angiogenesis on the CAM and mitogenesis of endothelial cells in vitro, indicated that the two assay systems were not measuring the same event. However, there were similarities; in both cases the WRC-256 cell extract had the highest activity and, with the exception of GDU-T, the normal adult cell extracts had low activities.

Stimulation of endothelial cell proliferation by extracts of GDU-T and NFU implied that TAF was probably not the only mitogenic factor in the cell extracts. It seems likely that in a crude extract many factors, which stimulate HUV non-specifically, might be present. Endothelial cell stimulation

in vitro cannot therefore be considered a measure of TAF activity alone, but of the much less specific event of endothelial cell mitogenesis (ECM), which itself might be a useful malignancy associated marker. Increased production of growth factors is a probable feature of many tumour cells, implied from their relative independence of serum in vitro (46, 47, 48). In order to use endothelial cell mitogenesis as a measure of TAF activity, purification of the latter component from the crude extract would be required

Conclusions

Several conclusions can be drawn from the cell characterisations and development of the marker properties associated with differentiation and malignancy:

1. Highly differentiated astroglial cells can be derived from malignant and foetal brain tissue and are able to proliferate in culture. In the malignant and foetal situation differentiation and proliferation may be uncoupled.

2. Markers of differentiation are not always co-ordinately expressed.

3. Markers of malignancy are also not co-ordinately expressed.

4. There is an inverse correlation between differentiation and production of plasminogen activator.

5. All glioma cell lines produce TAF as detected by vasoproliferation on the chick CAM.

PART II

Environmental Manipulation of Phenotypic Expression

6. Serum Effects

The normal adult, foetal and malignant cell lines used in this project were dependent on serum for growth in F10 medium. Serum undoubtedly affects many aspects of cellular phenotypic expression. Particular examples in the glial cell system were the variation with serum-batch in the detection of high affinity GABA uptake by rat C_6 cells, and the variation in detectable levels of plasminogen activator produced by the glioma line G-ATA. The latter observation, although a consequence of serum plasmin inhibitors bound to the cell surface during the PA assay, rather than a direct effect on phenotypic expression, probably contributed to the characteristics of the cultured cells. Serine proteases are involved in the post transcriptional processing of membrane proteins and glycoproteins. Their inhibition by serum components in the immediate environment of the cells might therefore change the cell surface properties e.g. cell adhesion and antigenic determinants, thus altering their biochemical and behavioural characteristics.

The complex nature of serum components and their effects on cells in culture, indicates the potential benefits of growing cells in serum-free, defined medium. Commercially prepared, pooled, foetal calf serum contains many undefined macromolecules, including growth factors and agents which regulate differentiation e.g. steroid and peptide hormones. It would be of considerable value, in studying proliferation and differentiation, to grow cells in serum-free medium and manipulate cell proliferation by addition and removal of specific

growth factors. Defined medium which supports the growth of rat astroblasts in culture, has been described (256). As discussed previously, growth medium constituents can determine the degree of differentiation of cells in the astrocytic lineage. There are many other reported examples of alterations in cell phenotype as a direct result of serum.

In a number of rat neuronal cell lines, the reversible formation of extended neurites was observed in medium with reduced serum, serum-free being the most effective (266). Similar observations were made with the C1300 mouse neuroblastoma (267). In the rat C_6 glioma, maintenance of the cells in serum-free medium resulted in a 3-fold induction of the oligodendroglial specific enzyme CNPase (268). These observations represent differentiation events as a result of serum withdrawal, and could, in theory, be a consequence of the cessation of growth or the removal of factors which block cell differentiation. Serum induced proliferation and reduced expression of differentiation might both be a result of growth factor activity. Evidence which suggests negative control of differentiation in rat granulosa cells,by growth related processes, has been put forward (269). These cells, maintained in serum-free medium supplemented with insulin, hydrocortisone, transferrin and fibronectin, produced 25-fold more progestin and oestrogen in response to FSH stimulation, than cells in serum supplemented medium. However, after 4 days in culture the granulosa cells showed a transient loss of their ability to produce steroids in response to FSH, which was regained after 35 days in culture. The transient loss of the FSH-induced function occurred at the same time as growth was initiated in these cultures.

The effect of serum on the phenotypic expression of cells in culture is considerable. In the phenotypic manipulation experiments, cells were maintained in a low (1 or 0%) serum concentration for seven days prior to determination of marker properties, and the same serum batch used throughout.

7. Cell Density

1. Rat C_6 Glioma

The C_6 glioma is a methylnitrosourea induced rat glial tumour often used in neurobiological research. The precise identity of the cells is unclear and they are reported to express both oligodendrocyte-specific features such as cortisol induced glycerol phosphate dehydrogenase (GPDH), and astrocyte-specific features such as GFAP (270). One possibility is that C_6 cells "transdifferentiate" from a predominantly oligodendrocytic to an astrocytic cell type. Another possibility is that the C_6 line represents a primitive stem cell i.e. a spongioblast, which has the capacity to express differentiated properties of astrocytes, oligodendrocytes or both.

The clone of C_6 cells used in this project expressed GFAP. Under standard culture conditions, 50% of the population contained GFAP and this was increased to almost 100% by the combination of dbcAMP and dexamethasone. The myelin associated enzyme CNPase (271) was also present in significant amounts and was further induced by the above drug combination (272). These observations showed that the C_6 cells were capable of expressing astrocytic and oligodendrocytic end cell characteristics at the same time, thus implying the

probable multipotent stem cell nature of the C_6 rat tumour.

The dramatic increase in the proportion of GFAP positive cells in C_6 cultures at the onset of confluence, was consistent with the reported accumulation of other differentiated products i.e. S100 protein (273), nerve growth factor (274) and GPDH (275), in this cell type at the early stationary phase of growth. This point in culture growth is characterised by a reduction in proliferative activity and an increase in the amount of membrane contact between cells. C_6 cells, isolated from one another in suspension, were found not to accumulate S100 protein when proliferation was stopped with metabolic inhibitors (273). This implies the important role of cell-cell contacts in the expression of differentiated properties in C_6 cells. The exact nature of the membrane contacts involved in the control of phenotypic expression is not known. C_6 cells have been shown to form gap junctions in culture (116) and it might be the increase in the number of communication channels between cells at confluence, allowing metabolic sharing, that is responsible for the increase in differentiated properties. Alternatively the effect might be a result of cell interactions at specific membrane recognition sites, causing fluctuations in metabolic signals which in turn alter expression of the differentiated phenotype

2. High Affinity GABA Uptake and Plasminogen Activator

In all the cell lines investigated there was an increase in high affinity GABA uptake with increasing cell density. As with GFAP in C_6 cells, this represented an increase in the expression of a differentiated glial property, most noticeable as the cultures reached confluence, at around 10^5 cellscm^{-2}.

The induction of cytostasis by withdrawing serum prior to confluence, and thus prior to the large increase in the number of cell-cell contacts which accompanies confluence, caused no increase in high affinity GABA uptake. Once again, the important role of increased cell-cell contact formation in inducing differentiation in glial cells was implied. In the case of both GFAP in C_6 cells and high affinity GABA uptake, cytostasis might have been necessary but was not sufficient for induction.

The cellular production of plasminogen activator, a malignancy associated marker, decreased with increasing cell density. A steady state level of production was reached at the onset of density dependent inhibition of growth. Experiments to determine whether or not the dramatic decrease in plasminogen activator was a result of reduced proliferation or increased cell-cell contact formation implied that both were important. Although increased contact formation played a part in reducing cellular plasminogen activator production, the decrease in the rate of cell growth was also important.

In the glial cell system, expression of differentiated properties increased with cell density, the opposite effect to that observed for plasminogen activator production. The roles of reduced proliferative activity and increased cell contacts in eliciting these effects have been discussed. It seems reasonable to postulate that rapid cell proliferation and low levels of "required" cell-cell membrane contacts, both of which might be prevalent in tumours, may favour repression of the differentiated phenotype and enhanced

expression of the malignancy associated phenotype.

3. Three Dimensional Perfusion Culture

The Vitafiber culture units provided a useful system for investigating cells grown in three dimensional, high density clusters. Two dimensional, monolayer culture has obvious disadvantages as a model system. Normal culture procedure, in which a large volume of medium is introduced with an initially small cell inoculum, results in exponential proliferation with the renewal of medium at appropriate intervals. Eventually, the cells reach plateau at which a confluent monolayer is formed. This series of events does not parallel any common biological process and the periodic changing of medium, a regime which has been described as "feasting and fasting" of cells, lacks the homeostasis found in a vascularised tissue where nutrients and oxygen are continually supplied and waste products removed.

In monolayer culture, cells only interact in two dimensions, whereas in tissues in vivo the cellular interactions are three dimensional. High density perfusion culture allows three dimensional interactions and might therefore provide a better model system, more accurately simulating the in vivo environment of cells and more closely reflecting their true physiological behaviour.

After inoculation and cell attachment, the normal adult cell line, NOR-F, and the glioma line, G-RAT grew on and between the hollow, polycarbonate fibres to a high density. The three dimensional clusters were observed in two main areas of the capillary bundles, opposite the top ports. The lack of uniformity in cell attachment over the entire surface

of the capillary bundles, implied the existence of a negative pressure pulling the cells to the particular regions where they settled and grew. Attempts to obtain uniformly spread cultures by mechanical manipulation of the chambers during the period of cell attachment were unsuccessful.

Despite its limitations, the Vitafiber system provided a means of growing cells in high density clusters with three dimensional cell-cell contacts. The continual perfusion of a large volume of medium provided a nearly constant micro-environment for cell growth. The two cell lines NOR-F and G-RAT exhibited an increase in high affinity GABA uptake after recovery from growth on the fibre bundles beyond that seen in high density post confluent monolayers. This increase was perhaps a result of the three dimensional interactions not formed in high density monolayer cultures. The role of cell-cell contacts in regulating the expression of high affinity GABA uptake was previously discussed. No detectable difference in cellular plasminogen activator production was observed, however, implying that the increase in membrane contacts between cells, as a result of three dimensional growth, did not effect this product. Plasminogen activator thus appears to be controlled largely by the proliferative state of cells.

8. Homologous and Heterologous Interactions

In the brain glial cells coexist with other cell types including neurones and both endothelial and smooth muscle cells from the blood vessels. Another aspect of recreating a more physiological environment for glial cells in culture was to introduce neurones, which are dependent on glial cells

for many aspects of their in vivo metabolism. In vitro, adult rat brain astrocytes have been found to promote the survival of both NGF-dependent and NGF-insensitive neurones, the former mediated by astrocyte derived NGF and the latter by another astrocytic diffusible product (276). In clonal neuroblastoma cells, process formation was induced by a macromolecular component of rat C_6 glioma conditioned medium (277). These observations suggest that diffusible factors from glial cells support and differentiate neurones in culture. However, Allin reported that C_6 cells and the mouse neuroblastoma cells NBU1 (derived from the C1300 tumour) were able to promote reciprocal morphological differentiation,which was not solely due to a diffusible factor (250). When the two cell types were maintained in co-culture, the differentiation was stable.

The effect of co-culture with neuroblastoma (TRK14 and IMR-32) on the phenotypic expression of a normal adult line, NOR-F, and a glioma line, G-UVW, was investigated. The results showed that NOR-F induced morphological differentiation of the neuroblastoma cells as observed by the extension of long, thin, dendritic processes. The morphological differentiation was not reciprocal. However, co-culture with neuroblastoma cells increased high affinity GABA uptake by NOR-F. These effects were unlikely to have been the result of diffusible products, as demonstrated by the lack of effect when the two cell types were cultured on separate coverslips, while sharing the same medium. Heterologous cell contacts were required. G-UVW was not affected by co-culture with neuroblastoma and had no effect on cell morphology. This might be due to a membrane alteration in the glioma, such as deletion of a

membrane component, as a result of which the appropriate contacts with the neuroblastoma may not have been formed. Plasminogen activator production by NOR-F and G-UVW was unaffected by neuroblastoma.

The observations discussed above indicate the importance of cellular interaction in regulating phenotypic expression. During embryonic development, the interaction of cells produce signals capable of triggering programmed cascades of sequential synthetic processes, resulting in membrane changes and altered cell function. Differentiation and development of cells in vivo is associated with the generation of, and response to, specific factors among neighbouring cells. In C_6 glial cells in culture, GFAP was induced by increased cell-cell contact formation at high density; high affinity GABA uptake was induced in several cell lines by increased membrane contacts at high density in monolayer culture, by three dimensional perfusion culture and, in the case of NOR-F, heterotypic interactions with neuroblastoma in co-culture. These findings suggest the importance of cell-cell contacts in the differentiation of cells of glial origin. The information for expression of the differentiated phenotype is contained within the cells but is regulated by the environment.

