A STUDY OF ASTROCYTOMAS

IN VM MICE

Thesis submitted for the degree of Master of Science

to the Faculty of Science at the University of Glasgow

by

SELINA J CRAIG

at the

University Department of Neurology
The Institute of Neurological Sciences
Southern General Hospital, Glasgow

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TABLE OF CONTENTS

LIST of TABLES and ILLUSTRATIONS

ACKNOWLEDGEMENTS

It is my pleasure to record my thanks to Dr. P.O. Behan for his help and guidance throughout this work. I wish also to thank Dr. D.I. Graham for his invaluable work on the histology; and Miss Diana Morgan for her help with tissue culture. In addition I would like to thank Dr. H. Fraser for helpful discussions, Professor Adam Curtis for helpful guidance in the earlier part of this work, Dr. R. Lyle Craig for constructive criticism of the written work and Mrs. Cathy Menzies for friendly assistance. Thanks are also due to the technicians of the animal house at the Southern General Hospital, Glasgow.

SUMMARY

This work consists of a study of the naturally occurring brain tumour which arises in the VM strain of mouse. This astrocytoma has clinical and pathological similarities to the human glioma. It is not known whether the appearance of such gliomas is due to pure genetic factors, the interaction of genetic and environmental influences, or the environment alone.

Clinical signs consist of ruffling of the fur, development of a hunched posture, loss of hind leg spreading reflexes, ataxia and weight loss. With progression of the disease there is exaggeration of these features, together with immobility and loss of righting reflexes. Pathologically the brain shows swelling and diffuse infiltration of anaplastic astrocytoma.

The VM astrocytoma can be maintained by passive transfer with intracerebral (i.c.) injections of fresh or frozen tumour homogenates into the VM and other mouse strains. Mice injected i.c. with tumour show clinical signs of severe disease about 21 days later. Passage of the astrocytoma is related to the cell density of the inoculum.

Attempts at transmission with killed cells occasionally proved successful, but the cell free filtrates were ineffective.

It was found that the tumour could be passaged in mice across the histocompatibility barriers into the SJL/J, C57BL 10/ScSn and BSC strains. The tumour was also successfully transplanted into neonatal hamsters and gerbils.

The VM astrocytoma was transmissible after being maintained for variable periods in tissue culture. The incubation period before clinical signs appeared was found to be dependent upon the time the tumour was maintained in culture and the cell concentration. Primary tumour cultures were effective in transferring tumours, whereas subcultures were found to be ineffective.

The effects of various drugs on the VM tumour were studied *in vivo* and *in vitro*. An antiviral agent, Ribavirin (1-β-D, Ribofuranosyl - 1,2-4-triazole - 3 carboxamide and testosterone had no effects on the tumour when administered *in vivo*. Prolonged treatment of the host with Levamisole, an immunostimulant drug, prior to

and following i.c. injection did not influence tumour growth. Treatment with the steroid drug Dexamethasone did reduce clinical signs and extend the survival time.

Ribavirin and P113 (angiotensin II antagonist - Saralasin acetate) were examined *in vitro* for any inhibitory effects on tumour transmission of the cultured VM astrocytoma. In this preliminary experiment, Ribavirin appeared to be without effect, whereas the angiotensin II antagonist, P113 showed a capacity to delay outset of clinical signs.

This study extends clinical and pathological data on this unique murine brain tumour.

ABBREVIATIONS

i.c.	Intracerebral
CNS	Central Nervous System
DMBA	Dimethylbenzanthrene
ENU	Ethylnitrosourea
MNU	Methylnitrosourea
RSV	Rous sarcoma virus
ASV	Avian sarcoma virus
ABRO	Animal Breeding Research Organisation (Edinburgh)
DMSO	Dimethylsulphoxide
i.p.	Intraperitoneal
i.m.	Intramuscular
i.v.	Intravenous

INTRODUCTION

Gliomas account for 45% of all primary intracranial tumours of man, and astrocytomas constitute 25-30% of all cerebral gliomas (Russel & Rubenstein, 1977). There is no treatment other than temporary alleviation of symptoms by drugs or major surgery and the prognosis is poor, with nearly all cases ending in death. Very little is known about the aetiology of astrocytomas and the study of these tumours is limited by the lack of a good animal model.

Tumours may arise from factors operative in embryonic life (Russel & Rubenstein). Genetic factors certainly account for some tumours, for example, in five out of seven families in a study there was a history of astrocytomas (Kjellin et al, 1960). Carcinogenic chemicals are responsible for some human neoplasms and have been shown to be capable of inducing experimental brain tumours in animals, but they have yet to be cited as specific causative agents of primary intracranial tumours in man. There is increasing interest in the modifications of the cell genome by oncogenic viruses but there is no definite evidence for supposing that bacterial or viral infections cause gliomas in man. Such viruses have however produced

experimental brain tumours in many different animals (Bigner & Swenberg, 1975), and have therefore provided useful tools for research.

Experimental Brain Tumours Induced by Chemical Carcinogens

Gliomas have been successfully induced by chemical carcinogens in mice and other animals using a variety of routes, i.e. direct intracerebral implantation (Zimmerman & Arnold, 1941), systemic injection (Schmidek et al 1971), oral administration (Strooband & Brucher, 1968), and transplacental tumour induction (Joshi et al, 1974).

a) Polycyclic Hydrocarbons (P C H)

The susceptibility of neuroectodermal tissues to chemical carcinogens was first demonstrated in 1939 (Seligman & Shear) by direct implantation of pellets of a polycyclic hydrocarbon, e.g. methylcholanthrene, into the brains of mice. This was confirmed by Zimmerman (1941 & 1955) who showed that the cytological identity of such tumours depended greatly on the actual site of implantation. For instance, an ependymoma results when the carcinogen impinges on the ventricular walls, or an astrocytoma when the agent is placed in the subcortical regions of the parietal white

matter. Another very important finding made by Zimmerman in this work was that the chemically induced ependymoma was easily transplantable both subcutaneously and intracerebrally into other mice, thus providing, for the first time, a reproducible brain tumour model.

Tumours of the central nervous system (C N S) have also been produced transplacentally by oral administration of dimethylbenzanthrene (DMBA) to pregnant female rats (Joshi et al, 1974). This resulted in various intracranial tumours arising in the offspring of female rats treated in this way.

b) Nitrosamines

The nitrosamines were first introduced as a group of effective carcinogens in 1964 (Druckey et al). Various malignant tumours of the CNS were produced transplacentally in the offspring when gravid rats were given a single dose of ethylnitrosourea (ENU) (Ivanovick & Druckey, 1968). A related chemical, methylnitrosourea (MNU) when injected systemically into adult or young rats was relatively successful in producing intracerebral tumours (Swenberg et al, 1972a). MNU has

also been found to induce brain tumours when given orally to rats (Strooband & Brudrer, 1968), and the resultant tumours were largely oligodendrogliomas.

These models have many inherent disadvantages, such as the long series of i.v. injections necessary when using MNU, and the trauma caused by intracerebral implantation of pellets in the case of polycyclic hydrocarbons. The tumour models employing systemic injections of nitrosamines have the advantage over PCH, in that the brain is not traumatised by rupture of the blood brain barrier or by tissue damage at the outset of the experiment. The major drawback however, to promoting any of these systems as experimental models, is the enormous variability in both the histological types of tumour produced and the long latency periods which can range from 68-750 days. Reproducibility is also poor, especially as the average success rate varies from 10% - 60% (Swenberg, 1976).

Experimental Brain Tumours induced by Oncogenic Viruses

Cerebral tumours have been induced in rodents and dogs by both human and animal viruses. Not all tumours

induced by viruses, however, are authentic neuroglial tumours and some of the previously reported ependymomas produced in rodents by i.v. injection of virus have since been identified as meningiomas or tumours of the choroid plexus (Russel & Rubenstein, 1977). Many of the experimental tumours that have been genuine ependymomas, have been produced in hamsters by various viruses, for example the simian virus SV40 (Gerber & Kirchenstein, 1962; Duffel & Hinz 1964), the avian adenovirus (Mancini et al 1969) and human J.C. papovirus (Walker et al, 1973). The value of such tumour models is limited as ependymomas are relatively rare in humans, accounting for less than 1% of all primary brain tumours.

The avian Rous Sarcoma Virus (RSV) has been reported to induce 'typical gliomas' (Ikuta & Kumanishi, 1971) but these represented only 50% of the tumours that occurred after 90 days incubation, and those tumours which developed earlier were of a wide variety of different tumour types. Puppies were injected with the Schmidt-Ruppin strain of RSV and reproducible brain tumours were successfully induced (Bigner et al, 1969); most of which were astrocytomas or the juvenile form - spongioblastomas. The most successful model for a

reproducible virus induced primary astrocytoma so far, has been presented (Bigner et al, 1973 and 1975/6) in which avian sarcoma visus (ASV) injected into rats of the Fischer 344 strain, resulted in 100% incidence of anaplastic astrocytomas about 110 days later.

Astrocytomas have also been induced relatively quickly and reproducibly by Shein (1968 & 1970) who used an ingenious technique to produce the tumours. He found that transformation in vitro of cultured foetal or neonatal hamster astrocytes by polyoma or SV40 viruses, and subsequent subcutaneous or intracerebral inoculation of such astrocytes into newborn or adult hamsters resulted in a very high proportion of astrocytomas.

The virus induced experimental brain tumours have two advantages over chemical tumour induction, i.e. the number of positive yields is considerably higher, usually being in the region of 75% of all animals injected, and in some models the incubation period is shorter, being as little as 30 days with polyoma viruses and even 15 days with Rous sarcoma virus (Russel & Rubenstein, 1977).

The above systems, however, produce tumours that are

often multifocal in origin and present a mixture of tumour types, for few are as consistent as the Fischer rat - ASV model. Studies on virus induced brain tumours in hamsters, rats and other animals demonstrate the ability of certain oncogenic viruses to cross species barriers, but they shed little light on the role of indigenous viruses causing spontaneous tumours.

A Spontaneously Arising Murine Astrocytoma

A spontaneous mouse brain tumour, first described by Fraser (1971) is a true astocytoma, arising in 1.6% of inbred VM mice by 500 days of age, being more frequent in males than in females (1.5 : 1). The astrocytoma was first noticed in the 6th generation of inbreeding of the VM strain, as an incidental discovery during a study on experimental scrapie, for which this strain is genetically suitable (Fraser & Dickinson, 1968). It was shown that this tumour could be reproduced by intracerebral passive transfer of viable tumour cells in the inbred host strain with 100% success (Fraser, 1974). This natural tumour provides an absolutely unique experimental model, with all the advantages that are lacking in artificially induced tumours. Here is an animal model where the tumours are consistently

of one type, that of an anaplastic astrocytoma, and in which the clinical and pathological features correlate very well with the human disease. It is an autochthonous tumour depending totally on neural tissue for successful proliferation. Several attempts to transfer this astrocytoma to extraneural sites have been unsuccessful (Fraser, 1975), and in this it resembles human gliomas which do not metastasise outside the CNS. Another major advantage of this model is the very short latency period, which from having been 60-90 days in the early passages, is now reduced to about 21 days following i.c. injection of maximum doses of tumour.

This tumour at first appeared to keep a close relationship with the genotype of origin, however, it was successfully passaged into two other strains, although with a very low incidence, eg, $^{1}/_{27}$ C57BL mice, and $^{1}/_{28}$ A_2G mice. The number of successful transplants increased in the F_1 hybrids eg, VM x C57BL, in which $^{10}/_{22}$ mice developed the tumour (Fraser, 1974). Fraser also found the VM tumour did not grow in the BRVR strain, which has an unrelated H-2 type, but which also develops a spontaneous astrocytoma with approximately the same frequency. It has since been found that it is possible to transfer the tumour by i.c. injection

into several other mouse strains, including BRVR (Fraser, 1980) with increased incidence of tumour in the F_1 hybrids. This work showed there was no relationship between H-2 type and susceptibility to the tumour.

Of the occasional VM survivors of the i.c. injection of tumour, not one out of nine developed tumour when challenged with a second i.c. injection (Fraser 1975), although peripheral injections provided no protection to reinoculation. This is of some immunological significance and gives yet another facet of tumour biology to which this versatile model could be applied.

In this study I have set out to establish the biological characteristics of the passaged VM astrocytoma, including the effects, in vivo and in vitro, of some of the drugs used in the treatment of human tumours. I have also attempted to show whether or not this tumour can be transferred to any other mouse strains or into other species.

MATERIALS AND METHODS

ANIMALS

A variety of mouse strains and other animal species were used in this study, see Table 1. With the exception of hamsters, all breeding animals were maintained in monogamous pairs and their offspring weaned at four weeks. All animals were housed in polythene cages, the mice in shoebox type cages and the gerbils, rats and hamsters in larger rodent cages. Sawdust and tissue paper were supplied as litter and bedding and all animals were fed ad libitum on rodent complete diet pellets (Diet 41B, Angus Milling Co., Perth) and provided with a constant supply of water. Hamsters and gerbils were given diet supplements of hay and also sunflower seeds twice weekly.

Table 1

Strain	H-2 type	Source
Inbred Mouse Strains		
VM	b	Animal Breeding Research Organisation Edinburgh. Courtesy of Drs. Fraser and Dickinson.
MM	a	
BSC	?	
C57BL/10ScSn	b	MRC Laboratory Animals Centre (LAC) Carshalton, Surrey.
B10Lp - a	b	
B10 D_2	d	
B10A	a	
AKR/J	k	
SJL	s	
C58/J	k	Imported from Jackson Labs Bar Harbour, Maine, U.S.A.
Inbred Rat Strains		
Osborne - Mendel		University Department of Neurosurgery at I.N.S., Glasgow. Courtesy of Dr. D.I. Graham
Sprague - Dawley		
AS		
Brown - Norway		
Hamsters		
Syrian - random bred		LAC Carshalton and Roundhill Rabbit Farm, Sheffield.
Gerbils		
Mongolian Random Bred		University Department of Dermatology Anderson College, Glasgow. Courtesy of Professor Milne.