9. Chemical Agents

The effects of cytostatic agents, inducers of differentiation, tumour promoter and a carcinogen on the balance between the differentiated and malignancy associated phenotypes, were investigated for a number of cell lines. The compounds used and reasons for interest in their action were discussed in 1.6.2. Changes in the quantitative levels of GFAP, high

affinity GABA uptake, glutamine synthetase, plasminogen activator and endothelial cell mitogenesis, were examined after prolonged treatment of the cells with the chemical agent.

Differentiated Properties

GFAP

The levels of GFAP detected by I^{125} binding for a variety of cell lines was consistent with the visual observations by immunoperoxidase and immunofluorescence. The results confirmed the high GFAP content of G-CCM and the foetal lines, and the low level or complete absence of GFAP in the remainder of the glioma and normal adult lines. There was no obvious manipulation of cellular GFAP by any of the agents, implying that in tissue culture, the expression of GFAP remains constant in human brain cells. GFAP is a structural protein thought to be important in the role of fibrillary astrocytes in providing support for central nervous axons. The structural nature of the protein might explain its apparent lack of adaptation to environmental conditions.

Evidence of manipulation of GFAP by chemical agents came from previous work with the rat C_6 glioma (116). In this case combinations of dexamethasone and dibutyrilcyclicAMP, dexamethasone and isoproterenol and dibutyrilcyclicAMP alone, stimulated the reversible induction of GFAP in C_6 cultures. Cycloheximide and actinomycin D inhibited the drug mediated induction, indicating that *de novo* RNA and protein synthesis are requirements of the stimulation. The probable stem cell nature of the C_6 tumour has been discussed and might explain the apparent ease with which it can be induced for differentiated astroglial features.

One of the agents used in the manipulation experiments, pig brain extract (PBE) contained pig GFAP. As a result, its effect on the expression of GFAP in human cell lines remains undetermined. In future studies on glial cell differentiation, it is intended to purify from PBE the component responsible for the observed morphological and biochemical differentiation of the normal human adult cultures. This purified component will then be used to examine its effect on GFAP expression.

High Affinity GABA Uptake

High affinity GABA uptake was stimulated in a number of cell lines by some of the chemical agents used. In particular isoproterenol alone (IP),dexamethasone alone (DX),dexamethasone plus isoproterenol, retinoic acid (Ret.Ac.),mitomycin C (Mit.C),methylnitrosourea (MNU) and pig brain extract (PBE), all increased the uptake of GABA by the high affinity transport system in the normal adult cells. The greatest stimulation was observed after treatment with PBE. Although the results for a range of glioma lines were more variable, the observed trends were similar to the effects of the drugs on normal adult cultures. In general IP, DX, DX plus IP, Ret.Ac., interferon (IFN), Mit.C.,MNU and PBE stimulated high affinity uptake, with PBE again the most potent inducer.The PBE stimulation in G-RAT cells was abolished by actinomycin D indicating the requirement for de novo RNA, and presumably protein synthesis for the induction.

The two foetal lines investigated, NFT and NFR, had relatively high constitutive levels and did not exhibit enhanced high affinity GABA uptake as a result of treatment with the above agents. With the exception of reduced high

affinity uptake by the phorbol ester TPA, and also by MNU in the case of NFT, the foetal cell lines were insensitive to manipulation of high affinity GABA uptake by chemical agents. PBE had no effect on the foetal cells, consistent with the view that Glia Maturation Factor is only active in maintenance of the differentiated glial phenotype in adult brain.

The stimulation of high affinity GABA uptake in adult cells, by IP, DX, IFN, Ret.Ac. and PBE is consistent with the hypothesis that these agents can promote increased expression of differentiated cellular properties. The stimulation by Mit.C. and MNU, both DNA alkylating agents, was an interesting observation, the signficance of which is discussed later.

Glutamine Synthetase (GS)

GS activities, determined in the absence of any repression by glutamine, were increased by DX in all the cell lines investigated. Although there was variation between cell lines, the stimulation was greatest for cells derived from malignant tissue i.e. 2 to 10 fold. In the cases of the foetal and normal adult cultures, the stimulation was considerably less i.e. 1 to 4 fold and 1.05 to 1.2 fold respectively. The DX stimulation of GS in G-UVW and NOR-F cells was abolished by actinomycin D, implying that the increase in activity was the result of de novo RNA synthesis i.e. an alteration in gene transcription rather than metabolic fluctuations as a result of steroid action.

In the normal adult and malignant cell lines investigated, PBE caused a small stimulation of GS activity i.e. 1.2 to 1.8 fold. The remaining chemical agents showed no common pattern of effect on GS. In some cell lines e.g. G-MCN and G-UVW

there were small stimulations as a result of treatment with IFN, Mit.C and MNU. However, these stimulations were not consistently observed for a range of cell lines.

The increase in GS activity in response to DX has been reported for a number of other cell systems, including the rat C_6 glioma (139) and CHO fibroblasts (140). The induction by glucocorticoids is thus a property not confined solely to glial cells in the body. However, the DX stimulation might still represent induction of differentiation in cells whose normal, mature phenotype includes expression of high levels of GS. Glial cells come into this category.

Properties Associated with Malignant Behaviour

Plasminogen Activator

The drug responses of normal adult and malignant cell lines were similar, with respect to PA production. DX reduced PA considerably in the normal cultures and almost completely abolished it in the gliomas. This steroid mediated reduction in PA is consistent with observations on the effect of DX on PA produced by human embryonic lung (HuEL) cells (193). Actinomycin D, cordycepin and α amanatin inhibited the steroid mediated reduction of PA in HuEL cells, indicating that the synthesis of mRNA is a requirement of the inhibition. The DX inhibition of PA is therefore a positive event requiring *de novo* transcription and translation (193). PBE also reduced PA in both normal adult and malignant cell lines, as did IFN although to a lesser extent. These agents, DX, PBE and IFN induced high affinity GABA uptake in the gliomas and stimulated GS activity.

Of the other agents used, IP, Ret.Ac, Mit.C, TPA

and MNU stimulated PA production. With the exception of TPA, these agents also induced high affinity GABA uptake in the glioma lines. The effects of IP and DX on cellular PA were opposite and the combination of the two agents in the glioma lines, inhibited PA almost as much as DX alone. TPA and MNU both stimulated glioma PA production although the effects were not additive.

The results of the drug studies indicated two types of effect. Agents such as DX, PBE and, to a lesser extent IFN, stimulated the differentiated glial properties of high affinity GABA uptake and glutamine synthetase, and switched off or reduced PA, a malignancy associated property. Agents such as IP, Ret.Ac., Mit.C and MNU, however, stimulated high affinity GABA uptake and in some cases glutamine synthetase, and also stimulated PA production.

The foetal cell lines, NFT and NFU, had slightly greater PA activities than the normal adult lines which were reduced by DX alone and in combination with IP. Mit.C and MNU caused slight increases in PA production. The other drugs investigated had no effect on the foetal lines, implying that, as with high affinity GABA uptake, the foetal cells were relatively insensitive to manipulation of PA by chemical agents. The lack of effect of PBE once again supported the view that GMF is only active in adult brain.

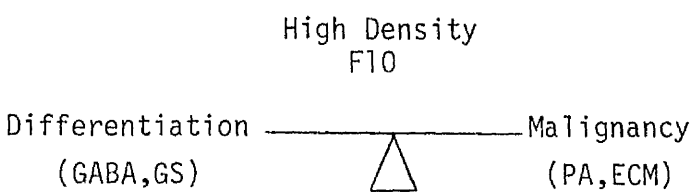
Endothelial Cell Mitogenesis (ECM)

The stimulation of endothelial cell mitogenesis by cell extracts was the least specific marker property associated with malignant astrocytes,as discussed previously. Despite its limitations as an *in vitro* criterion of malignant behaviour,

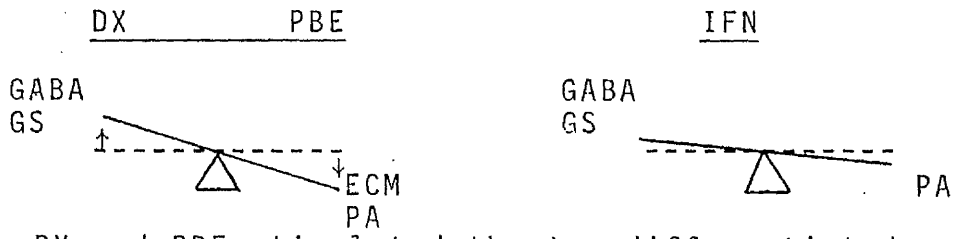
the drug mediated fluctuations in endothelial cell stimulation by extracts of two astrocytoma derived cell lines, were investigated. DX and PBE reduced endothelial cell mitogenesis activity in G-CCM and G-RAT cells, although the fluctuations were small in comparison to the effects of these agents on the PA activity of glioma cells. Fluctuations in specifically endothelial cell mitogenesis were perhaps masked by the more general cellular growth factors not affected by the drugs. Mit.C, TPA and MNU caused increases in mitogenesis activity, similar to the effects seen on PA activity of gliomas.

Drug Actions : Conclusions

Models can be drawn which describe, in general terms, the effects of the drugs investigated on marker properties associated with glial differentiation and malignant behaviour.



The model represents the hypothetical balance or equilibrium between the differentiated and malignancy associated phenotypes in astrocytoma derived cell lines. The absolute levels of expression of the individual markers varied between cell lines and are not related to the increases or decreases indicated on the models. However, the assumption is made that there is a steady state in vitro, which represents an equilibrium between differentiated and malignancy-associated properties under standard culture conditions.



DX and PBE stimulated the two differentiated properties and reduced the properties associated with malignant behaviour. DX has a growth inhibitory effect on glioma cells in culture (185) and is used in the treatment of patients with brain tumours to relieve intracranial pressure (184). Evidence from this investigation implies that DX might also have a considerable effect on the phenotypic expression of tumour cells, which is advantageous to the host i.e. stimulation of normal, differentiated glial function and reduction in the expression of malignancy-associated properties. The effect of PBE on the glioma cell phenotype was similar to that of DX. It would be of considerable interest to purify the component in PBE responsible for the phenotypic modification and investigate its potential use in reducing the malignant behaviour of glial tumours in vivo. The studies on the timing of the DX and PBE modulation of phenotypic expression implied that the two agents exerted their effects in different ways. From biochemical considerations of the mechanisms of action of steroid hormones and peptide factors on cell behaviour, possible mechanisms for the two agents can be postulated. It seems likely that DX would act by crossing the cell membrane, combining with a cytoplasmic receptor, translocating as a complex to the nucleus and directly or indirectly affecting gene transcription. The peptide maturation factor reported to be present in PBE, probably acts via membrane receptors resulting in the modulation of biochemical signals or second messengers within the cell, responsible for regulating many

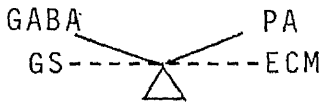
aspects of cell behaviour. In the case of Glia Maturation Factor the nature of the proposed second messenger is unknown; it is believed not to involve cyclicAMP (202).

The combination of DX and PBE gave rise to enhanced responses over those caused by either agent alone. Although their *in vitro* effects were not additive there was a degree of synergism which might be advantageous *in vivo*.

IFN, like DX and PBE, stimulated high affinity GABA uptake and in some cases glutamine synthetase, while decreasing PA production. The effect of IFN was less in magnitude than that of DX and PBE. However, its potential as a cytostatic agent which advantageously alters the phenotypic expression of glial tumour cells is recognised.

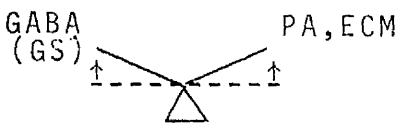
Clearly, the three agents, DX, PBE and IFN, which induced differentiated and reduced malignancy-associated properties, would be potentially the most useful of all the chemical agents tested in the treatment of gliomas *in vivo*. The active component of PBE, presumed to be the Glia Maturation Factor described by Lim *et al* (202), and IFN are both natural products. DX, a synthetic corticosteroid, probably acts in the same way as physiological glucocorticoids, which it mimics. One implication is that the most beneficial agents in the treatment of malignant disease might be natural physiological products such as putative tissue regulatory peptides or recognised hormones. Such agents should ideally be able to gain access to the tumour, be growth inhibitory to the malignant cells and reduce their capacity for malignant behaviour. In future experiments, it is hoped to study the effects of these agents, alone and in combination, on malignant gliomas *in vivo*.

IP, Ret.Ac.



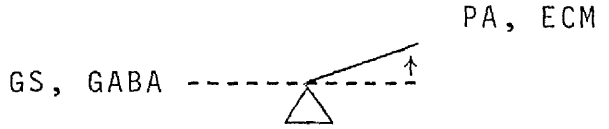
The effects of these agents were less general in terms of differentiation and malignancy-associated properties. High affinity GABA uptake and PA were stimulated, whereas glutamine synthetase and endothelial cell mitogenesis were unaffected.

Mit.C. MNU



Mit.C and MNU have common properties in that they both alkylate DNA and are known to be carcinogenic. Mit.C. is used in the treatment of malignant disease. Nitrosoureas are used in the clinical treatment of brain tumours. In malignant glial cells in culture these agents had profound effects on phenotypic expression. They not only stimulated the properties associated with normal differentiated function but also increased the properties associated with malignant behaviour. Although Mit. C and MNU are advantageous as cytotoxic agents and are apparently able to stimulate differentiation in malignant glial cells, they also stimulate malignancy-associated functions in these cells. This might assist the ultimate survival and advance of the tumour.

TPA



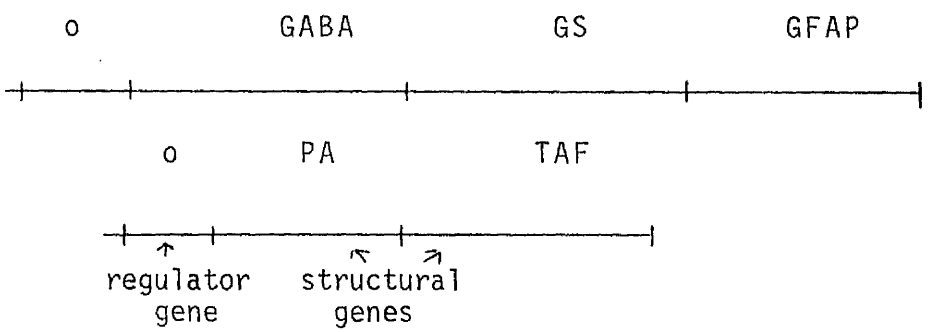
The tumour promoting phorbol ester TPA stimulated the

malignancy-associated properties of plasminogen activator and endothelial cell mitogenesis. However, there was no effect on the differentiated properties investigated. This observation differs from previous findings on the inhibition of differentiation by a phorbol ester in mouse preadipose cells (242), C1300 neuroblastoma cells (243) and cultures of newborn mouse epidermal cells (244). In malignant glial cells in culture the phorbol ester appeared to affect only malignancy-associated properties.

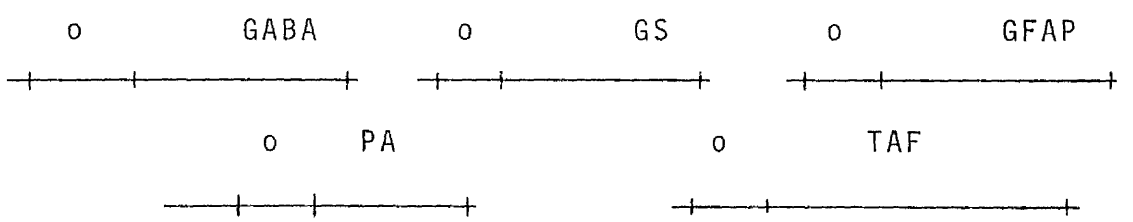
10. Concluding Remarks.

The results obtained in this investigation provided interesting findings about aspects of phenotypic expression, and its control in astroglial cells. Speculative hypotheses can be put forward for the mechanism of control of gene expression for the properties examined

One possibility is that the three differentiated properties and the two malignancy-associated properties form part of "batteries" of genes, coming under the control of single regulators, (as found in the lac operon system in bacteria) i.e.



Another possibility is that the genes are distributed throughout the genome, each with its own regulator gene, i.e.



On the basis of the results from the characterisation studies, it appears that the differentiated and the malignancy-associated properties were not always co-ordinately expressed. Cells which had the capacity for high affinity GABA uptake, for example, did not necessarily express GFAP; similarly TAF-producing malignant cells did not necessarily produce high

levels of PA. This implied that the battery of genes hypothesis was less likely, although battery gene transcription, followed by post-transcriptional processing allowing some products to appear in the final cell phenotype, cannot be excluded. Regulation of these products in response to changing environmental conditions, was co-ordinate. Agents such as dexamethasone and pig brain extract, stimulated expression of the differentiated functions and reduced expression of both the malignancy-associated properties. These agents have the capacity to specifically switch on the expression of some genes, and switch off or reduce the expression of others. It is difficult to envisage how this could occur for independent genes, with their own individual regulators distributed throughout the genome. However, it is known that normal development involves a large number of specific changes in gene expression occurring in sequence, and involving genes distributed throughout the genome. The molecular mechanisms responsible for this complex series of events, are also not understood. A clue to the mechanism of specificity of particular drugs in stimulating expression of differentiation and reducing expression of malignancy-associated functions, might come from a better understanding of the control of gene expression in normal developmental processes.

There are many known examples of activation of developmental genes occurring in neoplastically transformed cells. Loss of differentiated properties of tumour cells is also a recognised feature, along with the acquisition of the properties of malignant behaviour. In the astroglial cell system, the malignant cell lines represented a gradation in states of biochemical differentiation. The less well differentiated cell

lines could have arisen as the result of either selection of immature cells from the original tumour, or a de-differentiation process in culture. Tumour formation itself may be a characature of tissue renewal, with the neoplastic event perhaps occurring during the proliferation of non-terminally differentiated cells e.g. during a process such as gliosis in the brain. De-differentiation implies the switching off of genes once expressed in a differentiated cell. If this is the case, then it would appear, from the characterisation studies, that the genes coding for differentiated products were switched off in the reverse order from the way they were switched on in the postulated astroglial differentiation pathway. However, although many of the astrocytoma derived cell lines resemble immature glial cells with respect to the expression of differentiation, an obvious difference lies in their expression of other properties. The tumour products, TAF in particular, were produced by malignant cell lines, but never by normal brain derived cell lines, thought to be precursor glial cells.

The differences between tumour cells and their normal counterparts lie in cellular products associated directly or indirectly, with the capacity for malignant behaviour. A bulk biochemical approach to look at total cell products of malignant and equivalent normal cells,would be of considerable value, and might eventually lead to identification of the product(s) of the postulated 'onc' gene(s). One possibility is that the 'onc' gene product might, itself be a regulatory factor, functioning in the nucleus at the DNA level and involved in the direct control of gene expression. As a result of its activity, genes commonly found to be 'altered'

in cancer such as these involved in growth control, DNA rearrangement, early embryonic development and expression of differentiation, might be affected.

Evidence for the mechanisms involved in neoplastic transformation remains sketchy while the existence of the hypothesised 'onc' gene is also uncertain. However, although a good understanding of the basic molecular biology of neoplasia is still missing, many advances have been made in cancer therapy using cytotoxic drugs. Another promising line of attack on neoplastic cells might come from trying to reverse the faulty cytoplasmic and nuclear controls in tumour cells, allowing them to regain a differentiated state and reduce their potential for malignant behaviour. It would be of interest to screen drugs used in chemotherapeutic regimes to determine their effect on tumour cell phenotype. This would allow selection of drugs which not only inhibited tumour growth, but also stimulated re-expression of normal differentiated function and reduced malignancy. Such drugs might result in a more beneficial response for the host after chemotherapy, than drugs which maniuplate the phenotype in other ways. Examples of the latter are provided by mitomycin C and methylnitrosourea, which, as well as being cytotoxic in their action, stimulate expression of malignancy-associated properties *in vitro*. If the same is true *in vivo*, it might be that treated cells are more malignant in their behaviour once the growth inhibitory block is overcome; ensuing invasion and/or metastasis might therefore be more rapid.

Future

It now seems important to determine whether the effects of drugs on tumour cell phenotype observed *in vitro* are significant *in vivo*. It is hoped to investigate this using human tumours grown in immune deprived mice. It is also hoped to extend these studies to a more common epithelial tumour cell system, probably lung, in parallel with other projects looking at the production of malignancy-associated factors *in vivo* and *in vitro*.

SOLUTIONS APPENDIX

1. Holding Medium

Hams F10 medium
Non-essential amino acids
Hepes buffer (20mM)
Sodium bicarbonate (0.0375%)
Glutamine (2mM)
NaOH to pH 7.4
Penicillin (250 units ml^{-1})
Kanamycin (100 $\mu g ml^{-1}$)
Streptomycin (250$\mu g ml^{-1}$)

2. Dissection BSS

Hanks balanced salt solution
Penicillin (250 units ml^{-1})
Kanamycin (100 $\mu g ml^{-1}$)
Streptomycin (250$\mu g ml^{-1}$)

3. Growth Medium

Hams F10 medium
Non-essential amino acids
Hepes buffer (20mM)
Sodium bicarbonate (0.0375%)
Glutamine (2mM)
NaOH to pH 7.4
Foetal calf serum (10%)

4. PBS/EDTA

EDTA (1mM) in Phosphate buffered saline

5. Tris/Saline

0.1N HCl
0.2M Trizma base
0.09% NaCl
pH 7.6

6. DAB Solution

Diaminobenzidine-HCl ($0.5 mg ml^{-1}$)
Hydrogen peroxide (0.001%)
in Tris-Saline as above.

7. Amino Acid Free Medium (AAFM)

Hanks balanced salt solution
Glucose (0.1%)
Vitamins
Sodium bicarbonate (0.15%)
NaOH to pH 7.4

8. Sodium Barbitone Buffer

28.5mM Sodium barbitone
21.5mM HCl
0.09% NaCl
pH 8

9. BSS/Vitamins/Glucose Solution

Made up as for AAFM (7) using BSS containing no phenol red to avoid interference in chromogenic plasminogen activator assay.

MATERIALS APPENDIX

Reagents were obtained from the following companies:-

BDH Chemicals Limited, Poole, England.

Acetic acid (Analytical Grade)
Acetone (Analytical Grade)
Bromophenol Blue
Dimethylsulphoxide (DMSO) (Analytical Grade)
DPX Mountant
Giemsa stain
Glutaraldehyde (Analytical Grade)
HCl (Analytical Grade)
NaCl (Analytical Grade)
NaOH (Analytical Grade)
Sodium Lauryl sulphate (SDS)
Trichloracetic acid (TCA)
Trizma base (Analytical Grade)

Sigma Chemical Company, Poole, England.

β Alanine
γ Aminobutyric acid (GABA)
L-Aspartic acid
Dibutyrilcyclic AMP
α-D-glucose
L-Glutamic acid
Isoproterenol (IP)
poly-D-Lysine
Mitomycin C (Mit.C)
Methylnitrosourea (MNU)
Phorbol 12-Myristate acetate (TPA)
Porcine Liver Extract
Retinoic Acid (Ret.Ac.)
Sodium Barbitone
Thrombin
Trypsin (type III)

Gibco Europe Limited, Paisley, Scotland.

Foetal calf serum
Glutamine
Hepes buffer
Kanamycin solution
Non-essential amino-acids
Sodium Bicarbonate
Trypsin

Flow Laboratories, Irvine, Scotland.

Foetal calf serum
Hams Flo medium
Hanks balanced salt solution
Trypan Blue dye
Vitamins
Vitrogen collagen

Kabivitrum Diagnostics, Uxbridge, England.

Fibrinogen
Plasminogen
Chromogenic substrate S-2251
S-2238
S-2302

Dako Laboratories, Denmark.

Horse raddish peroxidase conjugated swine anti-rabbit serum
Rhodamine conjugated swine anti-rabbit serum
Rabbit anti-human factor V111 serum

Radiochemical Centre, Amersham, England.

$^{3}_{H}$-Glutamic acid
$^{3}_{H}$-γ Aminobutyric acid (GABA)

New England Nuclear, Boston, Massachusetts, U.S.A.

I^{125}-labelled Goat anti-rabbit lgG

Collaborative Research, Waltham, Massachusetts, U.S.A.

Endothelial cell growth supplement (ECGS)

Hoescht, Hounslow, England.

Hoescht 33258 stain

Worthington Biochemical Corporation, New Jersey, U.S.A

Collagenase

Glaxo, Ware, England.

Benzyl Penicillin

Oxoid Limited, Basingstoke, England.

Phosphate buffered saline (PBS) tablets

Evans Medical Limited, Greenford, England.

Streptomycin sulphate

Merck, Sharp and Dohme Limited, Hoddesdon, England.

Dexamethasone sodium phosphate (Decadron)

Leo Laboratories, Hayes, England.

Urokinase

Serva, Heidelberg.