The inbred VM mouse strain originated from sib matings of the random bred Moredun Institute stock mice obtained by Drs A.G. Dickinson and V.M.H. Miekle. The breeding nucleus for this study was obtained at the 41st generation of inbreeding and has progressed to the 45th generation. Inbreeding was continued by successive matings of siblings at weaning age, which were maintained together throughout breeding life, arbitarily to the seventh litter for prolific pairs.

PASSAGE OF VM ASTROCYTOMA

1. Source of Astrocytoma:

Six female VM weanlings were kindly injected by Dr. H. Fraser (at ABRO Edinburgh), with the VM astrocytoma, at the 54th passage. These mice served as the first source of tumour for this study. Since then serial passage has been maintained up until the 67th passage and from frozen stocks of tumour prepared from the 64th, 65th and 66th passages.

2. Preparation of Tumour Inoculum:

A. Fresh Tumour: Mouse donors exhibiting severe clinical symptoms were killed. After removing ears and fur over the head, the brain was excised (as aseptically as possible), by carefully cutting around each side of the cranium, starting with the point of fine scissors through the foramen magnum. After removing the cranium, the whole brain was lifted out into a sterile petri dish. A small piece,

usually the frontal lobes or cerebellum, was cut off and fixed in 10% formalin for histological examination. The remainder of the brain was homogenised, using a manual glass homogeniser, in 5ml sterile tissue culture fluid (RPMI - 1640 + L-glutamine, Gibco Biocult Ltd., Abbotsinch, Paisley.), with added penicillin and streptomycin, but without serum. Gross particles were allowed to settle out, and the cell suspension was then decanted into a sterile universal container. The cell density was adjusted to approximately 10^7 cells/ml diluting, if necessary, with tissue culture fluid, and this suspension was used as the standard tumour inoculum.

B. Standard Frozen Tumour Suspension: Several brains from mice showing severe clinical signs of tumour were excised as previously described, and homogenised in 10ml tissue culture fluid containing 10% dimethylsulphoxide (DMSO), which helps maintain the integrity of the cells during freezing. The cell concentration was adjusted to 2.10^7 cells/ml and the suspension aliquoted into plastic screw-top ampoules containing 0.5ml each. These ampoules were frozen to $-70^{\circ}C$, inside a polystyrene container which allowed the cell suspension to freeze at a rate of $1^{\circ}C$ per minute. The ampoules could then be thawed as

required at 37°C, and diluted to the required concentration. Stocks were tested before and after freezing by injecting the maximum dose (2.10^5 cells) into weanling mice. The freezer life of these stocks was about 6 months at -70°C, after which time the latency period before signs of tumour were evident, became unreliable.

3. Method of Intracerebral Inoculation

Intracerebral (i.c.) injections of exactly 20µl standard tumour inoculum containing 2.10^5 cells were made using a 1ml luer tip glass syringe with a repeating dispenser (both from the Hamilton Syringe Company.) Disposable hypodermic needles size 26G, $\frac{3}{8}$" long (Yale Microlance, Becton Dickinson, UK Ltd., Wembley, Middx.) were used for all i.c. injections. A needle guard was devised by cutting off the open end of the plastic needle container and retaining it on the hub of the needle, such that only half the needle was exposed, i.e. 4mm. This permitted more rapid and reproducible

injections, without risk of mortality due to very deep penetration of the needle into the brain.

Injections were always made into the right hemisphere, midway between the eye and the right ear; whilst holding the mouse firmly by the scruff, with one finger along the left side of the head to prevent it moving. Care was taken not to inject near the midline of the head in order to avoid the sagittal vein. Using a 26G needle, injections could be effected easily by direct penetration through the relatively soft cranium of weanling mice without 'drilling', as had been necessary when using a 25G needle for earlier passages and in adult mice, rats and gerbils.

CHARACTERISATION OF PASSAGED VM ASTROCYTOMA

1. Method of Clinical Grading

Clinical features, according to severity, were arbitarily graded A,B,C,D or E as follows:

A - mild disease - Seizures were observed with slight ruffling of the fur over the head and neck. Hind leg spreading and grip reflexes were weak.

B - moderate disease - Ruffling of the fur was more pronounced and mice started to take on a 'pop-eyed' appearance. Other trophic changes occurred, including slightly hunched posture when resting, hind leg reflexes were poor and mice had seizures, were jumpy and tended to hold the tail erect when startled, but they still had normal mobility.

C - severe disease - Trophic changes were very marked and the mice were now permanently hunched, often with the nose touching the ground.

Weight loss was noticeable although mice fed normally if offered food on the floor. Hind leg reflexes were absent. The mice were conspicuously jumpy and seizures were common, especially when disturbed. The mice at this stage were mainly stationary, but had periods of activity. Mice in most experiments were sacrificed at this stage.

D - very severe disease - All symptoms were exhibited to a greater degree. The mice were immobile and seizures could be provoked by disturbance. They did not eat, the eyes were usually closed and mice often felt cold to the touch. Weight loss was very marked at this stage and all mice were emaciated.

E - death.

Weight loss: Normal weight gain in weanling mice was followed up to the age of 12 weeks by weighing control mice. Weight loss was monitored for passage mice and for those experimental mice receiving drug treatments.

2. Preparation of brain tissue for histological examination

Brains for histological examination, from both control and experimental mice were placed in 10% formalin. Routine sections were stained with haemalum and eosin and examined for evidence of tumour.

3. Electron Microscopy

Mice, anaesthetised with ether, were perfused with a fresh solution of 2% glutaraldehyde by first exposing the heart and then inserting a blunted 19G needle through a cut at the base of the left ventricle. The needle was carefully eased into the aorta and clamped in position. The glutaraldehyde was expelled at an even pressure from a 20ml syringe into the aorta. The right atrium was cut to allow drainage. Perfusion was observed to be complete when the skin of the mouse had become yellowish and the animal was completely turgid, usually after injection of about 5 - 10 ml glutaraldehyde. The perfused brain was

removed as previously described and further fixed in 2% glutaraldehyde, then dehydrated in alcohol and blocked in epoxy resin and cured. Grids were then cut and stained with uranyl acetate and lead citrate and examined on a Philips 201 electron microscope.

PASSIVE TRANSFER OF VM TUMOUR

1. Passage of tumour by live cell suspensions

A survival curve was constructed for 92 mice injected ic with fresh standard tumour inoculum. Using standard frozen tumour suspension, dose response curves for both tumour incidence and period of latency were established.

2. Passage of tumour by killed cells

Various methods of killing cells were employed and the resultant dead cells injected intracerebrally into weanling mice. Fresh standard tumour suspension was prepared in each case; half was treated to kill the cells, and the remaining live tumour suspension was used as a control.

a. A series of killed cell preparations were made by freezing and thawing (from $-70^{o}C$ to $37^{o}C$) from one to four times.

b. Ultrasonic rupture of cells was effected using a sterilised untrasonic probe. This was immersed in the tumour suspension in a glass beaker resting on ice. The cells were exposed to 3.5 amperes for a total of 3 minutes in 30 second bursts.

c. Tumour cells were disrupted using sterile distilled water. The cell fragments were then concentrated by centrifugation at 1500 rpm and used for i.c. injections.

d. Tumour cells were incubated at 37°C in a 1% solution of tannic acid in tissue culture fluid for periods of 10, . and 30 minutes; the cells were then centrifuged and washed three times prior to i.c. injection.

Mice which were apparently unaffected by tumour were left for 6 months before sacrifice.

3. Passage of tumour by cell free extracts

Cell free extracts of disrupted tumour cells were prepared using a tissue homogeniser, live tumour cells,

and dead tumour cells as per a,b, and c above. The cells were contrifuged at 1500 rpm for 5 minutes and the supernatant filtered through a washed 0.45μm or 0.22μm sterile disposable Millex filter (Millipore UK Ltd., Abbey Road, London.). This filtrate was injected i.c. into weanling mice, and control mice received live standard tumour inoculum. These mice were left for 6 months before sacrifice.

4. Attempts to induce tumour with normal brain suspension

Tumour free brain suspensions were prepared from a) old VM mice (> 16 months old) and b) weanling VM mice. These cells were injected i.c. into 1) adult VM mice (> 16 months old); 2) weanling VM mice; 3) weanling MM mice; and 4) weanling BSC mice. These mice were left for 6 months before sacrifice.

PASSIVE TRANSFER OF VM ASTROCYTOMA INTO OTHER MOUSE STRAINS and OTHER SPECIES

1. Transmission into other inbred mouse strains:

Mice of all the strains and histocompatibility types previously listed in Table 1, were injected i.c. with 2.10^5 astrocytoma cells and were all left for 6 months unless any clinical symptoms of tumour were observed.

2. Tranmission into gerbils:

Four weanling gerbils, seven ten-day old gerbils and 30 neonatal gerbils were injected i.c. with standard tumour suspension. The weanling and 10 day old gerbils were kept one year before sacrifice.

3. Tranmission into hamsters:

Hamsters were injected i.c. with standard tumour

inoculum as neonates, generally one day after birth. These animals were left for 3 to 6 months before sacrifice.

4. Tranmission into inbred rat strains:

Standard tumour suspension was injected into 12 Sprague Dawley and 12 Osborne Mendel suckling rats 10 days old. These rats were kept 6 months before sacrifice.

5. Tranmission into immunosuppressed rats and gerbils:

The VM astrocytoma was injected i.c. under anaesthetic into ten adult AS rats and ten adult BN rats and fifteen gerbils aged six months. Half of the rats in each group and eight of the gerbils were immunosuppressed three days previously with 300mg/kg cyclophosphamide (Endoxana®, W.B. Pharmaceuticals, London) by intraperitoneal injection. The animals

that were not immunosuppressed stood as controls for this experiment. These rats were left for 6 months before sacrifice.

TISSUE CULTURE OF VM ASTROCYTOMA

1. Method of tissue culture:

Tumours bearing brains from severely affected mice were collected as aseptically as possible, and placed in tissue culture fluid + 1% penicillin and streptomycin (Gibco Biocult Ltd) in a sterile universal container. The brains were transferred to a sterile petri dish and washed in 25ml dissecting fluid (see Appendix 1) and then transferred to another dish containing 20ml fluid and chopped into $2mm^2$ pieces using fine scalpels. The brain tissue pieces were then pipetted into a universal container and the gross particles allowed to settle out, and the supernatant, containing mainly necrotic tissue, was discarded. The pieces were resuspended in 15ml dissecting fluid and again allowed to settle. This washing procedure was twice repeated and the particles finally resuspended in culture medium and 1ml injected into each 5ml plastic culture flask. The volume in each flask was made up to 4.5ml with culture medium (see Appendix 1) and 0.5ml collagenase added to break up the tissue. The cultures were then

pipetted up and down to break up any remaining lumps and the collagenase washed off by centrifugation at 200g for five minutes. The cell pellet was resuspended in culture medium and reinoculated into 5ml flasks and plated at 37°C. At this stage the brain cells were found to adhere to the base of the flask, and to grow as a monolayer. The cells were then cultured for the required period of time or until the cells became confluent, the culture medium was renewed every four days.

The cells were recovered from culture by trypsinisation as follows: after discarding the culture medium, enough PBS/1mM EDTA (see Appendix 1) was added to just cover the monolayer, in order to wash off the serum which contains antitrypsin. Enough 0.25% Trypsin (Gibco Biocult) was added to cover the monolayer and then poured off after 60 seconds and the flask incubated at 37°C for 15-30 minutes, until the cells were loosened. The cells were then resuspended in culture medium for experimental use, or diluted and subcultured at a concentration of 10^4 cells/ml.

2. Transmission of tumour from tissue culture into mice.

The cultured tumour cells were tested for oncogenicity after varying periods of tissue culture (1-5 five days and 3 weeks) by reinjection into VM weanlings. The supernatants from cultures were centrifuged and filtered and also injected into mice as cell free extracts of cultured tumour.

EFFECT OF DRUGS ON TUMOUR GROWTH IN VITRO

1. Ribavirin: This was added at a concentration of 100µg/ml to 48 hour VM astrocytoma cultures. The cultures were started for 48 hours without the drug to allow the tumour cells to commence normal culture metabolism. After two days culture with the drug, the astrocytoma cells were harvested and 2.10^7 cells injected ic. into each mouse. Control mice were injected with tumour cells treated in the same manner, but without drugs. Cell viability was tested by subculture.

2. P 113 (Angiotensin II antagonist - Saralasin Acetate) (Eaton Laboratories, Morton Norwich Products Inc., Norwich, New York) was added to 48 hour old cultures as for Ribavirin. The drug was added at three concentrations : 0.7µg/ml, 0.07µg/ml and 0.007µg/ml. After 48 hours incubation with the drug, the astrocytoma cells were harvested and 2.10^7 cells injected ic. into each of six mice for each dose. Control mice received tumour cells which had been cultured without the drug. Cell viability was tested by subculture.

EFFECTS OF CHEMOTHERAPY

A variety of drugs were administered orally, intraperitoneally (i.p.) or intramuscularly (i.m.) to VM mice to determine whether or not they affected the tumour. All mice were regularly weighed and assessed for clinical signs and compared to controls.

1. Hormone treatment: Mice of both sexes were given oestrogen or testosterone.
a) Ethinyloestradiol (lynoral ®) was given by suspending ground 1mg tablets in the drinking water to give a daily dose of 9µg per mouse, assuming a 12g mouse drinks 3ml water/day.
b) Testosterone was given by intramuscular injection of 2µl Primoteston Depot (Shering A.G., Berlin/Berghamer) giving a dose of 20mg/12g mouse/week. Both drugs were administered from the day of tumour injection until sacrifice.

Two other experiments were performed in which ethinyloestradiol was given for 2 weeks before and after tumour inoculation.

2. Treatment with Levamisole: The effect of Levamisole (ICI Pharmaceuticals Division, Macclesfield, Cheshire) was assessed. The drug was administered by allowing free access to a stock solution containing 125mg per litre of drinking water (dose ≡ 30mg/kg). The drug was given in some experiments from the day of tumour injection and in others as pretreatment for 2 weeks prior to i.c. injection.

3. Treatment with Dexamethasone: This drug was administered either on the day of tumour inoculation or 12 days afterwards. Dexamethasone phosphate (Decadron ®, Merck, Sharp and Dohme, Hoddesdon, Herts.) 4mg/ml was diluted and given in a dose of 0.14mg/kg/day (equivalent to 10mg/72kg man) in the drinking water.