$3^{1}3^{1}$-Diaminobenzidine HCl

Beechams Pharmaceuticals, Epsom, Surrey.

β-Interferon (Gift)

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chromatography against accepted metabolites as standards.

The cultured tumour cells were relatively insensitive to both MTX and DNR. However ADR in doses equivalent to DNR showed dose-related cell killing. There was complete cell killing if ADR was not removed from the culture before day 3. Metabolism of both DNR and ADR occurred, predominantly yielding side-chain derivatives.

The conclusion from this study so far is that these virally-induced tumours are sensitive to ADR but can distinguish this molecule from the very closely related DNR. The relationship between cell sensitivity and ADR metabolism, and between relative resistance and DNR metabolism is currently being investigated.

M. FRAME — D. MORGAN — R.I. FRESHNEY — R. SHAW

Glial fibrillary acidic protein and Plasminogen Activator in cultured normal and malignant brain cells

Glial fibrillary acidic protein (GFAP) and Plasminogen Activator (PA) production have been studied in a number of early passage cell lines derived from normal brain and malignant astrocytoma. These cells have been characterised by glutamine synthetase activity (1) and high affinity GABA uptake (2), known markers of glial differentiation. Previously, cells derived from malignant astrocytoma have been shown to have high labelling index at terminal cell density (3) and abnormal karyotype (4).

Only one astrocytoma cell line screened for GFAP has proved to be 100% positive (G-CCM) while some other lines have a small number of positive cells. The established rat glioma line C6 has 50% GFAP positive cells in control cultures and can be induced to almost 100% by known inducers of glial differentiation such as Bt_2 cAMP and dexamethasone. Increasing cell density in C6 cultures also increases GFAP. Many cell lines, including those derived from normal adult brain, are completely negative and are not induced by the above agents. Normal foetal brain cultures are up to 100% GFAP positive without chemical induction.

Plasminogen activator, often associated with malignant behaviour (5), is produced by a number of glioma lines all of which are GFAP negative. Increasing cell density reduces PA production, the opposite effect to that seen with increasing cell density on GFAP in C6 cultures. PA is not detectable in cultures of G-CCM and is very low in C6 (both GFAP positive). Normal brain derived cells are also very low in PA activity.

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J.D. PITTS — R.R. BÜRK — J.P. MURPHY — A.E. HAMILTON

Retinoic acid inhibits junctional communication between animal cells

Most types of animal cells, *in vivo* and in culture, form intercellular junctions which are freely permeable to small ions and molecules but not to macromolecules. These permeable junctions (gap junctions) provide pathways of direct communication between all the cells in a coupled population. They allow the spread of action potentials through excitable tissues. The rapid equilibration of metabolite pools between coupled cells results in the cordination of enzymic activity and can

MARKERS OF DIFFERENTIATION IN GLIAL CELLS

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The kinetics of uptake of glutamate and γ-aminobutyric acid (GABA) by normal glia and glioma cell lines have been used as marker properties of differentiation. With respect to glutamate uptake, all glia and glioma lines tested showed biphasic Lineweaver-Burk plots, within the range of glutamate concentrations used, indicating the presence of both low affinity (Km 0.3 - 0.5mM) and high affinity (Km 30 - 50μM) uptake mechanisms. The high affinity mechanism is sensitive to competitive inhibition by aspartate. Demonstration of high affinity transport and specific inhibition by aspartate may prove useful as a marker of differentiation in these cells. Of the other cell types tested, only melanoma lines showed the presence of two different affinity mechanisms of uptake. Foetal Human Intestine (FHI) cells, 3T3 mouse fibroblasts and MRC-5 human diploid fibroblasts gave rise to monophasic Lineweaver-Burk plots with only a low affinity mechanism for glutamate uptake. Another cell line (GMS) which was derived from a human astrocytoma but was thought to be predominantly endothelial from morphological evidence, its loss of GFAP positive cells with passage number, and its high level of alkaline phosphatase, also showed only a low affinity glutamate uptake system. All the glial lines tested and some glioma lines have both high and low affinity mechanisms of GABA uptake. In some cases the presence of the high affinity system was only evident after treatment of the cells with known inducers of glial differentiation such as dibutyryl-cyclic AMP (in the absence of serum) or β-methasone. These observations imply that high affinity transport of putative neurotransmitter amino acids may be restricted to neurectodermal cells.

Glial fibrillary acidic protein (GFAP) has been shown to be a specific marker for astroglia and can be demonstrated in both normal amd malignant glia, *in vivo* and *in vitro*. GFAP was induced spontaneously in cultures of human and mouse foetal brain. It is also present in rat C_6 glioma cultures, being maximally expressed in early stationary phase cultures. This increase in GFAP production coincides with the onset of cell - cell contact and density dependent inhibition of proliferation. Induction of GFAP in C_6 cultures above control values can be achieved by agents such as isoproterenol, dibutyryl-cyclic AMP and dexamethasone with maximal induction being obtained using a combination of these agents. The protein synthesis inhibitor, cycloheximide, and the transcriptional inhibitor, actinomycin D, both prevent this induced increase in GFAP.

It may be possible to exploit these marker properties of glial cells (with the help of inducing agents) in future studies of differentiation and malignancy in cultures of normal glia and glial tumours.

Acknowledgements

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GLUCOCORTICOIDS, PROLIFERATION AND THE CELL SURFACE

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ABSTRACT

An attempt has been made to correlate the induction of differentiation in glial cells with a reduction in cell proliferation to determine whether the cytostatic effect of glucocorticoids may be related to a normal regulatory role. Growth of cells at high cell densities using cell counts and labelling indices with (^{3}H)- thymidine has been compared between cultures exposed to glucocorticoids and cyclic AMP, and contrasted with the effect of these agents on differentiation, monitored by induction of glial fibrillary antigen and uptake of neurotransmitter aminoacids. The effect of glucocorticoids on (^{3}H)- and (^{14}C)-glucosamine incorporation into protease digests of the cell surface has also been examined. These results show that glucocorticoids are cytostatic to malignant glia and can promote differentiation and a normalisation of the cell surface. Isoproterenol (or cyclic AMP), while promoting a higher level of differentiation, does not appear to be cytostatic. Its effect on the cell surface has not been examined.

KEY WORDS

Glioma, glial fibrillary antigen, γ -aminobutyric acid, cell surface, glucocorticoids, isoproterenol, cyclic AMP.

Glucocorticoids are well established as antineoplastic agents in the treatment of leukaemia, lymphoma, breast carcinoma and many other malignancies. Just as normal physiological regulation by glucocorticoids exhibits site specificity, the effect on different tumours may vary according to the target cell. While the effect of glucocorticoids on leukaemia and lymphoma is apparently cytotoxic (Kondo and Co-workers, 1975), mediated by specific receptor binding, the effect on other tumours is less well characterised. Unlike haemopoietic tumours, the effect on solid tumours such as breast (Braunschweiger & Co-workers, 1978), and lung (Jones & Co-workers, 1978) carcinoma may be cytostatic.

Glucocorticoids and Differentiation

Glucocorticoids have long been known to induce differentiation in many different cells including neural retina (Piddington & Moscona, 1967), liver parenchyma (Greengard, 1970), hepatoma (Granner & Co-workers, 1968), rat cerebral cell

cultures (Breen & de Vellis, 1974) and the rat C_6 glioma (Bennett & Co-workers, 1977). It is possible that steroids may have a cytostatic effect by promoting the differentiation of a more normal phenotype, including the expression of cell surface features normally recognised in contact mediated density limitation of cell proliferation.

Glucocorticoids and the Cell Surface

Ivarie and O'Farrell (1978) demonstrated the induction of a new sialoprotein in hepatoma cells induced by glucocorticoids and Furcht & Co-workers (1979) have shown that fibronectin, often deleted in malignant cells, is accumulated on the surface of SV40-transformed human fibroblasts following glucocorticoid treatment. This may be due to induction of de novo protein synthesis or repression of extra cellular protease activity (Wigler & Co-workers, 1975).

Malignancy and the Cell Surface

Glimelius and co-workers have suggested that malignant transformation is accompanied by an increase in sialation of cell surface glycopeptides and they showed that continuous lines of human glioma have more heavily sialated glycopeptides than their normal counterparts. Malignant transformation may result in a loss of some cell surface glycopeptides, such as fibronectin (Vaheri & Co-workers, 1976), and a redistribution of glycosylation of others.

Glucocorticoids and Malignancy

We would like to propose that part of the action of glucocorticoids is to cause the re-expression of the cell surface properties of the equivalent normal cell by inducing the accumulation of adhesive glycoproteins such as fibronectin and, as a result, or additionally, causing the distribution of carbohydrate residues to return to normal. This could produce a cell more capable of recognising adjacent normal cells, increasing contact mediated density limitation of growth and motility, i.e. a cytostatic and anti-invasive capability mediated by one general phenotypic modification.

Glucocorticoids and Human Glioma

The present approach, to study the effect of glucocorticoids on glioma, was encouraged by the extensive use of these steroids to reduce oedema associated with brain tumours. It had been suggested also that glucocorticoids would halt glioma in the G_1 phase of the cell cycle (Wilson & Co-workers, 1972), and others (Mealey, Chen & Schantz, 1971) had suggested a cytotoxic effect. Our own observations (Guner & Co-workers, 1977) showed that glucocorticoids could stimulate cell survival (cloning efficiency) and proliferation (colony size) in a clonal growth assay and that cytostasis did not occur until either the colonies reached a certain size (50-100 cells) or a high density monolayer was used (Freshney & Co-workers, 1980). Treatment of monolayer cultures showed that a lower terminal cell density was attained in the presence of glucocorticoids (Fig 1) and that the labelling index with (3H)-thymidine at this density was about one third of untreated controls (Fig 2). That this was not due to cytotoxicity, peculiar to this cell concentration, was demonstrated by cloning the cells following prolonged treatment (5 days) with β-methasone at terminal cell density. Cloning efficiency was unimpaired and the steroid gave the induction of cloning efficiency previously found (Freshney & Co-workers, 1980).

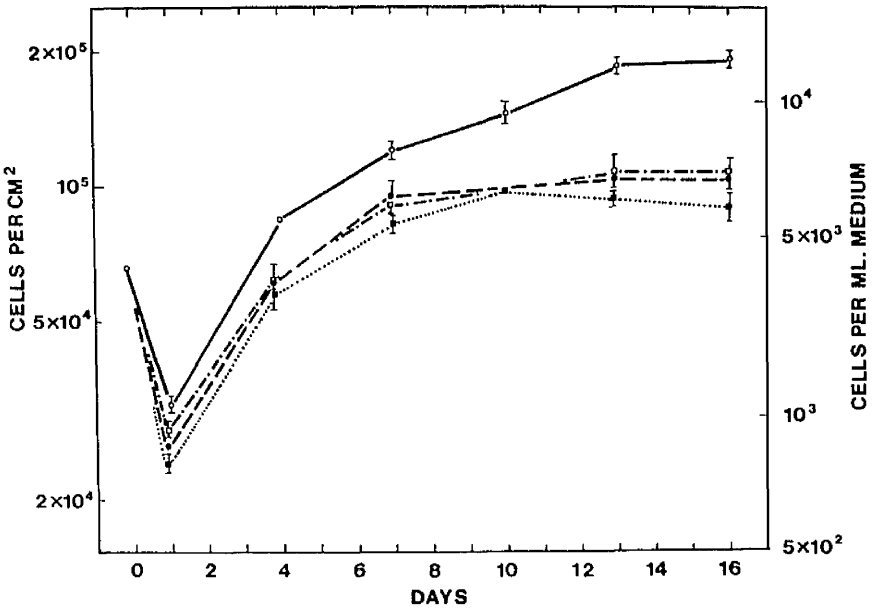

Fig 1 Reduction in terminal cell density by glucocorticoids. Cell line LET 11 was trypsinised in the third passage and seeded on to 15mm coverslips grown in 24-well Linbro dishes (Flow Laboratories). The next day the coverslips were transferred to 9cm bacteriological grade petri dishes containing 20ml medium and culture continued for a further 15 days with medium changes every 2-3 days. Cell counts were performed at intervals by disaggregating the cells in 0.125% trypsin and 1000 u/ml collagenase (Worthington, CLS). o———o Control; ●- - -● 10 µg/ml B-methasone; □-·-·-□ 10 µg/ml dexamethasone; ■·····■ 10 µg/ml methyl prednisolone.

Glucocorticoids and Rat C_6 Glioma

A similar cytostatic effect of dexamethasone has been noted in the rat C_6 glioma. Monolayer cultures treated for two weeks with dexamethasone reached a lower terminal cell density than controls (Fig 3). Isoproterenol, a cyclic AMP mediated adrenergic drug had no effect on terminal cell density. C_6 cells were examined because (1) they express the normal differentiated marker of astroglia, glial fibrillary antigen (GFA), (see below), and (2) are inducible for glycerol phosphate dehydrogenase synthesis by dexamethasone (Bennett & Co-workers, 1977). This second feature is in some conflict with the first as glycerol phosphate dehydrogenase is an oligodendrocytic marker while GFA is astrocytic. Either C_6 can express both or contains sublines which can differentiate down either route.

Induction of GFA in C_6 Glioma

Dexamethasone alone was capable of only slight induction of GFA in C_6 (Fig 4(a)) but cyclic AMP alone or in the presence of dexamethasone caused an increase in

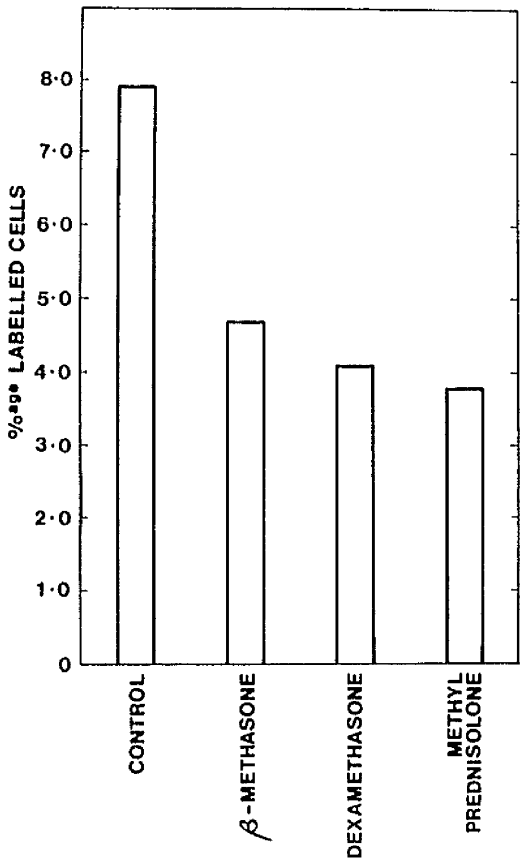

Fig 2. Inhibition of labelling index at terminal cell density by glucocorticoids. Conditions as in Fig 1. Duplicate coverslips were labelled for 24 hours with (^{3}H)-thymidine (200 Ci/mMol, 5 mCi/ml) at 16 days, and the cells disaggregated and seeded at approximately 1:5 dilution on to fresh coverslips. After 24 hours the monolayer was washed, fixed, acid soluble precursers extracted, and autoradiographs prepared. They were developed after 2 weeks' exposure and the % labelled cells determined.

the proportion of GFA positive cells. This effect could also be demonstrated by isoproterenol (Fig 4(a)). Increasing cell density alone will increase GFA expression although less than cyclic AMP or isoproterenol (Fig 4(b)). Maximum induction was obtained with cyclic AMP in combination with dexamethasone when almost 100% of the population expressed GFA.

Glucocorticoids, Cyclic AMP, and Uptake of Neurotransmitter Amino Acids

The high affinity transport of γ-aminobutyric acid (GABA) has been shown to be a differentiated function of glial cells (Schon & Kelly, 1974, Schousboe & Co-workers, 1979). This may be induced by dexamethasone and cyclic AMP in cultures from normal human brain but so far we have not demonstrated this in glioma. We have also demonstrated high affinity glutamate transport in several normal and malignant lines but not in cell lines of non-neurectodermal origin.

Differentiation and Cytostasis

The original hypothesis above suggested that the induction of the normal differentiated phenotype might bring about cytostasis. However, promotion of differentiation by isoproterenol or cyclic AMP had little effect on terminal cell density while dexamethasone alone, although limiting terminal cell density, had less effect on differentiation (Figs 3 and 4 and unpublished observations).

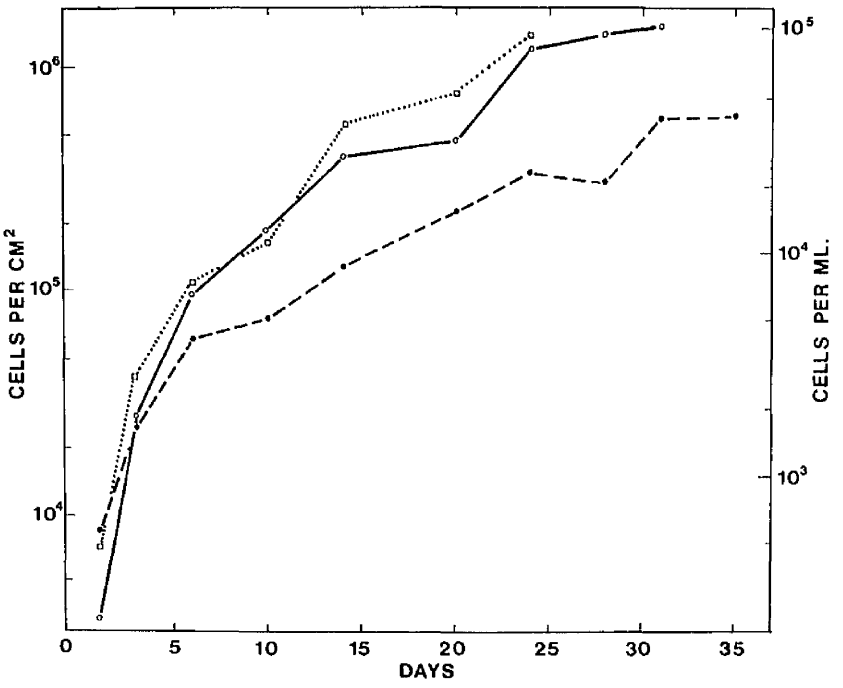

Fig. 3 Reduction of terminal cell density in rat C_6 glioma. Conditions as in Fig 1 except that C_6 cells (Benda et al (1968)) were used instead of human glioma.
o———o control; ●– – – – – – ● 2.5×10^{-5} M (10 mg/ml) dexamethasone; □·····□ 10^{-5} M isoproterenol.

Glial Cell Surface

The model also suggests that one important phenotypic change induced by glucocorticoids may be at the cell surface. Glimelius & Co-workers (1979) showed that continuous glioma lines differed from normal glial cells by an increase in the proportion of high molecular weight glycopeptides in protease digests of the cell surface. Since continuous lines of glioma, though undoubtedly malignant, may have undergone further transformation in vitro (eg. the karyotype shifts from near diploid to subtetraploid), we have investigated cell lines from glioma at lower passage levels. These lines show a similar increase in the proportion of high molecular weight glycopeptides when compared to cell lines from normal brain (Fig 5(a)).

When glioma cultures were treated for two weeks with dexamethasone and then the cell surface glycopeptides compared (Fig 5(b)), the high molecular weight component was reduced and two low molecular weight peaks appeared. These were also present in very small amounts in the normal cells and may be mainly glycosaminoglycans.

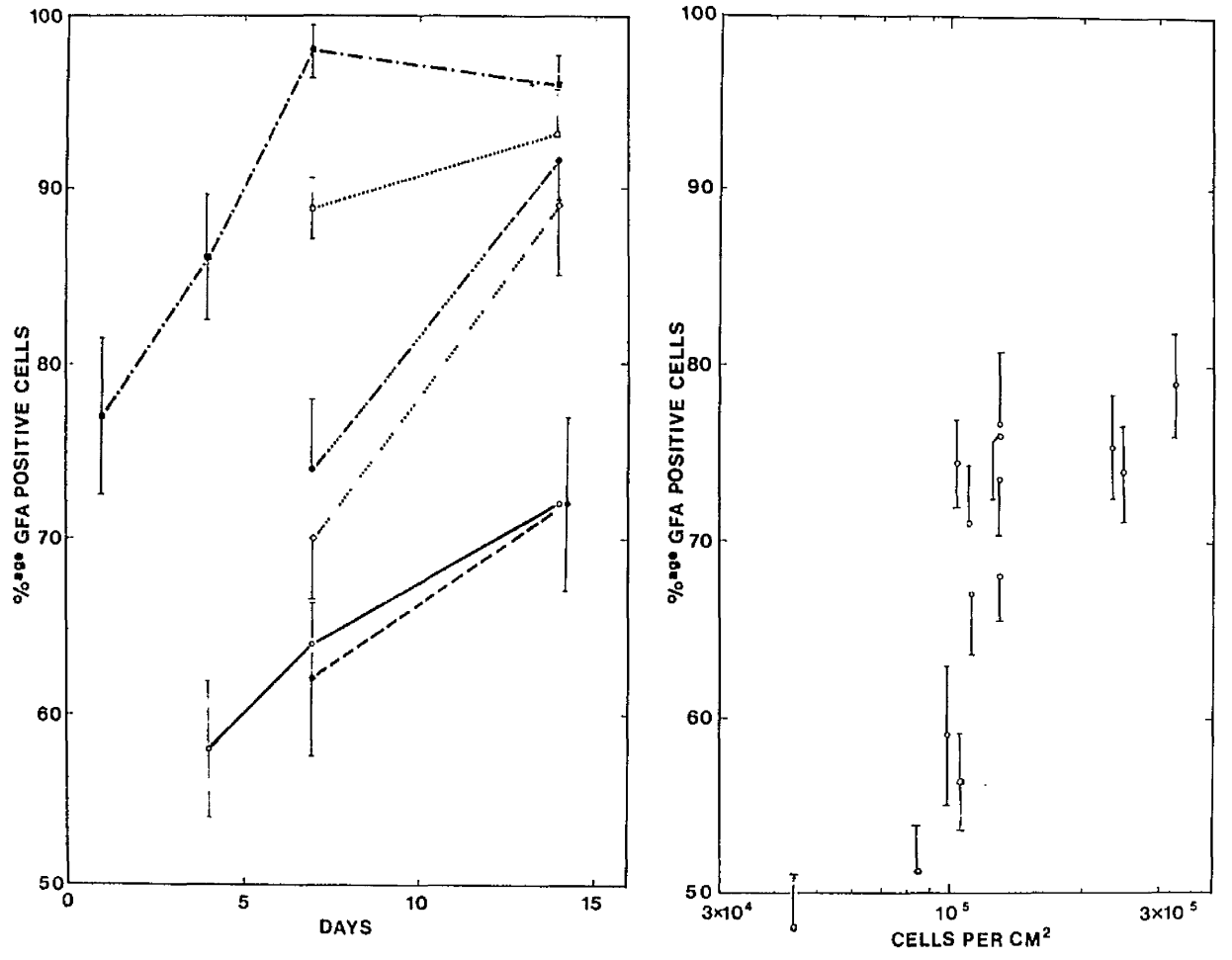

Fig.4 Induction of GFA in C_6 cells. This represents the accumulated data of several experiments.

(a) Cultures were grown in 75cm^2 flasks and stained for GFA by the immunoperoxidase method at the times indicated by cutting out portions of the flasks. Between 200-500 cells were scored and the percentage GFA positive cells determined. Samples without anti-GFA antibody, used as controls, were uniformly negative. Each point is the mean and standard deviation of 10-20 replicate fields from duplicate samples. o——o control, ● - - - ● 2.5 x 10^{-5} M dexamethasone; □·····□ , 10^{-4} M dibutyryl cyclic AMP; □ ·—·—·—· □ dexamethasone plus dibutyryl cyclic AMP; ◊ ... ... ◊ 10-5 M isoproterenol; ◆ —···—··· ◆ isoproterenol plus dexamethasone.

(b) Summary of control observations plotted against cell density.