4. Treatment with Ribavirin: Ribavirin (1-B-D Ribofuranosyl 1,2,4 - Triazole -3- carboxamide, ICN Pharmaceuticals, Nucleic Acid Reasearch Institute, Irvine, California) was given by i.p. injection. The dose regime was 75mg/kg/day for 14 days following tumour inoculation and was given dissolved in 0.2ml saline.

Controls: Control mice for these experiments were injected i.c. with tumour on the same day and were given no treatment, except for those experiments involving injections, where the same volume of saline was injected at the appropriate site.

The results were treated throughout to a Student's t test to determine the mean latency periods, standard deviations and probability (P) values.

RESULTS

CHARACTERISATION OF PASSAGED VM ASTROCYTOMA

1. Clinical characteristics

Clinical signs of tumour were evident 15 days after intracerebral inoculation. These consisted of mild subtle signs which gradually intensified until the animals death about 12 days later. Mice were usually sacrificed at 'stage C' of the disease, about 21 days following ic injection. See Plate 1.

Some mice with the tumour showed additional clinical features to those previously listed. These included ataxia, in which the mouse, when held by the tail, revolves very fast; and walks in circles, holding its head to one side. Tumour bearing mice that were rotated two or three times by the tail showed severe exaggeration of their ataxia and often developed seizures.

The weight loss found in tumour bearing mice injected i.c. at 21 days and 32 days of age can be seen in Fig.1, and the weight gain in normal weanlings is shown in Fig.2.

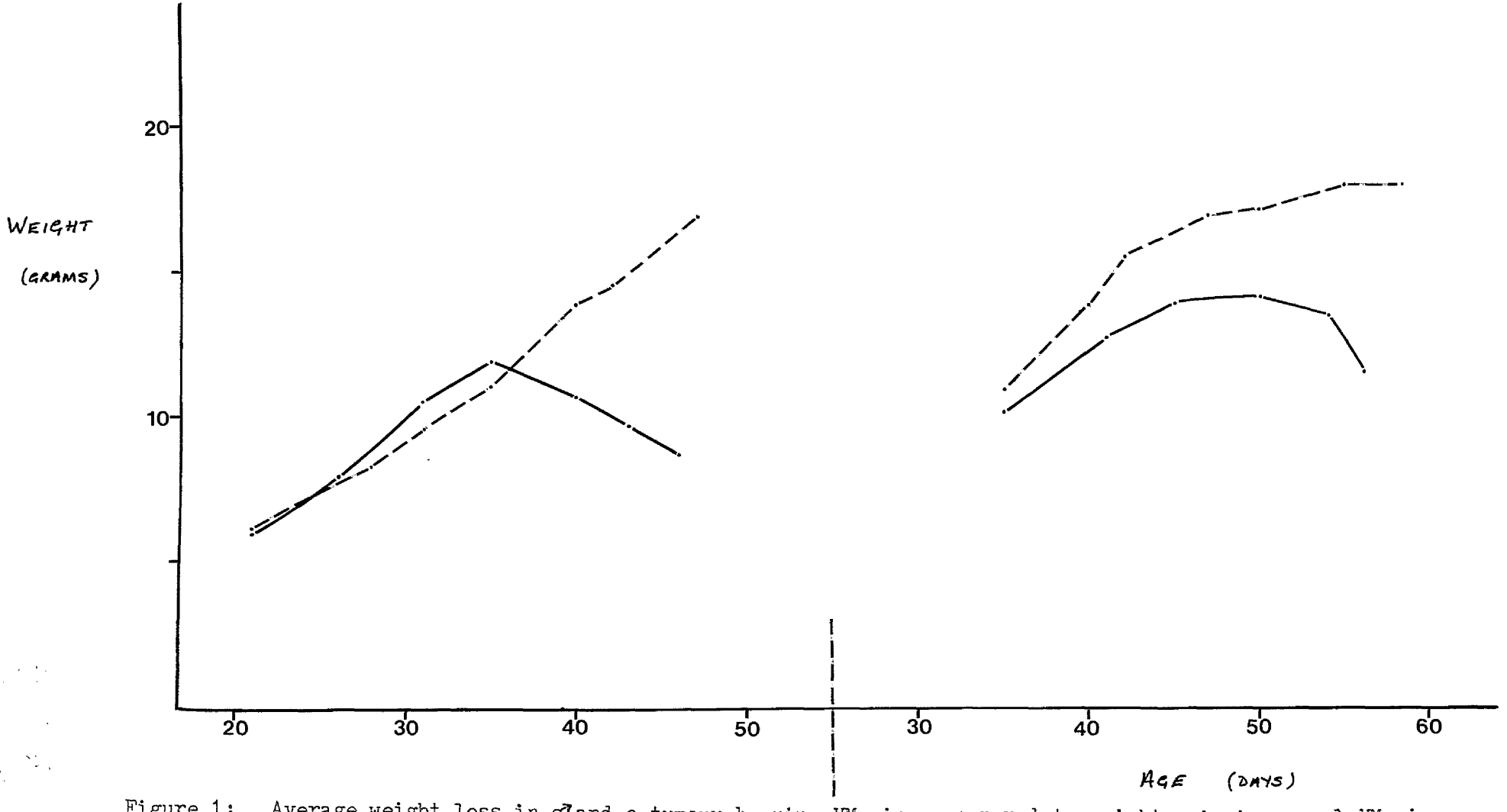


Figure 1: Average weight loss in ♂ and ♀ tumour-bearing VM mice, compared to weight gain in normal VM mice.

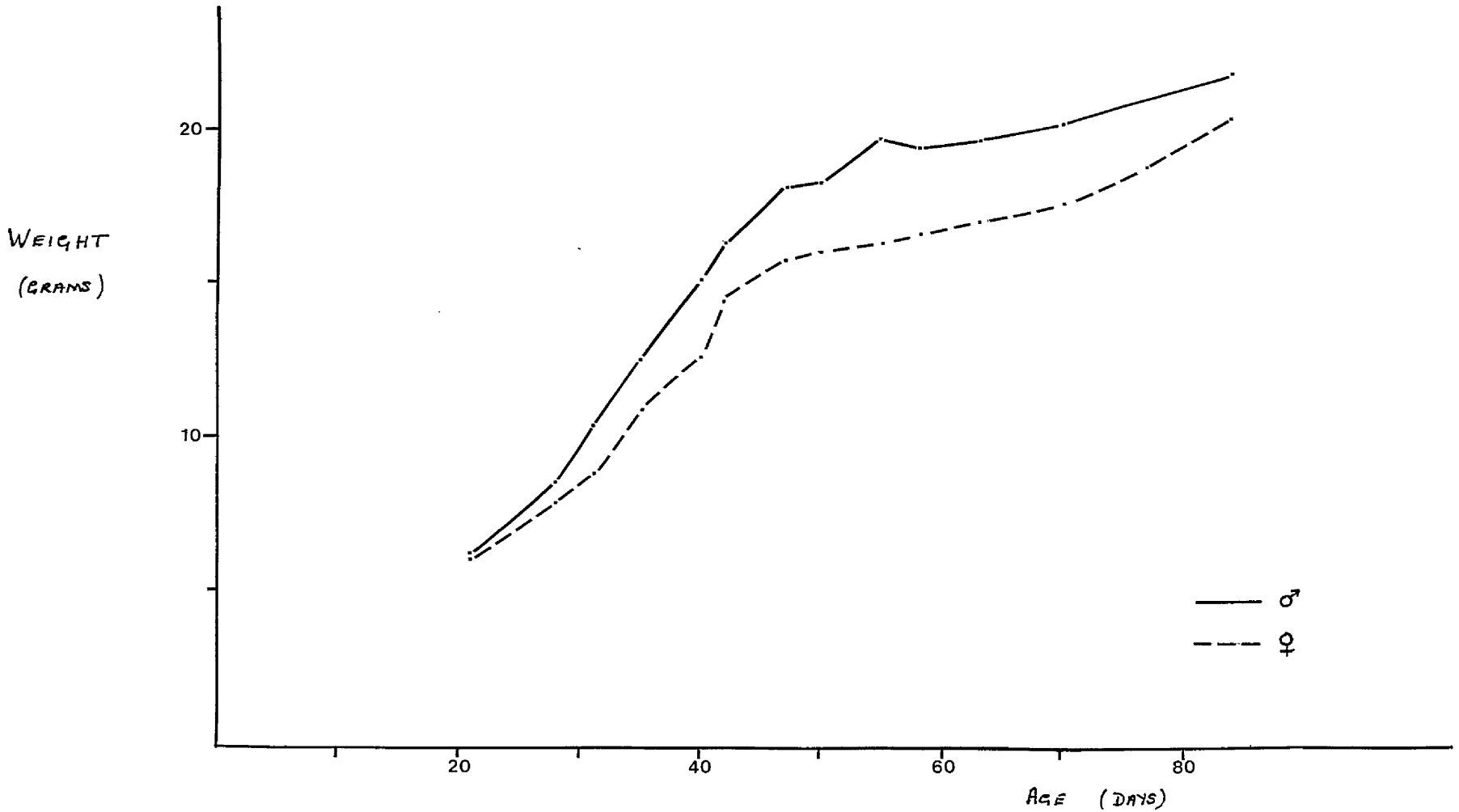


Figure 2: Weight gain in 26 ♂ and 26 ♀ normal VM weanlings.

2. Pathological characteristics

a) Distribution of the tumour The tumour was largely confined to the white matter, at least in the early stages, but at late stages invaded the cerebellum and brain stem.

At about 5 days following i.c. inoculation the tumour was observed in small foci in the corpus callosum, the septal area and the fimbria. By ten days the tumour had spread to the fornix, corpus striatum and anterior commissure. From ten days onwards the tumour spread to the thalamus and extended along the peduncles into the cerebral hemispheres, and along the descending fibres to the brain stem. Later, in mice with C or D stage disease, the tumour may spread into the ventricles and subarachnoid space. No extracranial extension was observed.

Brain swelling was conspicuous (see Plate 2) in mice sacrificed at 'stage C'. The distribution of the tumour, which was often asymmetrical, can

be seen in Plates 3a and b, showing the coronal section of a brain from severely diseased mouse (stage C) compared with that of a normal mouse.

b) Histology of the tumour Light microscopy

Virtually no variation in histological features of the tumour was observed in all the mice, gerbils and hamsters that were examined. Microscopic examination of the tumour showed variable cellular pleiomorphism ranging from apparently well differentiated pilocytic to anaplastic forms. Nuclei were often either small and darkly staining or larger with a more open structure (Plates 4a and b). Multinucleated giant cells were observed and cellular cytoplasm was present in only moderate amounts (Plates 4a and b). Moderate but variable numbers of mitoses were found. Areas of suppiginous necrosis with pseudo-pallisading of surviving cells were found in the most anaplastic regions of the tumour.

The tumour type was identified on general morphological features and was further characterised using special staining techniques. Phosphotungstic

acid haemotoxylin positive stains showed the presence of glial fibres in some cells, but silver stains for reticulin were negative. The histological appearance of the tumour was in keeping with that of an intermediate grade or anaplastic astrocytoma.

3. Electron Microscopy:

As can be seen from Plate 5, the nuclei showed irregular morphology often with a prominent nucleolus and clumps of heterochromatin along the nuclear membrane. In better differentiated areas of the tumour , bundles of neurofilaments could be observed in the cytoplasm of the cells but microtubules were scarce (Plates 6 and 7).

In some cells, normal mitoses were observed adjacent to the areas of necrosis, cell debris was found along with some phagocytosis and the occasional presence of polymorphonuclear leukocytes and macrophages. No inclusion bodies were found and nor was there any evidence of viral particles.

The general features were again consistent with those of an astrocytoma.

Plate 1a - Normal healthy 5 week old VM mouse.

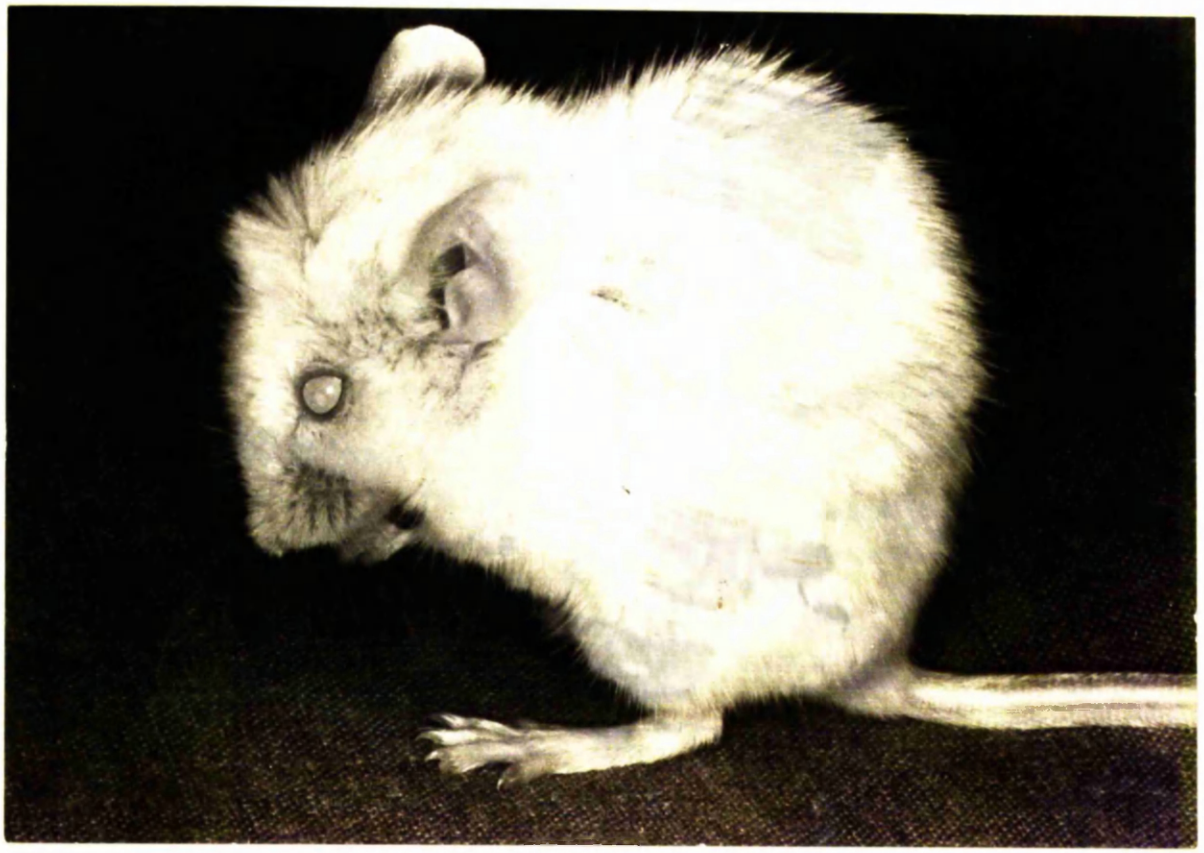


Plate 1b - VM mouse (6 weeks old) 21 days following i.c. injection with standard tumour inoculation exhibiting signs of stage '6'.