Hence treatment of glioma lines recreates a cell surface glycopeptide pattern on elution from Biogel P10, more similar to normal cells. Treatment of normal cells and a continuous line from human anaplastic astrocytoma with dexamethasone

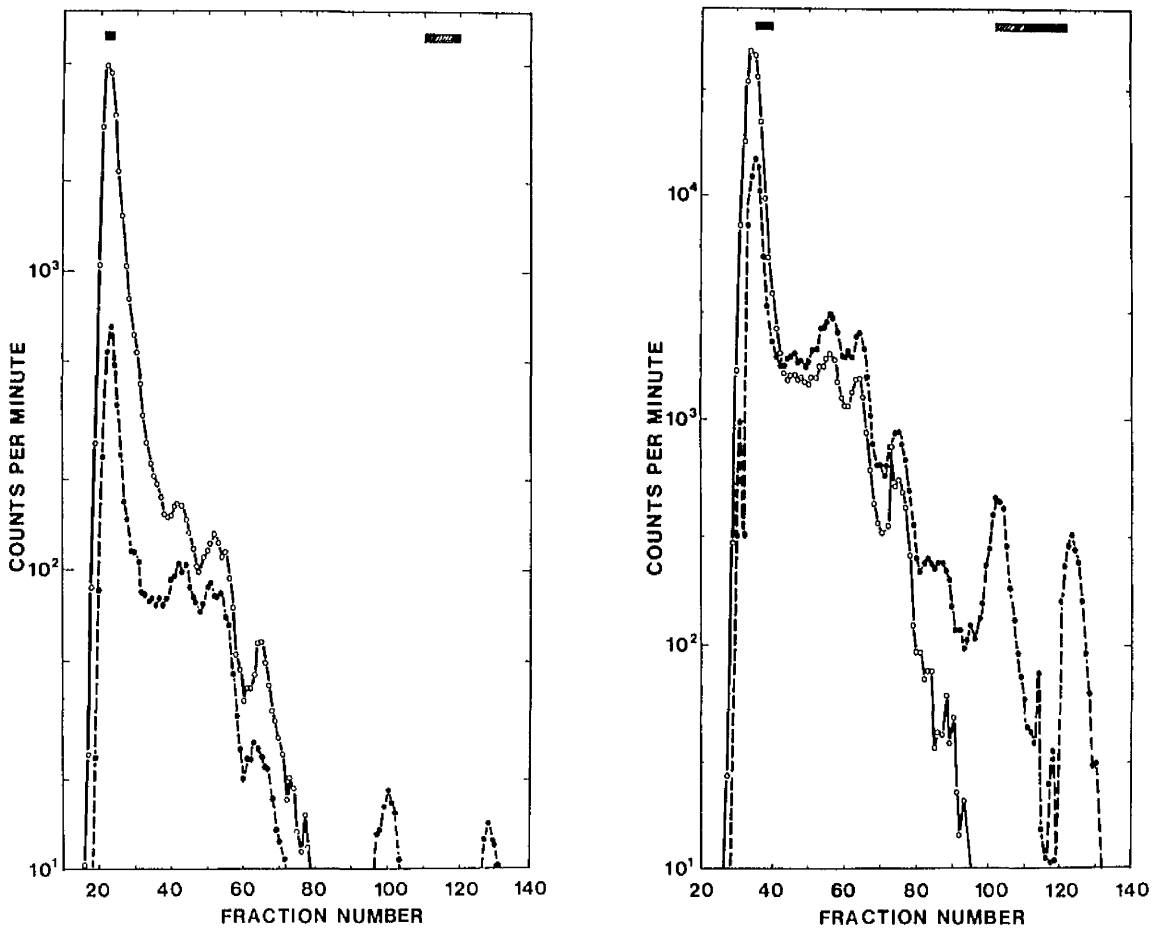

Fig 5 Cell surface Glycopeptide Digests. (a) Cultures from a glioma line CLD and a normal brain line NAB. Cells were grown to confluence in 120cm^2 glass flasks and labelled with (^{3}H)-glucosamine (4Ci/mMol) or (^{14}C)-glucosamine (61 mCi/mMol), 0.2 uCi/ml for 5 days. The cultures were then treated as in Glimelius & Co-workers (1979). Briefly, the supernatant from trypsinised cells was treated with pronase, dialysed, and the combined supernatants from normal (^{3}H-labelled) and malignant (^{14}C-labelled) run on a Biogel P10 column. The eluates were collected and counted. o———o glioma; ●----● normal brain. ▬ Blue Dextran marker (void colume, > 20,000 daltons); ▨ phenol red marker. (b) Effect of glucocorticoids. Conditions as in Fig 5(a) except that CLD cells were used with and without 2.5 x 10^{-5} M dexamethasone, labelled separately with (^{3}H) or (^{14}C) glucosamine and chromatogrammed. o———o control; ●— — —● dexamethasone.

had no effect on their glycopeptide pattern. It is of interest in this context that glucocorticoids have less effect on the terminal cell density of normal cells and no effect at all has been recorded on the continuous cell line from astrocytoma.

In conclusion, while differentiation and cytostatis may not be totally linked in glial cells, glucocorticoids to favour the induction of a more differentiated

phenotype and apparently reconstitute at least one normal cell surface characteristic. Whether this is related to the cytostatic effect of the hormone, and whether this will affect the invasive properties of the cells, remains to be seen.

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CULTURE OF ENDOTHELIAL CELLS

FOR THE STUDY OF ANGIOGENESIS IN VITRO

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Running Title: Culture of Endothelial Cells

Key Words: Endothelium, Culture, Angiogenesis, Tumour, Glioma.

Contents

Abstract

Endothelial cells can now be cultured from a number of different sites and show certain common characteristics in vitro. They provide useful models of density-dependent growth control and differentiation and are of interest in studies of vascular disease and neoplasia.

We have prepared endothelial cell lines from a biopsy of cerebral astrocytoma (GMS) and from human umbilical vein (HUV) by digestion in collagenase. Both were found to be factor VIII positive and responded to mitogenic stimulation with an intracellular extract from Walker 256 carcinoma cells.

Cultures of HUV cells grown on plastic display signs of morphological differentiation if maintained at saturation density in the presence of endothelial growth supplement, simultaneously shedding cells into the supernatant medium. Transmission electron microscopy of these cultures has shown Weibl Palade bodies, tight junctions and the production of extracellular matrix.

Extracts of human gliomas, and Walker 256 cells, have been shown to promote angiogenesis in the chick chorioallantoic membrane in ovo, but this system presents certain problems of quantitation so the potentialities of cell culture assay are now being explored.

Human umbilical vein cells (HUV), grown to confluence on collagen coayed dishes, undergo density limitation of growth at a lower cell density than cells grown on plastic. Addition of crude

extracts of cultured Walker 256 cells and one human astrocytoma cell line elicits a mitogenic response resulting in an elevated saturation density. Experiments are now underway to determine whether this response can be used to monitor the production of angiogenesis factor by human astrocytoma cultures.

CULTURE OF ENDOTHELIAL CELLS

R. Ian Freshney and Margaret C. Frame.

Introduction

The successful culture of functional, characterised cell lines from vascular endothelium has generated an upsurge of interest in many different disciplines from the basic cell biology of growth control to the potential value of differentiated endothelial cell cultures in the regeneration of diseased blood vessels.

Considerable interest has also been expressed in the interaction of malignant cells with vascular endothelium in vitro (a) as a model of invasion and metastasis (Kramer and Nicolson, 1979; Zamora et al., 1980; Jones et al., 1981) and (b) as a model for tumour-induced angiogenesis (Fenselau and Mello, 1976; Schor et al. 1980; Folkman and Haudenschild, 1980; Taylor and Folkman 1982). The reassociation of monolayers of endothelial cells with underlying multilayers of smooth muscle cells in vitro (Jones, 1979) also suggests an interesting model for the study of vascular diseases such as atherosclerosis and thrombosis, as well as a three dimensional model for tumour cell invasion (Jones et al., 1981).

Although some care is required during disaggregation and subsequent culture to select out endothelial cells from smooth muscle and connective tissue they are not difficult cells to grow, given the correct culture conditions. Cultures have been prepared from tissues from a number of different sites (Table 1) and human tissue is often readily available from umbilical cord, and foreskin, avoiding most ethical problems. It is possible to obtain/

obtain homogeneous cultures either by cloning (Clark and Pateman, 1978; Folkman et al, 1979; Jones, 1979), or by carefully selecting the starting material by dissecting out small capillary segments of pure endothelium under the microscope (Folkman et al, 1979). It is also possible to eliminate smooth muscle cells by selective adhesion.

The cultures that are produced are very sensitive to density limitation of growth and tend to arrest as a confluent monolayer providing a good model for the study of growth control. A number of specific markers are available to identify the cultures and the expression of some of these markers, e.g. Factor VIII and angiotensin -converting enzyme is influenced by cell density (DelVecchio and Smith, 1981) implying that full differentiated function is not expressed until the cultures reach confluence. Spontaneous morphological maturation has been observed at this stage (Folkman and Haudenschild, 1980) and capillary-like structures may be formed without any heterologous cell interactior

While monolayer growth is a rather artificial situation for many cultured cells it is highly appropriate for endothelium. Given that the cells are supported on an appropriate extra-cellular matrix (which they appear to secrete themselves) (Gospodarowicz et al, 1980), with access to the nutrient medium at both surfaces, they may make a good model for characterising in vivo behaviour.

Isolation and Culture

In some of the first studies of well characterised endothelial cultures, cells were derived primarily by collagenase digestion of the inner surface of major blood vessels, such as bovine aorta or human umbilical cord (Table 1,Fig. 1). Collagenase digests the basal lamina but does not totally dissociate the endothelium and cultures arise from small islands of undis-aggregated endothelium. Isolation from other sites has also relied on the resistance of endothelial cells to dissociation to provide small sections of capillary blood vessels, from brain, adenal cortex and other sites (Table 1), which can be physically isolated and grown as explants or as a monolayer after further enzymic dispersal. DeBault et al, (1979) found brain dis-aggregated readily on a fine mesh sieve and the blood vessels were retained when the neural cells were washed through the sieve.

Endothelial cells may also survive tumour disaggregation in collagenase, and proliferation may be enhanced by the mitogenic action of factors released by the tumour cells.

Endothelial monolayers may be passaged by conventional treatment with trypsin and cell lines may be maintained for around 40 generations in media such as Dulbeccos DME, α-MEM or Ham's F10 (Table 2) supplemented with human or bovine serum. The use of FGF (Vlodavsky et al, 1979), endothelial growth factor(s) (Maciag et al, 1979; Folkman and Haudenschild, 1980) or tumour angio-genesis factor (TAF) (Fenselau and Mello, 1976; Folkman et al, 1979; Schor et al, 1980) was decisive in enabling serial propagation/

propagation to be possible for more than a few generations. Although treatment of the substrate with gelatin or collagen may be necessary for full phenotypic expression it does not seem to be necessary for serial propagation. This may be due to the ability of the cells to produce their own extracellular matrix, which has been shown to be capable of stimulating the growth of other cells as well (Gospodarowicz et al, 1980).

We have found that the proliferation of human umbilical vein endothelial cells (HUV) (Fig.2) is dependent on supplementation of the medium with endothelial growth supplement (Collaborative Research/Uniscience) without which cell proliferation ceases in about 7-10 days.

Characterisation

A number of substances involved with the formation and dissolution of blood clots are associated with vascular endothelium. Amongst these, Factor VIII, a coagulation factor, has been found to be a useful specific marker for endothelial cells in culture. (Booyse et al, 1975; Jaffe, 1977; Folkman et al 1979). It may be demonstrated in endothelial cells by immunofluorescence or immunoperoxidase staining of fixed preparations (Fig.3).

The activity of angiotensin - converting enzyme (EC3.4.15.1) also seems to be localised specifically to endothelium and its presence can be used to confirm endothelial cell identity in vitro (Caldwell et al, 1976; DelVecchio and Smith, 1981). Other enzymes associated with endothelium are alkaline phosphatase, butyryl/

butyryl cholinesterase and γ-glutamyl transpeptidase (Spatz et al, 1980). In our hands it has not been possible to demonstrate alkaline phosphatase in endothelial cells in vitro (Doyle, Vaughan, Morgan and Freshney - unpublished observations) although no attempt was made to induce the enzyme.

At the cytological level, transmission EM reveals characteristic structures known as Weibel-Palade Bodies (Weibel and Palade, 1964) as well as the presence of tight junctions. While the latter can be demonstrated in other cell types in vitro, the former are regarded as quite specific to endothelium.

Collagen (types III and IV) is produced by endothelial cultures during the monolayer growth phase. The characteristic structural modification which arises in post-confluent cultures (see below) is accompanied by an alteration to the production of type I collagen (Cotta-Pereira et al, 1980). Expression of the differentiated phenotype is also associated with a reduction in platelet binding capacity (Zetter et al, 1978) and is dependent on the presence of growth factor. FGF was used in these studies but the relationship of FGF to endothelial growth supplement is not clearly established.

Tumour Angiogenesis

Experiments in this laboratory have been aimed at using the induction of mitogenesis in endothelial cell monolayers as an assay of TAF production by tumours. We have used a cell line (HUV) derived from human umbilical vein by collagenase digestion as a target monolayer. About 10cms of cord (full term) was/

was rinsed and the umbilical vein flushed out with PBSA (PBS lacking Ca^{2+} and Mg^{2+}). Collagenase, 2000u/ml, CLS grade (Worthington), was introduced into the vein which had been clamped at one end. The other end was then clamped and the cord incubated at 37°C for 30 mins. The cord was then gently massaged and the collagenase suspension collected. The vein was then washed out with medium and a second isolate collected. Both isolates were centrifuged and the cells resuspended in fresh medium (Hams F10 + 10% Foetal bovine serum), and cultured separately.

The primary disaggregate consists of small groups of cells, not fully dissociated, which eventually attach and form colonies of endothelial cells (Fig. 4). In the presence of endothelial growth supplement, 0.1mg/ml (Collaborative Research/Uniscience) the colonies grow out to form a monolayer which can be sub-cultured conventionally with 0.25% crude trypsin in PBSA. When allowed to grow past confluence on plastic, a morphological alteration of the monolayer is observed ("sprouting") reminiscent of the morphogenetic transition observed by others (Cotta-Pereira et al, 1980; Folkman and Haudenschild, 1980) (Fig.5). Accompanying this is a substantial release of cells into the medium.

When cells are cultured on collagen they arrest at a lower cell density, the same morphological transition occurs but it takes longer and fewer cells are released into the medium. (Fig.6)

Examination of cells in monolayer shows that they stain positively/

positively for factor VIII antigen (Fig.7). Transmission EM (by courtesy of David I.Graham, and Shahida Abraham) of superconfluent cultures reveals the intracellular presence of Weibel Palade Bodies and tight junctions between cells (Fig.8). There is also evidence of extracellular matrix between the cells and the underlying substrate.

When crude extracts of Walker 256 carcinoma or human glioma are absorbed on filter paper and placed on the chorioallantoic membrane (CAM) of a, 10 day, embryonated hen's egg, neovascularisation can be observed 5 days later. Similar extracts added to cultures of HUV cells, which have been grown to confluence and have arrested in "plateau", stimulate further proliferation (Fig.9). This can only be demonstrated satisfactorily with monolayers grown on collagen due to the higher plateau level and excessive cell loss observed when cells are grown on plastic. Schor et al (1980) have observed that endothelial cell mitogenesis could not be induced in cells grown on plastic and that anchorage to native collagen gel was necessary.

Neovascularization on the chicken CAM implies that angiogenesis may be induced by extracts of cultures from human and animal tumours as has been previously reported (Klagsbrun et al, 1976). Mitogenesis in endothelial monolayers is a less specific event and we have yet to demonstrate that its induction is specific to extracts from tumour cells. Nevertheless, either alone or in conjunction with the CAM assay, this cell culture assay may yet provide the degree of quantitation which has so far been lacking in the assay of angiogenesis.

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TABLE 1 EXAMPLES OF ENDOTHELIAL CULTURE

Tissue	Isolation	Species	Reference
Aorta	Collagenase	Cow	Schwartz, 1978
Foetal Heart and Aorta	"	Cow	Fenselau & Mello, 1976
Umbilical Vein	"	Human	Jaffe et al, 1973 Gimbrone et al, 1974
Pulmonary Artery	"	Cow	Del Vecchio & Smith,19
Brain	Trypsin	Rat,Cow	Phillips et al, 1979
"	Homog., Seive	Mouse	DeBault et al, 1979
"	Homog.,Coll. Trypsin	Rat	Spatz et al, 1980
Adrenal Cortex, Foreskin, Spleen	Collagenase	Cow, Human	Folkman et al, 1979
Glioma	Collagenase	Human	Freshney, Morgan & Shaw
Ovarian Ca, Neuroblastoma, Rhabdomyoma	"	"	Folkman et al, 1979
Kuppfer Cells, Liver	Cloning	Hamster	Knook et al, 1977 Clark & Pateman,1978
Cornea	Scraping	Cow	Gospodarowicz et al, 1977 Gospodarowicz & Greenburg, 1979

TABLE 2 CULTURE CONDITIONS

Medium	Dulbecco's DME M199 Eagle's MEM -MEM Ham's F10
Serum	10% Calf. 10-20% Foetal Bovine 15% human
Growth Factors	FGF. (Vlodavsky et al, 1979) ECGF (Maciag et al., 1979) ECGS (Folkman & Haudenschild, 1980) TAF (Schor et al., 1980; Folkman et al., 1979 Fenselau & Mello, 1976)
Conditioning Medium Substrate	Bovine aortic cells (Folkman & Haudenschild, 1980) Bovine aortic cells (Gospodarowicz., 1980) Gelatin (Folkman et al., 1979) Collagen I (Schor et al., 1980)

TABLE 3 MARKERS

ENDOTHELIUM SPECIFIC

Factor VIII	Jaffe 1977 Booyse et al, 1975
Angiotensin Converting Enzyme	Del Vecchio & Smith, 1981 Caldwell et al., 1976
Collagen III & IV in mondayer, converting to I in "sprouting"	Cotta-Pereira et al., 1980
Weibel-Palade bodies	Weibel & Palade, 1964

ENDOTHELIUM ASSOCIATED

Tight junctions	
Alkaline Phosphatase	Spatz et al., 1980
Butyryl Cholinesterase	" "
γ-glutamyl transpeptidase	" "
L-DOPA uptake	" "
Platelet Binding	Zetter et al., 1978

Figure Legends

Fig.1 Variations in methodology for the disaggregation of endothelium for culture.

Fig.2 Human umbilical vein cells (HUV) were seeded at 10^4 cells/m in 24 well plates (Linbro) (1ml/well), grown in Ham's F10 medium supplemented as indicated in the right hand columns, and trypsinised and counted at the times shown. ECGS = endothelial growth supplement (Collaborative Research/Uniscience). WRCE = Walker rat carcinoma extract and was used at 1mg/ml. Points are means of four replicate wells.

Fig.3 Factor VIII antigen present in an endothelial cell line (GMS), at the 10th passage. This cell line was derived from a human glioma. Note characteristic granular staining. Immunoperoxidase. Scale bar 100μm.

Fig.4 (a) Colony of endothelial cells from human umbilical vein, derived as described in text. Scale bar 100μm. (b) Endothelial cells from trypsinised rat brain. (c) Putative smooth muscle cells from same preparation as (b). All on same scale as (a).

Fig.5 Morphological alteration with increasing cell density. (a) Subconfluent HUV cells (b) HUV multilayered cells two weeks after seeding at 5×10^4 cells/ml (~1.5×10^4 cells/cm^2) (c) Approximately 3 weeks after seeding. Loss of cells into medium, retraction of monolayer and formation of secondary structures ("sprouting"). Cells grown on plastic (Falcon, 75cm^2 flasks). Scale bar 100μm.

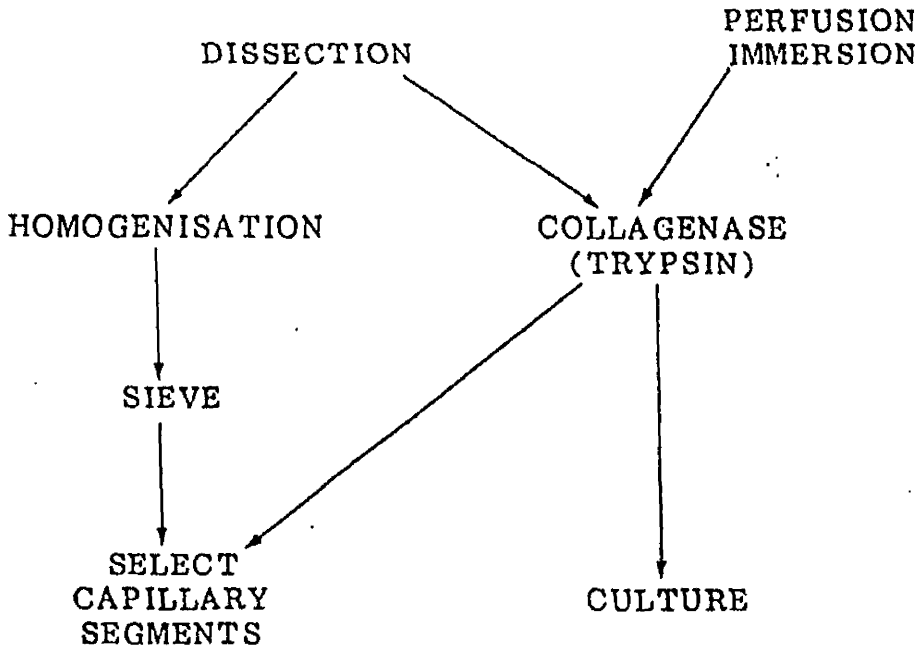
Fig.6 Growth of HUV cells to saturation density on plastic and collagen. HUV cells were trypsinised (0.25% crude trypsin in PBS without Ca^{2+} and Mg^{2+}), and seeded at 5×10^4 cells/ml on plastic/

plastic microtitration phase (Linbro) or on collagen plate coated with Vitrogen (Flow Labs) and allowed to dry. Cultures were fed every 2 days and cells counted by trypsinisation at the times indicated. Points and means and SD's of six replicate wells.

Fig.7 HUV cells stained for Factor VIII antigen by immuno-peroxidase method. Scale bar 100μm.

Fig.8 Transmission electronmicrographs of HUV cells prepared after spontaneous retraction of the multilayer and detachment from the substrate. (a) Weibel Palade bodies (b) Tight junction (c) Extracellular matrix (lower half of photograph). Scale bars: (a) 1μm, (b) 200nm, (c) 100nm.