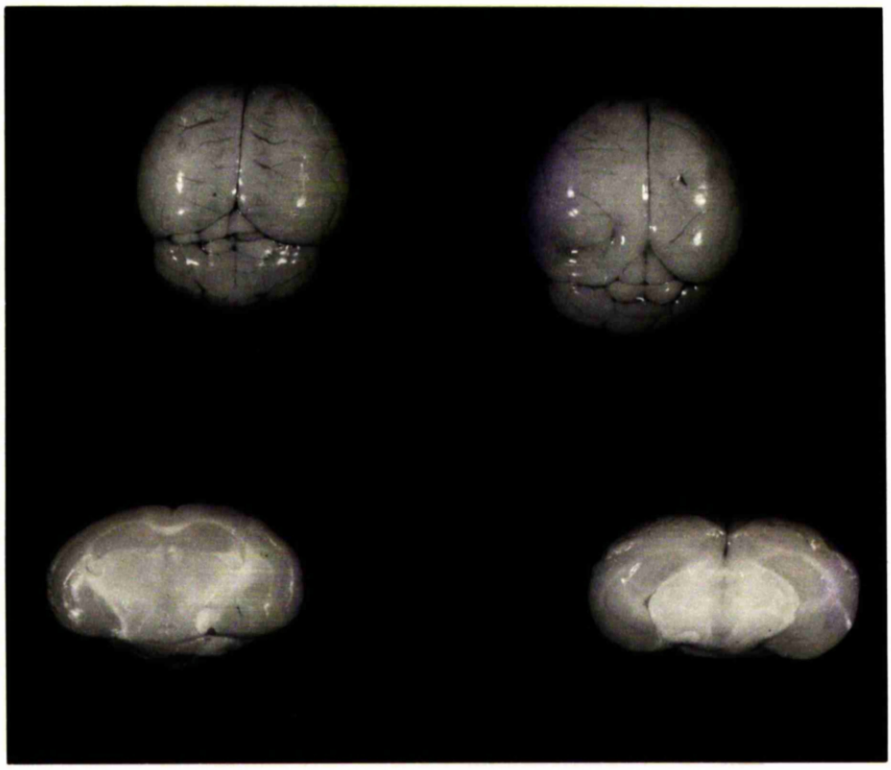
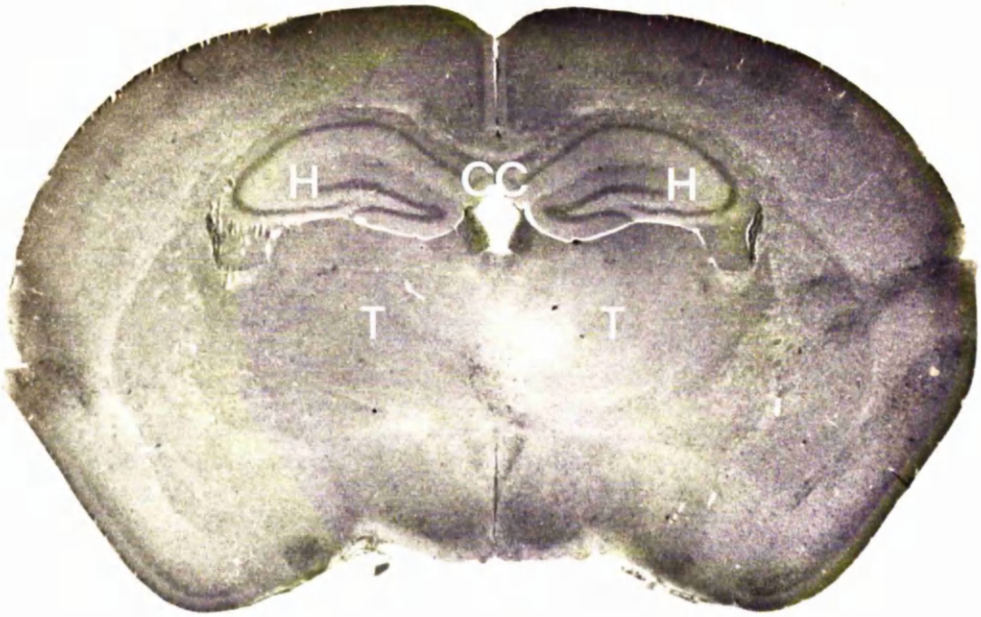


Plate 2 - Gross comparison between the brains of a normal VM mouse (left) and a tumour bearing VM mouse (right). Note swelling of cerebral hemispheres in the tumour bearing brain.

Plate 3a - Coronal section of cerebral hemispheres of a normal VM mouse. x 16.

Plate 3b - Coronal section of cerebral hemispheres of tumour bearing VM mouse. Tumour is present in the deep white matter, including the corpus callosum (CC), hippocampus (H) and thalmus (T). x 16.


H
CC
H
T
T

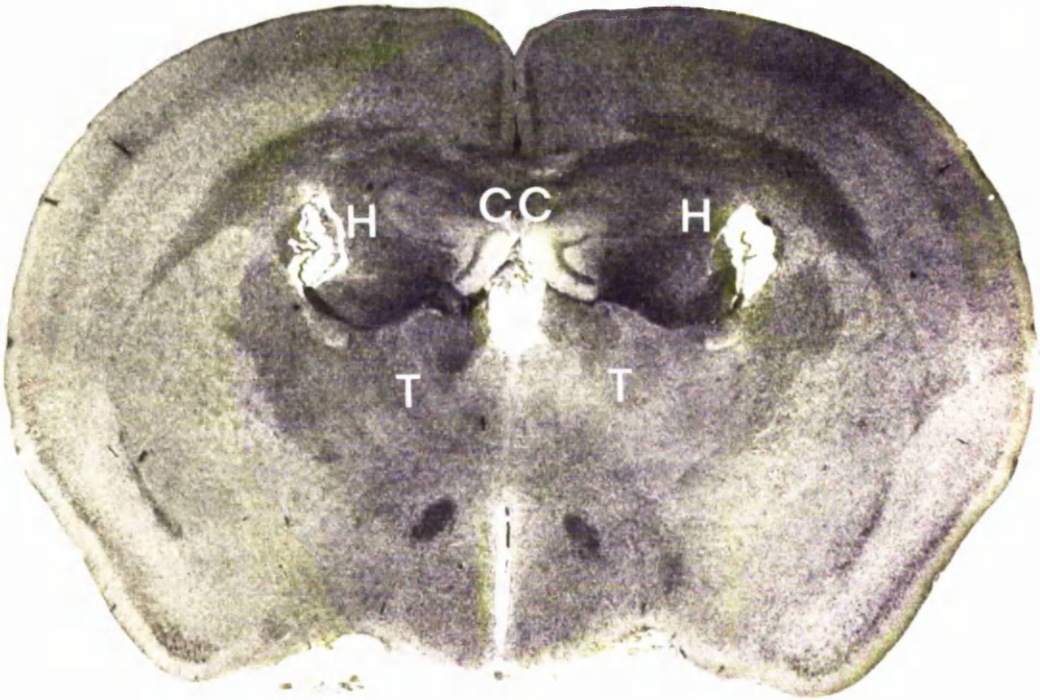
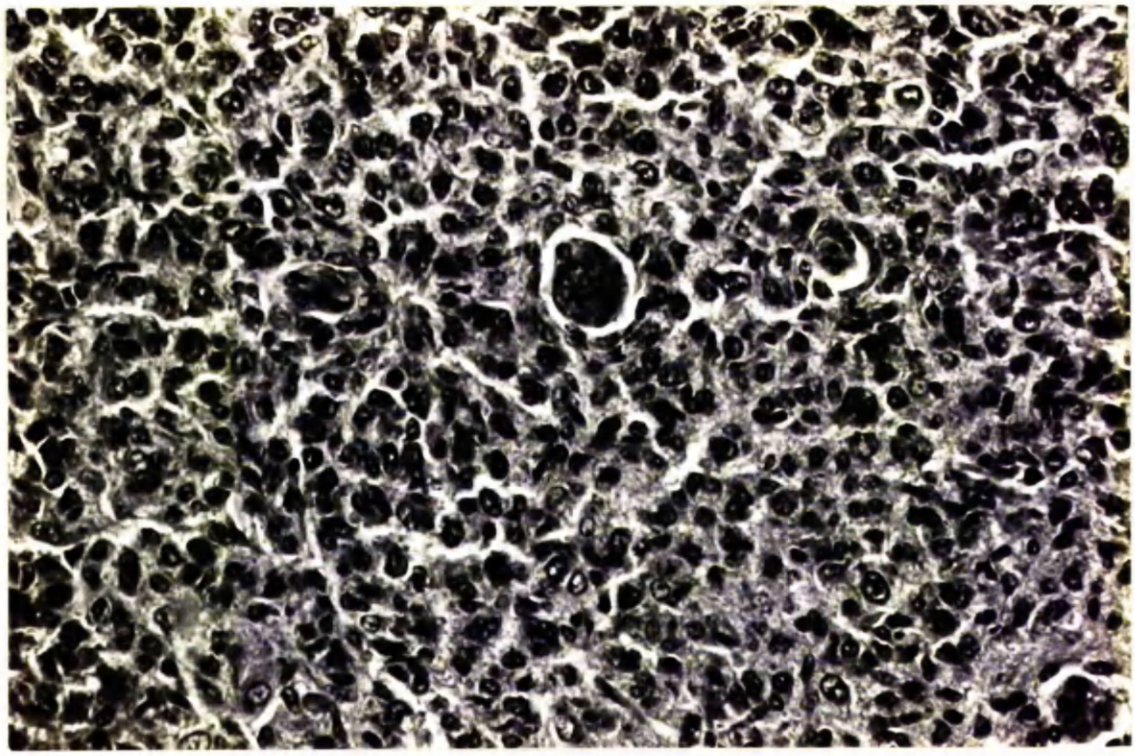

H
CC
H
T
T

Plate 4a - Moderately anaplastic astrocytoma. There is some nuclear pleomorphism and the occasional multinucleated giant cell is seen.

Haemalum and eosin x 420.

Plate 4b - Anaplastic astrocytoma showing focal necrosis, nuclear pleomorphism and the occasional giant cell formation.

Haemalum and eosin x 420.



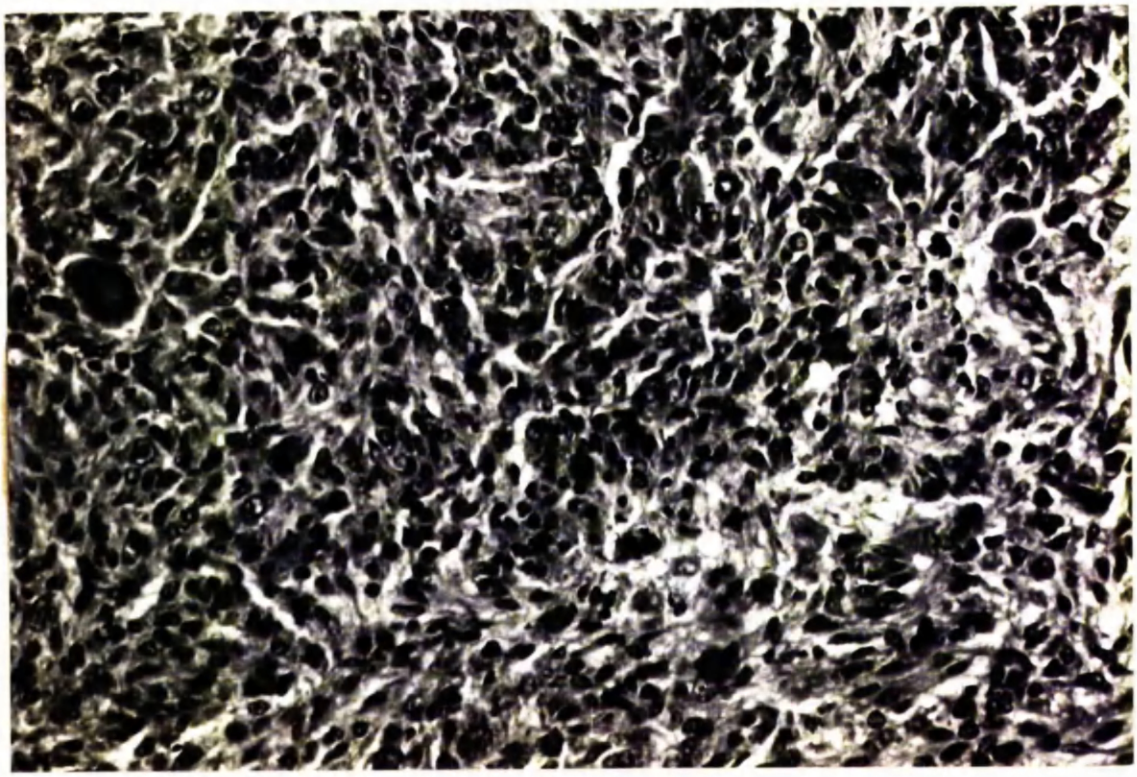


Plate 5 - Low power electron micrograph to show tumour infiltrating the white matter of the corpus callosum. Note variation in size and shape of nuclei (N), the surviving bands of myelinated fibres (MY) and normal capillary (C).

Uranyl and lead x 4,000.

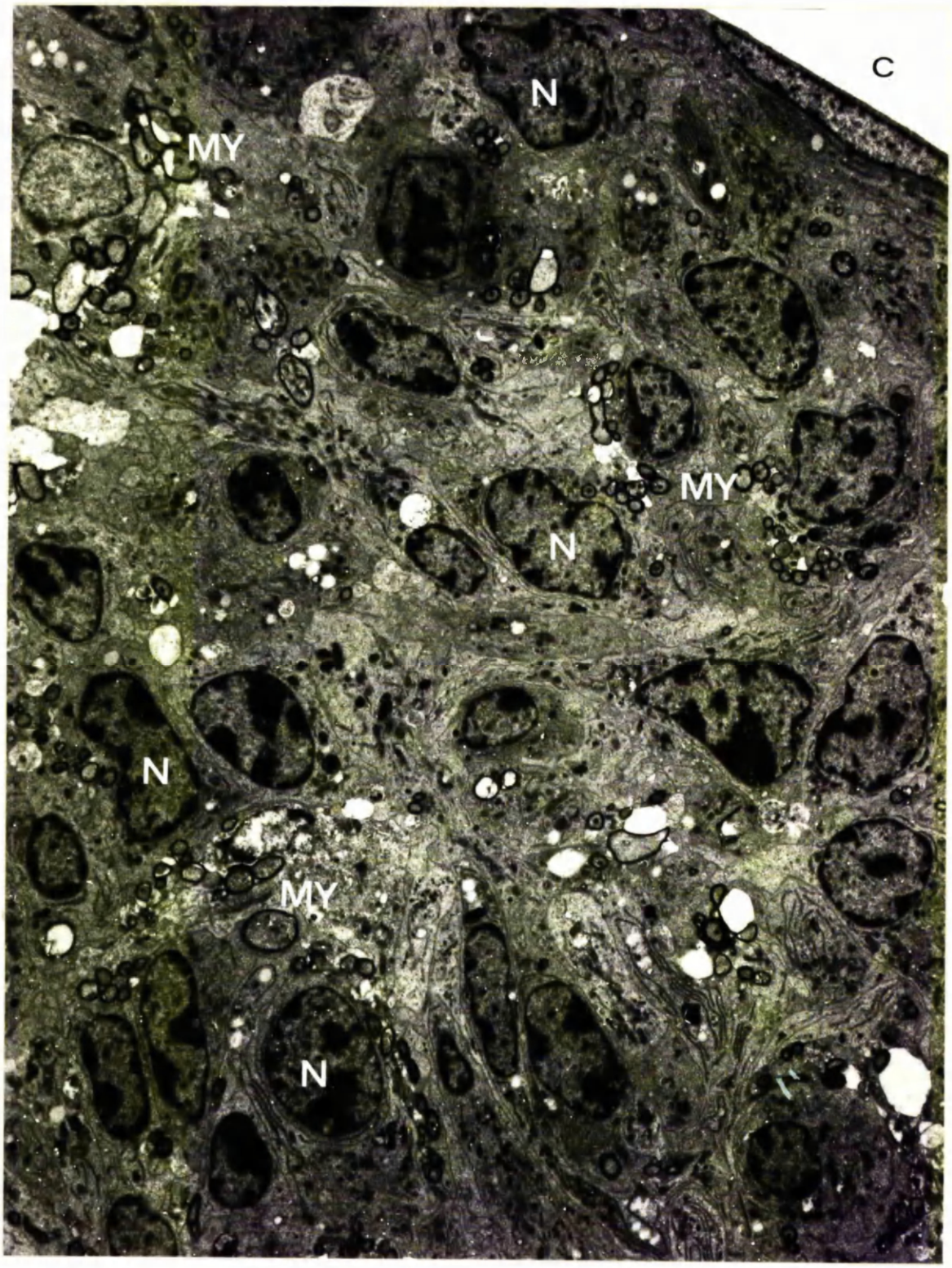
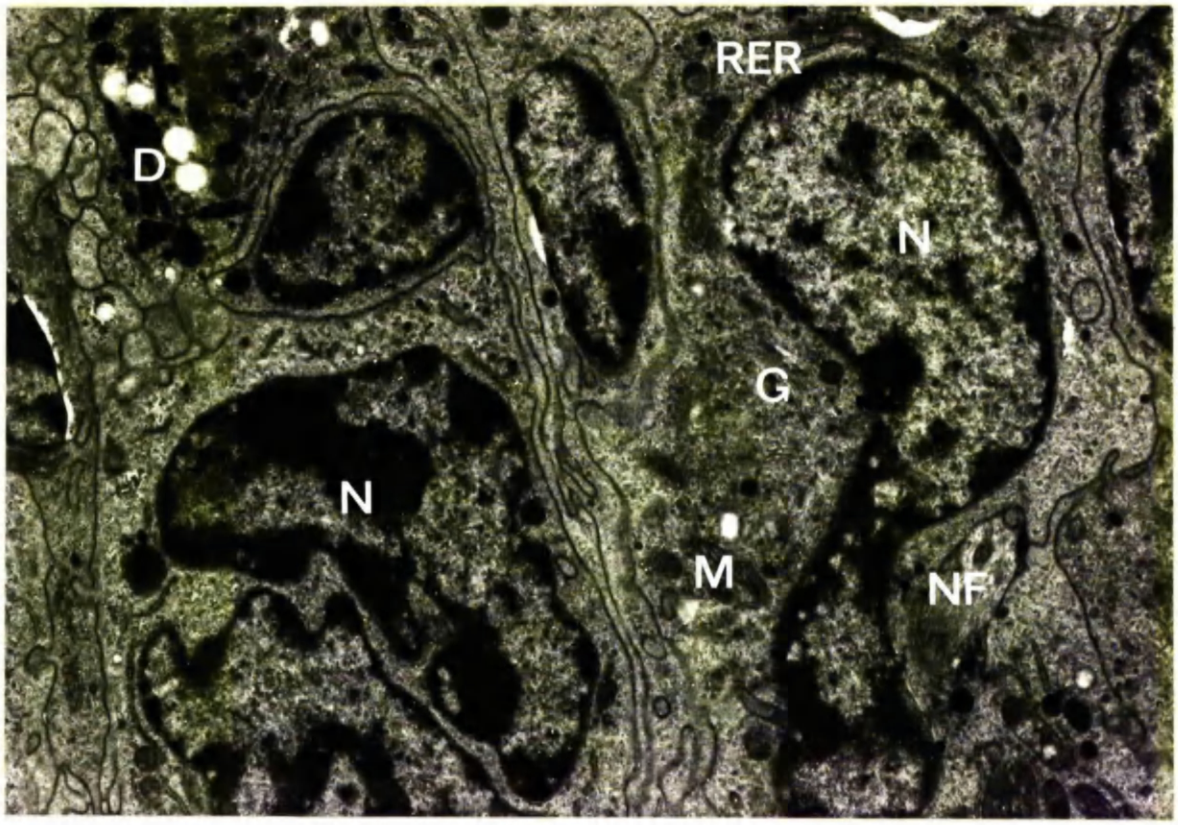

C
N
MY
MY
N
N
MY
N

Plate 6 - Higher power electron micrograph of tumour cells. There is considerable irregularity in shape and size of nuclei (N) in which clumps of heterochromatin are seen. The cytoplasm is moderately abundant and contains the usual organelles. M = mitochondrion; G = Golgi apparatus; RER = rough endoplasmic reticulum; D = dense bodies. Note bundles of neurofilaments (NF).

Uranyl and lead x 12,000.

Plate 7 - Edge of tumour invading neurophil of corpus striatum. N = astrocytic nuclei; F = focus of necrosis; NF = bundles of neurofilaments.

Uranyl and lead x 12,000.


RER
D
N
G
N
M
NF

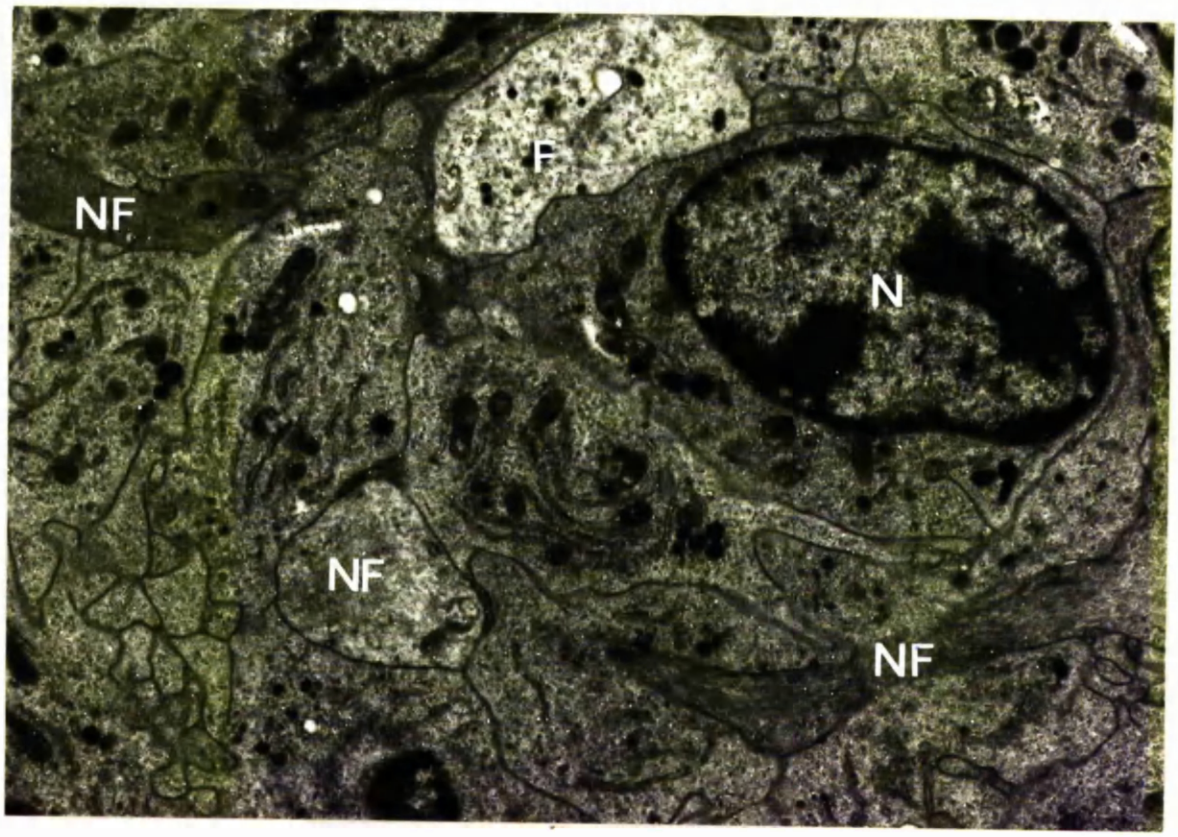

F
NF
N
NF
NF

Plate 8.

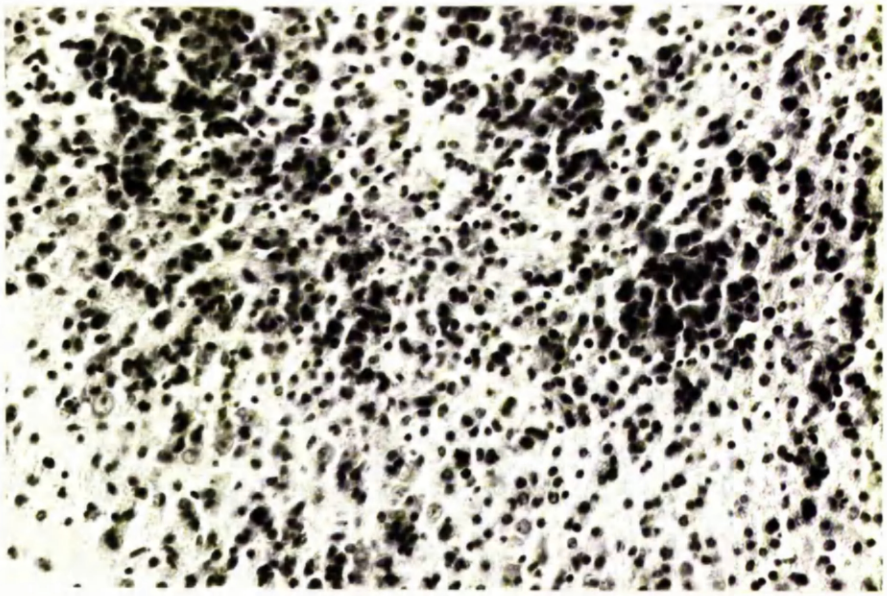
Anaplastic astrocytoma passaged in gerbil from tissue culture. The appearances differ somewhat from those of Figure 4 as the photomicrograph has been taken from the edge of the tumour.

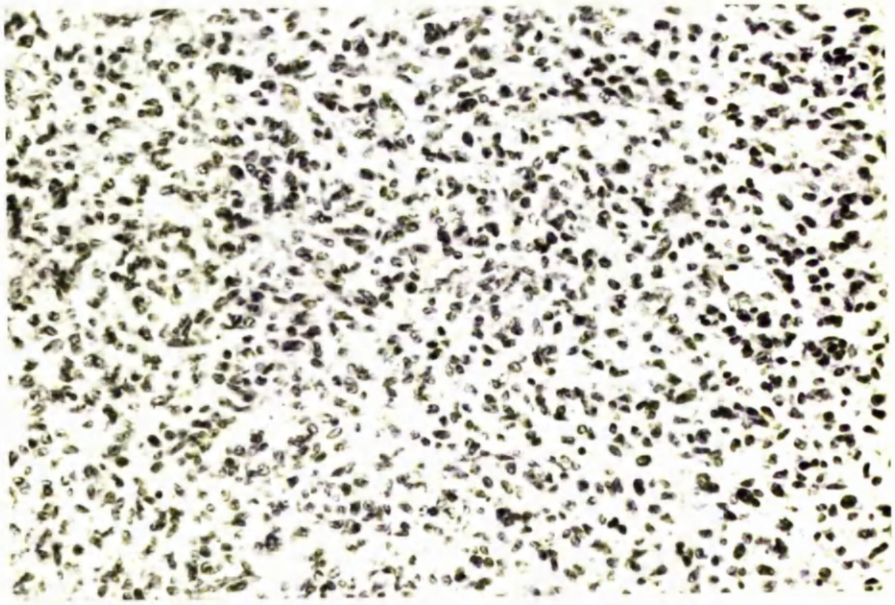
Haemalum and eosin x 420.