Fig.9 Induction of mitogenesis in density limited cultures of HUV cells. Replicate samples from plateau phase on collagen (from Fig.6 above) were treated as indicated in the right hand columns. Extracts were prepared by freezing and thawing three times in PBS without Mg^{2+} and Ca^{2+}. The lysate was centrifuged at 4°C at 48,000 xg for 30 mins. and the supernatant was filtered through a Millex (Millipore) 0.2μm porosity filter. Protein estimations were made by absorption at OD_{280nm} and the extracts were used at the protein concentrations indicated. Points are means and standard deviation of six replicates.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(TRYPSIN)
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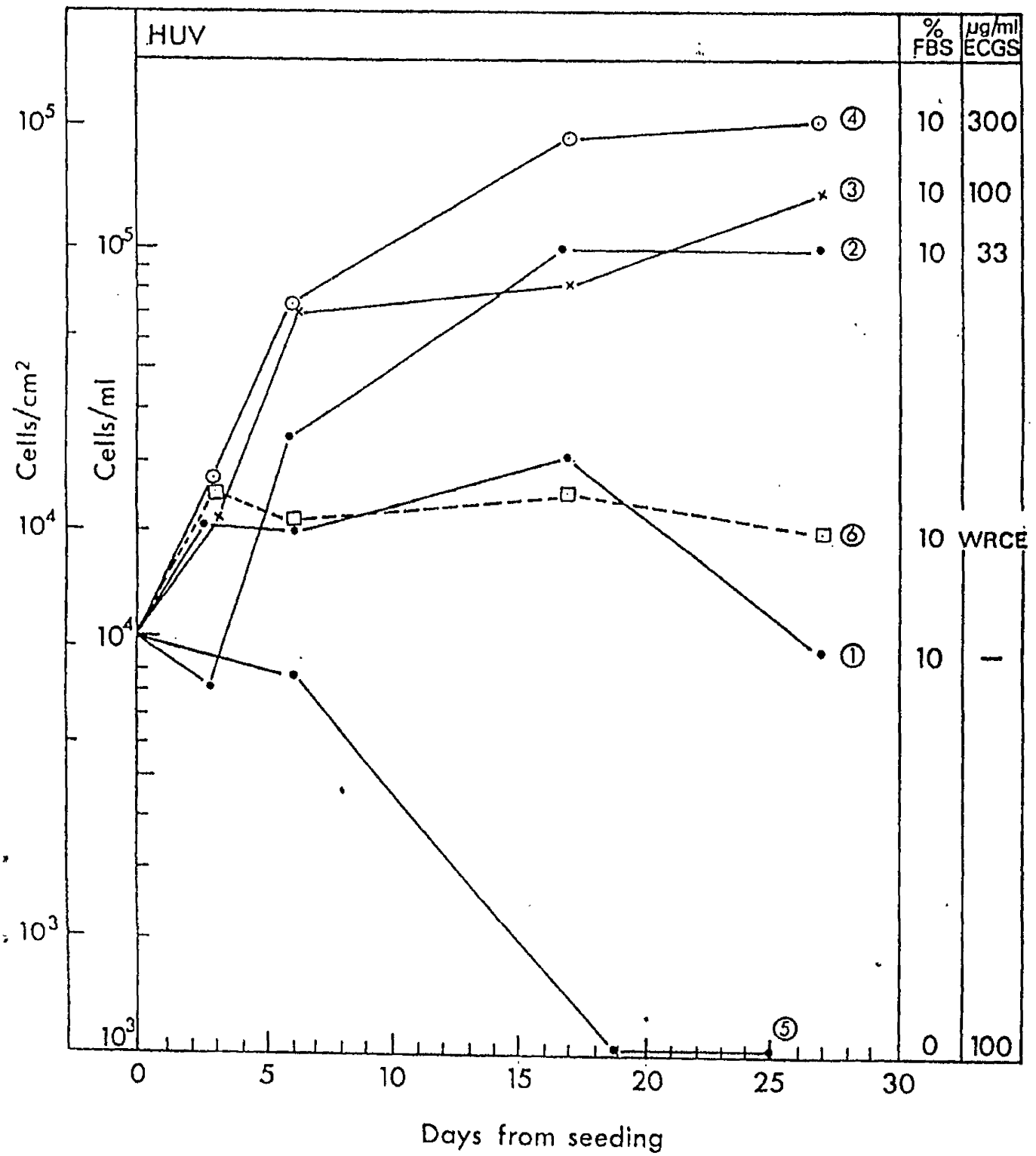
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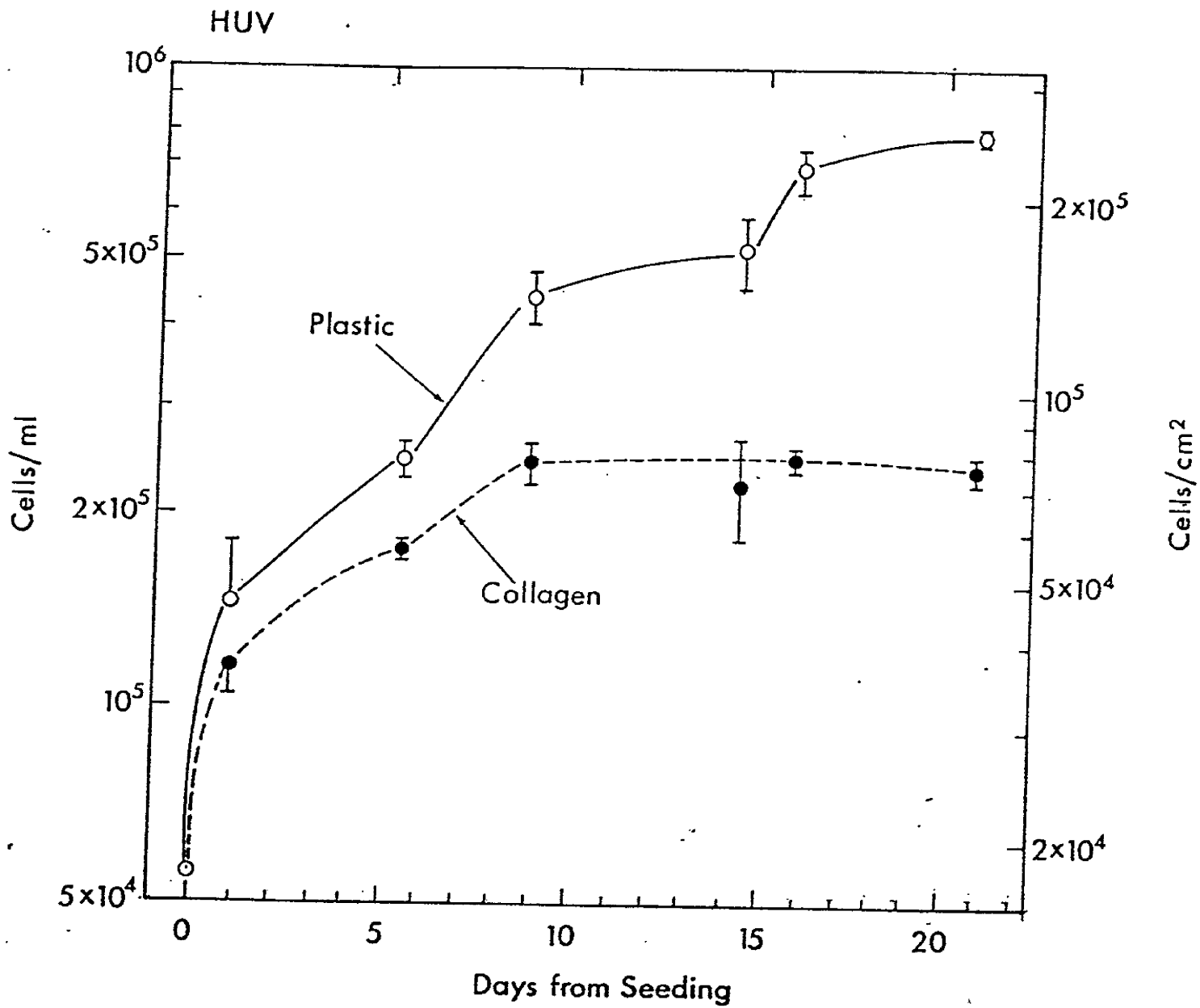
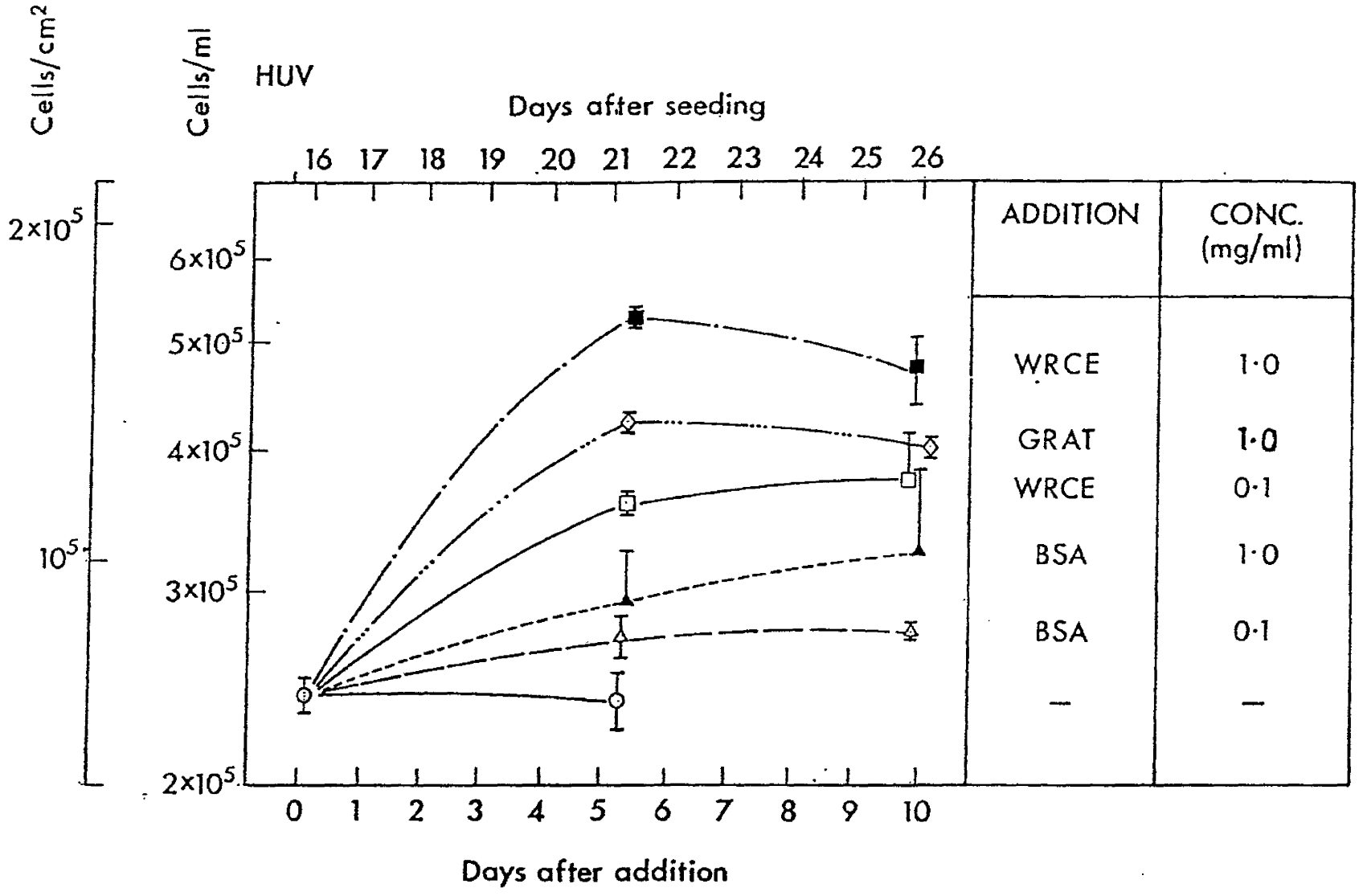
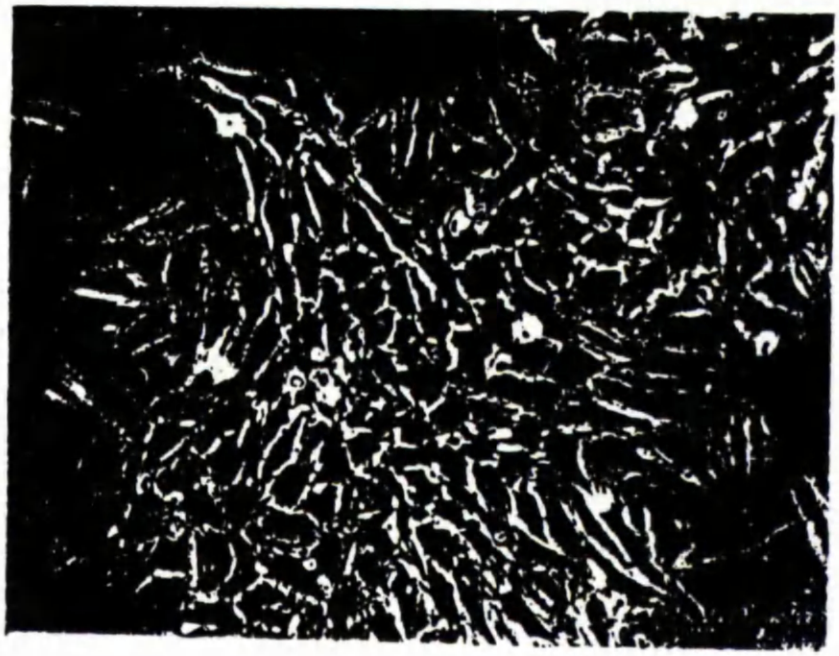
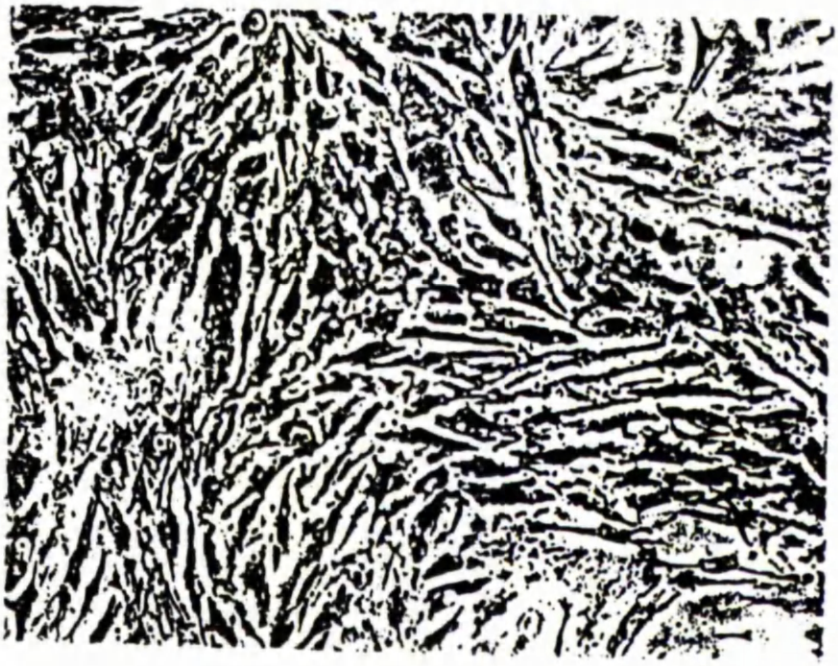
% FBS
µg/ml ECGS
Cells/cm²
Cells/ml
10⁵
10⁴
10³
④ 10 300
③ 10 100
② 10 33
⑥ 10 WRCE
① 10 —
⑤ 0 100
0
5
10
15
20
25
30
Days from seeding


HUV
10^6
5×10^5
2×10^5
10^5
5×10^4
Cells/ml
2×10^5
10^5
5×10^4
2×10^4
Cells/cm²
Plastic
Collagen
0
5
10
15
20
Days from Seeding








100 µm

(a) (b) (c)

(a) (b) (c)