Plate 9.

Anaplastic astrocytomas passaged in hamster from tissue culture. The appearances are similar to those seen in Figure 4.

Haemalum and eosin x 420.





PASSIVE TRANSFER OF VM TUMOUR

1. Live cell passage:

a) Fresh tumour preparations

The mean latent period, i.e. the average time taken for mice injected with tumour to reach stage C and be sacrificed, was found to be 22.4 ± 2.43 days (see Table 2). Of the 111 mice that were used for maintaining the tumour passage, there were four which fell beyond this latent period and only three mice which did not develop tumour after three months, giving a 97.3% incidence of tumour take. The three survivors were rechallenged with tumour and were found to be again unaffected after a further three months.

Table 2

Tumour Incidence and Latency

Latency - (duration from i.c. injection of tumour until C disease and tumour incidence in passage mice injected i.c. with standard tumour inoculum ($2x10^5$ cells/mouse)

Passage No.	Tumour Incidence	Latency (days)
54	7/8	26
55	7/7	23 a
56	8/8	22
57	8/10	22
58	12/12	19
59	6/6	22
60	8/8	21
61	6/6	24
62	5/5	18
63	12/12	25
64	4/5	27
65	6/6	20
66	3/3	23
67	4/4	25 b
64	3/3	21
65	4/4	22
66	3/3	21 c
	97.5%	= 22.4 ± 2.43

a 2 mice not included in calculation had tumour at 41 and 60 days

b 1 mouse not included in calculation had tumour at 32 days

c 1 mouse not included in calculation had tumour at 34 days.

As can be seen from Fig. 3 the 50% survival period on the standard survival curve is 20.47 $\pm$ 2.4 days. The survival values were obtained from the number of mice sacrificed at any given time taken as a percentage of the total number of mice injected with tumour.

b) Frozen Tumour Preparations:

Frozen tumour stocks were titrated out to various cell densities from 500 up to 2.10^5 cells/ 20µl dose and it was clearly shown that both latency and tumour incidence were dependent upon dose, i.e. the number of cells injected (Fig.4). The threshold dose for inducing 100% tumour incidence appears to be about 7.10^3 cells. At high doses, i.e. 1.10^5 or 2.10^5 latency is similar to that shown by fresh cell suspensions.

The age of recipient mice had no effect on the latency or take of the tumour. Three aged VM females ($>$ 16 months old) injected with the standard inoculum were sacrificed with stage C disease at 21.33 $\pm$ 2.53 days ($P \geq 0.005$).

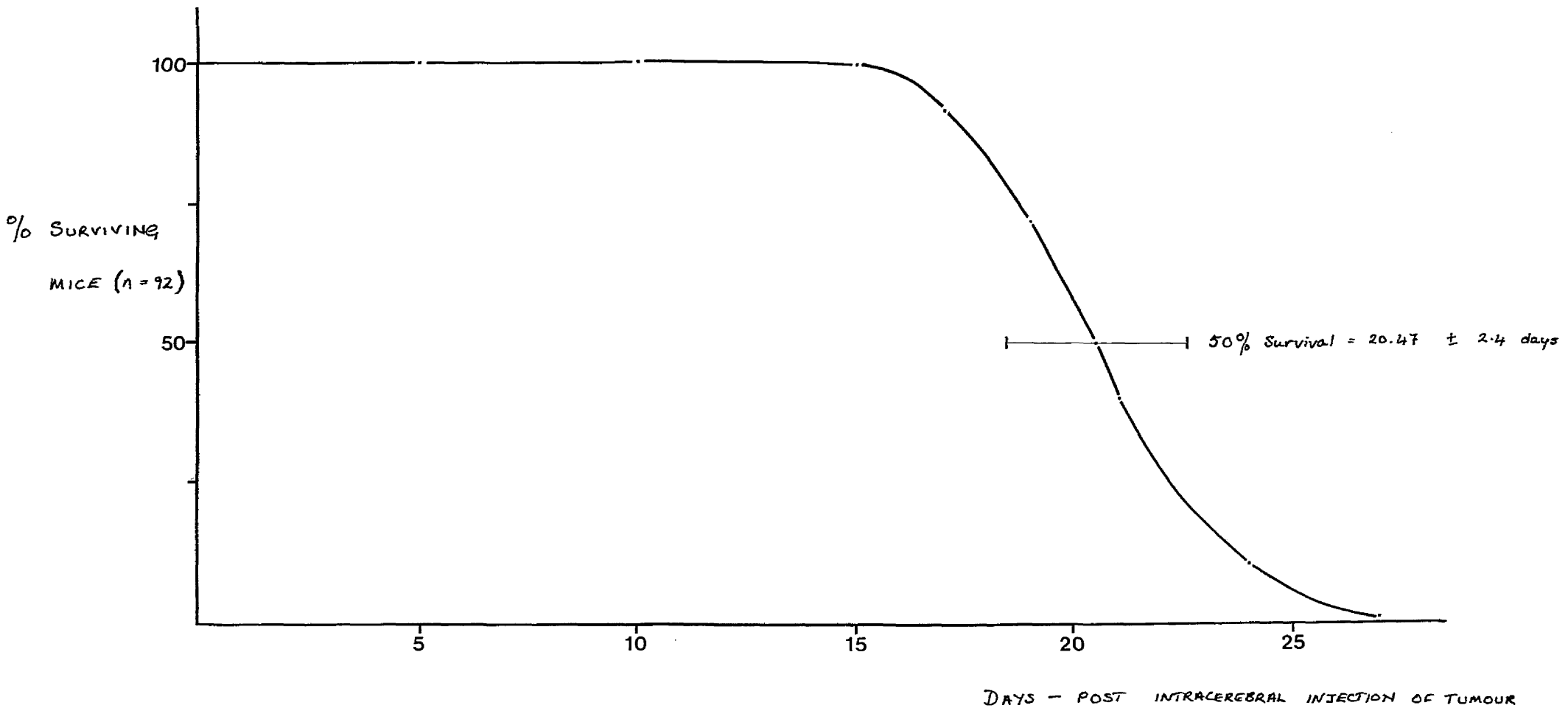


Figure 3: Standard survival curve showing % survival in time following injection with tumour in standard passages (54th - 67th)

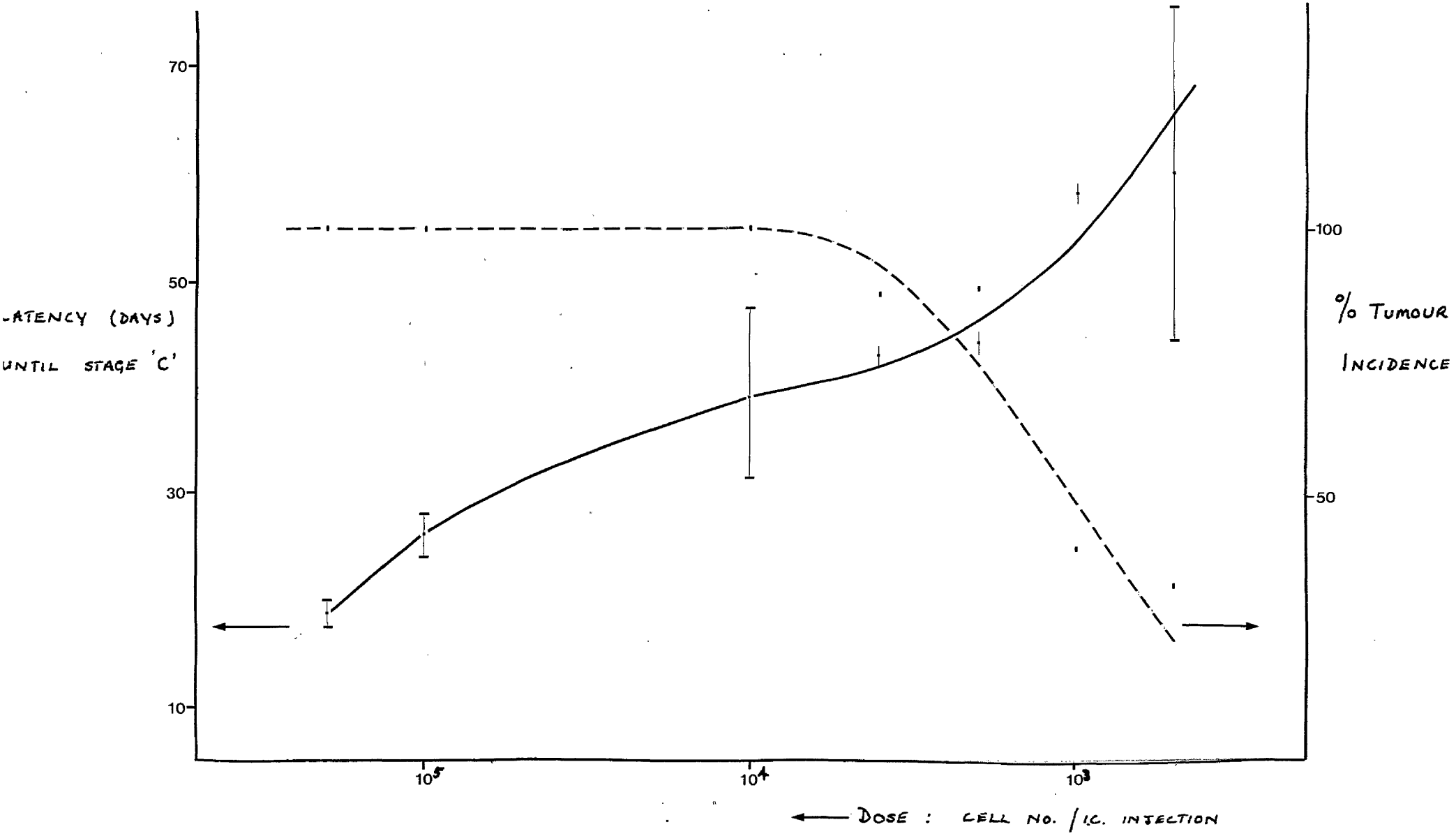


Figure 4: Relationship between tumour dose and the latency and incidence of tumours induced by standard frozen inoculum

2. Killed cell passage

Rupture of cells with hypotonic solution and tannic acid treatment did not appear to interfere in any way with successful tumour passage. Cells killed by freezing and thawing several times, were sometimes successful in transmitting tumour. Mice so treated with killed cell preparations survived longer than mice treated with standard tumour suspensions (see Table 3). In one experiment using cells which had been frozen and thawed four times, and which were used at three times the usual concentration, the recipient mice developed severe tumour, on average, at 18 days following i.c. inoculation. Tumour cells disrupted by ultrasonic treatment failed to produce tumour in VM mice after 6 months observation.

Microscopy of frozen and thawed preparations revealed no intact cells, but some intact cells were seen in hypotonically ruptured cell preparations.

Table 3

Transmission of VM astrocytoma by killed cell preparations.

Method of cell kill	Tumour incidence	Mean survival (days)	P
Freeze & thaw	0/9	-	
x4	9/9	18.33 ± 0.5	< 0.005
	7/14	48.29 ± 1.8	< 0.005
x1	3/3	41.33 ± 1.15	< 0.005
x2	0/3	-	
x3	0/5	-	
x4	0/4	-	
Ultrasonic rupture	0/22	-	
Hypotonic rupture	4/4	21.75 ± 1.5	> 0.2
Tannic acid			
at 37°C 10 mins	5/5	33.8 ± 1.64	< 0.005
at 37°C 30 mins	1/1	33 ± 0	-

3. Cell Free Passage

Cell free extracts of tumour cell preparations failed to induce tumour in any of the 83 recipient VM mice.

Table 4

Transmission of VM Astrocytoma by cell free extracts prepared from killed cell suspensions

Method of cell rupture	Tumour incidence
Freeze and thaw	0/10
x4	0/9
	0/9
	0/7
Mechanical - homogenisation	0/12
Ultrasonic rupture	0/12
Hypotonic rupture	0/7
"	0/7

4. Passage with Normal Brain Suspensions

As can be seen in Table 5, attempts to induce tumour by i.c. injection of aged VM normal brain into aged and weanling VM mice were unsuccessful. Similarly using young VM normal brain suspension, tumour could not be produced in aged or weanling VM mice.

Table 5

Attempts to transmit VM tumour with normal Brain homogenates

Inoculum	Recipient	Tumour incidence
VM old normal brain homogenate	VM > 16 months	0/3
	VM weanlings	0/6
	BSC "	0/5
	MM "	0/6
VM weanlings normal brain homogenate	VM > 16 months	0/3
	VM weanlings	0/6
	BSC "	0/4
	MM "	0/8

PASSIVE TRANSFER OF VM ASTROCYTOMA INTO OTHER MOUSE STRAINS AND OTHER SPECIES

1. Inbred mouse strains

Tumour was successfully passaged into the C57BL/10ScSn strain, having the same histocompatibility type as the VM ($H\text{-}2^b$), SJL/J strain ($H\text{-}2^s$) and BSC strain of unknown H-2 type. Attempts to produce tumour in various other inbred mouse strains (see Table 6) were unsuccessful.

Table 6

Transmission of VM Astrocytoma into mice of other strains and histocompatibility (H-2) types.

Strain	H-2	Tumour incidence	Latency (days)
C57BL/10	b	2/10	46,73 days
B10Lp-a	b	0/5	
B10 A	a	0/15	
B10 D2	d	0/5	
C58	k	0/14	
AKR	k	0/6	
MM	a	0/30	
BSC	?	4/23	25,25,43,52
SJL	s	2/12	36,65
VM controls	b	12/12	25

2. Gerbils

Out of a total of 56 gerbils injected i.c. with standard tumour inoculum, five of those which had been injected as neonates were found to have developed tumour (see Table 7). One of these five gerbils was found to be very wasted after 28 days, but did not otherwise exhibit any of the clinical signs previously described for VM mice with the tumour. Three siblings of this gerbil did not thrive and were subsequently found dead, but cannibalism by the mother prevented histological confirmation of tumour. These three gerbils were excluded from the figure of incidence. The other four gerbils which developed tumour were also siblings,but of a different gerbil family. They showed no clinical signs other than slow development,and at sacrifice were found to have very mild tumours. The fifth member of this litter had no tumour and was considerably larger than its siblings. Histology showed the tumours in the gerbils to be astrocytomas, although the sections looked slightly different from the usual passaged tumour in the VM mouse (see Plate 8).

Table 7

Transmission of VM Astrocytoma into other species

Species	Age at i.c. injection	Latency (days)	Tumour incidence
Mongolian gerbil	> 10 days	-	0/26 *
	neonatal	28,44,44 44,44	5/30
Syrian hamster	neonatal	19,27,30 33,54	5/40
AS rat	adult	-	0/10 *
BN rat	adult	-	0/10 *
OM rat	10 days	-	0/12
SD rat	10 days	-	0/12

* 8 Gerbils
5 AS rats
5 BN rats

Immunosuppressed with 300mg/kg cyclophosphamide 3 days prior to i.c. injection of VM tumour.

3. Hamsters

The VM tumour was also successfully induced in a second species, the Syrian hamster. Five hamsters, three of which were siblings, showed signs of weight loss and poor development. On sacrifice and histological examination these hamsters were found to have tumours which looked very similar to the passaged material in the VM (see Plate 9). These hamsters had been injected as neonates with standard tumour inoculum and developed tumour within four weeks (see Table 7) except for one which was found to be severely affected by tumour after 54 days.

4. Rats

No tumours were found in any of the inbred rats that were injected with VM tumour suspension.

5. Immunosuppressed Rats and Gerbils

An unsuccessful attempt was made to induce VM tumour in 5 AS rats, 5 BN rats and 8 gerbils which had been immunosuppressed with

cyclophosphamide (300mg/kg) three days prior to i.c. injection with tumour. The control rats and gerbils did not develop tumours either.

TISSUE CULTURE OF VM ASTROCYTOMA

The VM tumour was successfully maintained in culture for periods of up to four weeks. It is not known how long it is possible to culture the tumour and retain its ability to reintroduce tumours in mice. Intracerebral inocula of primary astrocytoma cultures, three weeks old, were found to successfully transmit the tumour back into VM mice. Secondary subcultures were unsuccessful in recipient mice that were observed for nine weeks.

As can be seen from Table 8, the incidence of tumour take in VM mice depended upon cell density and how long the tumour had been cultured. Duration from the time of i.c. inoculation to reach stage C in the recipients, also bore relation to the time the tumour had been in culture, e.g. after one day, i.c. inoculation of 2.10^5 cultured cells produced clinical disease C in 25.5 days, whereas using cells cultured for four days, the latency was 34.4 days.

Table 8

Transmission of tumour with tissue cultured VM Astrocytoma

Period of culture	Dose cell no./mouse	Tumour incidence	Latency (days)	P
1° 3 weeks	2000	5/19	94.4 ± 19.23	<0.005
2° 6 weeks	100	0/12		
	1000	0/16		
	10^4	0/16		
	10^5	0/12		
1° 0 day	2.10^5	4/4	25.5 ± 1.89	
1 "	"	6/6	25.5 ± 1.97	<0.2
2 "	"	5/6	27.2 ± 2.17	<0.005
3 "	"	6/6	30 ± 1.26	<0.005
4 "	"	5/5	34.4 ± 7.8	>0.05
5 "	cultures unhealthy	0/4		
7 "		0/4		
cell free extracts				
1° 1 day		-		
2 "		0/3		
3 "		0/3		
4 "		0/3		
5 "		0/1		

Tumours taken from mice injected i.c. with cultured tumour inocula, were found to be successful in further inducing tumour in VM mice, with the same incidence and latency as the standard inocula.

EFFECT OF DRUGS ON TUMOUR GROWTH IN VITRO

1. Ribavirin

Attempts to reduce tumour growth in recipient mice by treating primary tumour cultures with Ribavirin at a concentration of 100µg/ml were unsuccessful. Both control and test mice reached stage C at the same time, i.e. within 30.5 ± 3.36 days ($P < 0.05$) following i.c. injection (Table 9). The treated and control tumour cultures were both successfully subcultured, showing that Ribavirin had not killed the tumour cells.

Table 9

Effect of Ribavirin in vitro on ability of cultured VM tumour cells to transmit tumour.

No. of mice	Treatment	Tumour incidence	Latency (days)
20	i.c. injection of 2.10^5 cultured tumour cells treated with 100µg/ml Ribavirin	20/20	30.3 ± 3.16 ($P < 0.05$)
10	Controls i.c. injection 2.10^5 cultured tumour cells	10/10	30.55 ± 3.36

2. P 113 (Saralasin acetate)

Control mice and those injected with cultured tumour treated with 0.7ug/ml and 0.007ug/ml all succumbed to the tumour within 40-45 days. Mice injected with tumour treated with 0.07ug/ml showed clinical signs of severe tumour on average after 66 days; this includes one mouse presenting tumour as early as 42 days and one mouse which survived until 134 days.

EFFECT OF CHEMOTHERAPY

1. Hormones

a) Oestrogen: In two experiments the administration of 9µg/mouse/day oestrogen in the drinking water was found to significantly alter the latency of tumour development in both males and female VM mice. As can be seen in Table 10 the effect was to slightly extend this period by a few days, and in one experiment by a week. In a repeat experiment involving pretreatment with oestrogen, there was no apparent effect.

Table 10

Effect of hormones on course of VM Astrocytoma

No. of mice & sex	Treatment	Tumour incidence	Latency (days)	P
5 ♂	control	5/5	20.6 ± 0.89	
7 ♀	"	7/7	19.7 ± 2.21	
9 ♂	testosterone i.m. 0.05mg/ mouse/week	9/9	21 ± 1.94	> 0.5
10 ♀		10/10	20.4 ± 0.96	> 0.5
10 ♂	oestradiol oral 1.2µg/ mouse/day	9/10	23.11 ± 1.83	< 0.05
10 ♀		10/10	24.1 ± 1.2	< 0.005
14 ♀	oestradiol oral 1.2µg/ mouse/day from 2 weeks prior to i.c. tumour injection	14/14	29.7 ± 2.2	< 0.005
8 ♂		8/8	20.5 ± 0.93	> 0.05
8 ♀		8/8	20.25 ± 1.04	> 0.5

b) Testosterone: This had no effect on the latent period or incidence of the tumour development in treated mice.

2. Levamisole

Levamisole did not prevent or delay the course of tumour development, even with a long preinoculation period of treatment at a dose of 30mg/kg/day. If anything Levamisole slightly shortened the latent period by a significant amount ($P = <0.005$) (see Table 11).

Table 11

Effect of Levamisole treatment on the course of VM Astrocytoma.

Treatment	Tumour incidence	Latency (days)	P
Controls	12/12	22.92 ± 1.88	
30mg/kg/day in drinking water	24/24	20.88 ± 1.87	< 0.005
30mg/kg/day in water from 2 weeks prior to i.c. injection of tumour	10/10	20.6 ± 0.84	<0.005

3. Dexamethasone

This drug was administered to try and control cerebral oedema in tumour bearing mice, at the dose of 0.14mg/kg/day. When administered on day 12, following i.c. inoculation of tumour, Dexamethasone was without apparent effect on the clinical condition or survival of mice. However five mice given the drug from the day of tumour injection until sacrifice, whilst still exhibiting stage B signs of the disease, survived for longer. These five mice (see Table 12) showed a latent period of 24.8 ± 1.2 days, as compared with 20.44 ± 0.73 days for control mice. Interestingly, the short treatment with the drug appears to very slightly reduce the latent period.

Table 12

Effect of Dexamethasone treatment on the course of VM Astrocytoma.

Treatment	Tumour incidence	Latency (days)	P
Controls	9/9	20.44 ± 0.73	
Dexamethasone from Day 12	10/10	19.3 ± 0.95	0.0093
Dexamethasone from Day 0	5/5	24.8 ± 1.2	0.00002

4. Ribavirin

Ribavirin administered at a dose of 75mg/kg/day, for fourteen days following i.c. inoculation of tumour had no significant effect on the clinical condition or survival period of the treated mice (see Table 13). The survival periods for both control and test mice is longer than the standard period of 22 days, but this was typical of frozen tumour stocks used for this experiment and which had been stored for more than six months.

Table 13

Effect of Ribavirin administered *in vivo* on the course of VM Astrocytoma

Treatment	Tumour incidence	Latency (days)	P
Ribavirin in 0.3ml saline - i.p. 75mg/kg/ day - for 14 days Tumour i.c. on 2nd day of drug treatment.	17/17	36 ± 3.45	> 0.1
Controls - i.p. injection 0.3ml 0.9% saline	11/11	34.36 ± 2.69	

DISCUSSION

The results in this study demonstrate the general behaviour of the VM astrocytoma under experimental passage conditions. Results are also given of attempts to transfer passively the tumour with killed cells. The effects of various drugs on tumour growth *in vivo* and *in vitro* and the ability of the tumour to grow in other mouse strains and in other species are also demonstrated.

The clinical presentation of the VM tumour is similar to that of many murine diseases in that affected mice show a general malaise and wasted appearance. It is however characterised by a number of specific neurological signs such as loss of reflexes, ataxia and seizures. Weight loss is primarily due to an increasing inability to reach the food hopper as even severely affected mice would eat readily if food was placed within reach. It was therefore accepted as an expression of clinical involvement of the tumour. Weight loss was first evident at approximately 15 days following i.c. injection with tumour and coincided with the first

clinical signs.

A single spontaneous astrocytoma was found in one 220 day old female from the breeding colony, ultimately totalling 96 mice. The spontaneous tumour showed a similar histological pattern to the passaged material.

The pathological features of VM astrocytomas found in this study were similar to the description given of the histology of spontaneously arising VM astrocytomas (Fraser, 1971). It was observed, as reported in Fraser's study (1980), that in severe cases the tumour invaded the ventricles and subarachnoid space. Some tumours included several giant multi-nucleated cells and bore a resemblence to glioblastoma multiforme. These features were also evident in some of the tumours derived from cultured VM astrocytoma cells. Giant cells with bizarre nuclear patterns typical of glioblastoma multiforme have also been reported in high grade astrocytoma induced by N-methylnitrosourea (Schmidek et al, 1971).

Passage of the tumour was standardised and the variability in latency period, as reported by Fraser (1975)

was minimised. The latency period was found to be approximately 21 days, which was shorter than the 25–30 days indicated by Fraser (1980). This was possibly a dose response effect as the standard dose in this study was 2.10^5 cells/0.2µl as compared with 10^4 cells/0.2µl used by Fraser. A definite relationship was found between the number of cells injected and the tumour latency period and incidence. The minimum latency period has yet to be determined, but it is possible that there is a maximum cell concentration above which there is no reduction in time for the tumour to manifest itself. Likewise, it is possible that there is a threshold number of cells below which tumour cannot be induced. The threshold dose would be below 500 cells, but it would be difficult to establish a very precise number, since the proportion of tumour to non-tumour cells may be variable. It would however, be useful to establish an approximate threshold dose of tumour inoculum for the investigation of the immunology and chemotherapy of the tumour. There were four cases in which the tumour presented severe signs after 30 days, one being as late as 60 days. This can be explained either by a low ratio of tumour cells to normal cells in the inoculum given to these particular mice or by a slight resistence to the tumour in these individuals.

Three mice, at the 54th, 57th and 64th passages (from the first passage carried out by Fraser, 1974) developed no tumour. These survivors were challenged i.c. with the tumour and remained unaffected. Resistence by VM mice to the passaged astrocytoma and to subsequent challenge has been reported at earlier passages (Fraser 1974 and 1980). It was observed that the latency period was slightly longer in those passages including survivors. This was also reported by Fraser (1974), who suggested that this was due to too few tumour cells being present in the inoculum. This does not explain, however, the resistance shown by these mice to further challenge. It is possible that a subpopulation of the VM strain is either privileged with an immunologic resistance to the tumour, or is genetically incompatible with the tumour causing agent. The genetically determined predisposition of VM mice to succumb or resist the passaged astrocytoma could be tested by observing the behaviour of the tumour in selectively bred offspring of apparently immune VM mice. Another possibility is that the surviving mice did, in fact, receive a subthreshold dose which was sufficient to invoke a cell mediated immunity to subsequent challenge.

Contrary to previous belief, the brain is not an immunologically privileged site; both inhibition and rejection of an i.c. implanted mouse glioma have been found following intradermal immunisation of mice with isologous glioma - adjuvant mixture (Scheinberg & Taylor, 1968). VM mice, however, immunised by various peripheral routes succumbed to intracerebral challenge with the tumour (Fraser 1980). It may be possible to stimulate an immune response in VM mice by i.c. immunisation of a subthreshold dose of tumour cells. Humoral antibodies cytotoxic for cultured astrocytoma cells are present in human glioma patients and have been studied for diagnostic use in a microcytotoxicity test (Philips et al, 1976). Cellular immunity in Fischer rats bearing primary brain tumours has been demonstrated by the specific inhibition of peritoneal macrophage migration by soluble extracts of avian sarcoma virus induced glioma tissue (Adams et al, 1977 and 1978). An antibody dependent cellular cytotoxicity reaction has been shown to cure a mouse neuroblastoma in vivo (Byfield et al 1976). This was however achieved on a tumour which will grow subcutaneously in syngeneic A/J mice. The cure was effected by first injecting antibody coated mouse neuroblastoma cells and then rat spleen cells into the flanks of mice.

It has previously been reported that the VM tumour failed to grow in any of a variety of sites outside the CNS, even using neonatally thymectomised mice (Fraser, 1974). The VM tumour has, however, been grown subcutaneously in totally immunodeprived CBA mice. Immunodeprivation was carried out as follows: 7 week old male CBA mice were neonatally thymectomised; 3 weeks later they were given 850 rads whole body radiation, followed immediately by an i.v. injection of 5.10^6 syngeneic bone marrow cells. Immunodeprived mice were injected s.c. with the standard VM tumour dose. After four weeks subcutaneous growth measuring 0.5cm across were found along a blood vessel. Histological observations were not available at the time of writing, but it is possible that these growths were astrocytomas, as tissue cultured cells have since been found to grow intramuscularly and subcutaneously in nude mice (Serano et al, 1980). (Work done at the National Hospital for Neurological Diseases, Queen Square in conjunction with Dr. N. Bradley, Royal Marsden Hospital.) Such immunodeprivation techniques should be explored to examine the possible extraneural growth of the tumour in VM mice.

Of the various methods of cell kill used, only

ultrasonic rupture consistently inhibited tumour transmission. It is possible that the tumour transmitting capacity of the cells was destroyed by heat, as generation of high temperature is an inherant problem in the sonication of liquids. It was attempted to minimise this effect by keeping tumour preparations on ice during sonication. Alternatively, as sonication disrupts the cell completely, it is possible that no sufficiently large fragments remained to allow a membrane associated agent to transmit the tumour.

Hypotnic cell rupture showed no effect on the tumour latency period or incidence. This is not surprising as a few intact cells were seen amongst the large cell fragments in the preparation. Whole intact cells may have persisted in the inoculum.

Cell preparations killed with tannic acid were still able to transmit the tumour, although there was a significant extension of the tumour latency period to 33 days. The incubation time with tannic acid did not vary the result, i.e. tumour latency period was the same from both the 10 minute and 30 minute incubations. Vital staining with methylene blue

showed that no viable cells were present in the inoculum. This suggests that intact killed cells were able to transmit the tumour and that the tumour causing agent was not inactivated by tannic acid.

Freeze and thaw killed cell preparations produced rather variable results. In some cases total tumour kill was achieved, and in others the oncogenicity of the tumour was retained. Where successive freezing and thawing was carried out only the X1 preparations produced tumour, the X2, X3 and X4 freeze and thaw preparations producing no tumours after 6 months observation. Two previous X4 preparations had, however, proved successful in producing tumours and one had not. It is interesting to note that in two experiments, a X4 and a X1 preparation, there was a significant delay in tumour latency period to 48 and 41 days respectively. In another X4 preparation, the latency period for both test and control animals to show severe tumours was only 18 days. Since this preparation contained three times the standard cell concentration, and therefore a greater concentration of the transmissible agent, a dose response effect may account for the

very short latency period. Intact cells were not found in any of the X2, X3 or X4 freeze and thaw preparations, but some were seen in the X1 preparation. It is not clear whether all cells were killed by this method. It is therefore possible that incomplete cell kill merely extends the tumour latency period by a dose response effect. It appears therefore that VM tumour cell fragments and intact dead cells can transmit the tumour, but that the damage caused by successive freezing and thawing, or by tannic acid may inhibit or delay the onset of tumours.

Cell free filtrates prepared from all the above methods of cell kill failed to produce any tumours in 73 VM mice. Previous attempts to induce VM tumours with i.c. inoculations of cell free filtrates were also unsuccessful (Fraser, 1975). Successful transmission of a spontaneous transplantable mouse neuroblastoma using cell free extracts has been reported (Prasad et al 1970) but another group (Myers et al, 1971) failed repeatedly to reproduce their work and cast doubts on the validity of Prasad's methods. The VM astrocytoma therefore requires intact cells or cell fragments in the inoculum in order to transmit the tumour successfully.

These results are difficult to interpret meaningfully as more work on the effects of cell kill is necessary. Three points may, however, be drawn from these experiments. First, the VM astrocytoma grows faster in the host from intact viable tumour cells. Secondly, the transmissible tumour causative agent is associated with cell membranes or other cell structures. Thirdly, it is possible that the tumour is transmitted, in the passage situation, by infection of the host's brain cells, rather than by proliferation of inoculated tumour cells; both mechanisms may however work concurrently. This last point would be clarified by establishing the sexual identity of tumour cells in male and female mice. The original spontaneous tumour, from which the passaged material was derived arose in a female VM mouse. Chromosome banding for example, with Giemsa or quinacrine staining of mitotic tumour cells, would demonstrate the VM karyotype.

The VM astrocytoma was transmitted across the histocompatibility barrier, into the SJL/J strain (H - 2^{s}), BSC strain (H - 2 unknown) and also into the C57BL/10 strain of mouse, which has the same H - 2 type as the VM (H - 2^{b}). The number of such

transmissions was very low and the tumour latency period extended. The tumour could not be reproduced in a number of other mouse strains (Table 6). It was reported by Fraser and McConnell (1975) that attempts to produce the tumour in other strains had been generally unsuccessful. Subsequent work, however, has shown that the VM tumour can be induced in other mouse strains, with increased tumour incidence in the F1 generation of VM hybrids e.g. VM/C57BL (Fraser 1980). No correlation was found in either study between tumour incidence and histocompatibility type. It was proposed by Fraser and McConnell (1975) that the success of the tumour depended on its maintaining a close relationship with the genotype of origin. The tumour has however been successfully transmitted across the species barrier into 5/40 hamsters and 5/30 neonatal gerbils. Three siblings of one of the tumour bearing gerbils showed similar wasting and poor development, but cannibalism by the mother prevented histological confirmation of tumour. The actual frequency of passaged VM tumour in gerbils could be as high as 8/30. The latency period was usually longer than in the VM host, being between three and eight weeks. The tumour could not be grown in four different rat strains.

Transfer of brain tumours to heterologous species in not unknown; human glioblastomas have been transplanted both directly and indirectly, via the anterior chamber of a guinea pig eye, into guinea pig and mouse brains (Greene 1951). Human ependymomas and astrocytomas treated in the same way failed to grow and Greene attributed the success of the glioblastoma to its extreme malignancy. Similarly the high malignancy of the VM anaplastic astrocytoma may account for its successful transmission into other species. Immunosuppression with cyclophosphamide did not aid transmission of the tumour into adult rats and gerbils. The possibility of transmitting the tumour into yet more mouse strains and species, however, should be examined in more detail. The use of immunosuppressive drugs and immunodeprivation techniques, such as irradiation and neonatal thymectomy would be helpful. The VM tumour may grow for example in the Fischer rat , in which artificially induced astrocytomas have been obtained by i.c. injection of RSV (Wilfgong et al 1973). The monkey would be an obvious candidate, as successful growth of the VM tumour would provide a useful parallel with human glioma research.

That the VM tumour is capable of transmitting tumour to other mouse strains, and more importantly to other species, is consistent with a viral aetology. It also supports the hypothesis that the tumour is transmitted by infection of the host cells, and not be proliferation of inoculated 'foreign' cells.

The VM tumour was successfully maintained in culture for short periods; up to three weeks in primary (1^{o}) culture and six weeks in secondary (2^{o}) culture. It failed however on subsequent subculturing. Fraser (1975) also found that the VM tumour could be maintained in tissue culture, although he found that it failed on the first subculture. It was found that tumour cells from 1^{o} cultures would transmit the tumour back into the host; this was also reported by Fraser (1975). Secondary cultures, however, did not transmit the tumour into VM mice. Intracerebral inoculations of three week old cultures of VM tumour at 200 cells/0.02µl/mouse produced tumours in 5/19 mice, after an average of 94 days, although one tumour developed as early as 75 days. This shows a significant delay and reduction in tumour incidence. A similar dose of standard inoculum produces severe tumours after approximately 44 days

in 75% of mice. Some 1^{o} cultures were maintained for only a week, and groups of mice were injected i.c. with 2.10^{5} cells from cultures harvested on each day. Although the groups of mice were small, there was 100% tumour incidence in those groups injected on days 0-4. It is interesting to note that the latency became progressively longer, being 34 days on day 4, as compared with 25 days on day 0. This culture unfortunately failed on day 5, but as tumour incidence had been 100% up to day 4, it is unlikely that this explains the gradual extension of the latency period. It is possible, that had this culture survived for three weeks, the latency period would have been comparable with that of the previous three week 1^{o} culture. A possible explanation is that the tissue culture environment favours growth of normal VM cells, and that with time the proportion of tumour cells diminishes. This might also explain the failure of 2^{o} cultures to transmit tumours. An alternative explanation for the failure of subcultures is that the incubation period had become so long that it fell outside the 6 month observation period. It has since been reported that 5 cell lines have been successfully established in tissue culture,

and that these cell lines retain their tumourgenicity after several subcultures and give rise to tumours *in vivo* not only intracerebrally but subcutaneously and intraperitoneally (Serano et al 1980). Successful growth of the VM tumour in tissue culture will be most useful for *in vitro* assays of antitumour and antiviral drugs and for immunological studies.

The drug Ribavirin was synthesised by Witowski et al (1972) and its broad spectrum antiviral activity *in vivo* and *in vitro* was described (Sidwell et al 1972, 1973 and 1975). The effects of Ribavirin on RNA and DNA viruses was also reported (Huffman et al 1973). Ribavirin has also been shown to be effective *in vitro* against Erlich ascites tumour cells (Smith et al 1974), and Friend leukaemia virus (Sidwell et al 1975); from these studies it was suggested that Ribavirin has direct antitumour properties. The effects of Ribavirin were therefore examined on 1^{o} VM tumour cultures. The Ribavirin treated cultures, when inoculated i.c. into VM mice, produced tumours with the same latency period as untreated control cultures, i.e. about 30 days. Tumour incidence was 100% in both cases. Ribavirin may have failed to

inhibit or delay VM tumour growth at the concentration i.e. 100µg/ml which was used. The VM tumour cultures should be treated with Ribavirin at a range of concentrations, before the efficacy of the drug can be dismissed. Another explanation can be offered in that Ribavirin affects the early stages of the virus cycle, by inhibition of synthesis of viral antigen and infectious virus when added within 4 hours of infection of cell cultures with influenza virus (Durr & Lindh 1975). Sidwell et al (1975) concluded that tissue culture studies had shown that Ribavirin was not virucidal at the cellular level, neither did it prevent penetration or absorption of virus to the cell. Ribavirin has been shown to inhibit intracellular synthesis of viral nucleic acids and their incorporation into the viral genome; it does this by inhibiting inosinate dehydrogenase which is required by synthesis of guanine ribonucleosides (Streeter et al 1973). Ribavirin was added 24 hours after placing the VM tumour cells in culture. These cells would already be infected with the putative virus, and therefore would not be affected by Ribavirin. It might be informative to infect normal VM brain cell cultures with viable tumour cells or cell fragments and to test the resulting cultures for

oncogenicity in the VM mouse. Newly infected cultures could then be treated with Ribavirin at various concentrations before reinoculation into VM mice.

The specific angiotensin II antagonist P113 was shown to suppress cell growth of mouse fibroblasts and Simian SV 40 virus transformed fibroblasts *in vitro* (Schelling et al 1975). This drug was therefore examined, in a preliminary experiment, on the VM tumour in tissue culture. P 113 appeared to delay the onset of tumour where it had been added in low doses to the tumour cultures prior to i.c. injection into VM mice. Cultured cells treated with 0.07μg/ml P113 produced clinical signs of severe tumour in recipient mice on average 66 days after i.c. injection. There was considerable spread in the results and one mouse showed signs of severe tumour as early as 43 days, and one as late as 134 days. The control mice injected with untreated cultured cells succumbed to the tumour within about 42 days. It is possible that the P113 suppressed cell growth *in vitro* and thus slowed down the proliferation of the inoculated tumour cells *in vivo*. The delay in onset of tumour may reflect the time taken for

cells affected by P113 to recover. This experiment should be extensively repeated to determine the most effective dosage of P113 to add to VM tumour cultures. The behaviour of both VM normal brain cell cultures and tumour cell cultures in the presence of the drug should also be determined.

A selection of drugs were tested in vivo for any effects on the course of the VM tumour. The use of steroids, i.e. oestradiol and testosterone was mainly without effect. In two of the trials with oestradiol, however, the tumour latency was significantly extended ($P = 0.002$) by up to a week in female mice only. This result was not consistently repeatable, a possible explanation is that solution of oestradiol tablets in drinking water did not give a constant dosage. The effect of oestrogen on the VM tumour should be further examined in view of the results obtained by giving oestrogen to rats with a transplantable glioma. Methyl- Prednisolone was found to increase survival, reduce cerebral oedema and dramatically decrease the weight of tumours in rats with intracerebral gliomas (Gurcay et al, 1970 and 1971).

Use of the immunostimulant drug Levamisole did not delay or inhibit the onset of tumour development. In fact mice treated with Levamisole succumbed to the tumour two days earlier than the controls, which is

significant in a latency period of only 20 days. The dose (30mg/kg/day) given was the equivalent of that given to humans and may have a slight toxicity for mice. Levamisole may have been ineffective if the immune processes of the VM are not affected by the astrocytoma. This seems unlikely in view of the documented decrease in immunological competence, as shown by diminished responsiveness of T cells to mitogens in human glioma patients (Thomas et al, 1975), and in Fischer rats with RSV induced astrocytomas (Roszman et al 1978). Another possibility is that those immune responses involved with the tumour are not affected by Levamisole. It is also possible that such immune processes are involved entirely within the brain; it may be significant that only those mice which were immunised i.c., survived challenge with tumour. Levamisole can potentiate an existing immune response; and as this tumour has arisen in genetically identical inbred mice, it is possible that there is no rejection of the isologous tumour. It would be interesting to see whether Levamisole has any effect on the VM tumour in heterologous systems.

Dexamethasone, when administered from the day

of i.c. injection with tumour was able to delay the onset of clinical signs of tumour by four days. When given from day 12 after tumour inoculation the drug had no beneficial effect. Dexamethasone is used to control oedema in human tumours and has been shown to increase survival of mice with ependymoblastoma, by inhibition of tumour growth and reducing oedema (Shapiro & Posner, 1974). The same mechanisms could account for the delay in latency period in the VM tumour. That the drug did not affect the onset of tumour when given after day 12, is possibly due to the growth of the tumour being too far advanced at that stage.

Ribavirin has been shown to be effective in vivo against various virus infections, including herpes induced encephalitis in hamsters (Sidwell et al 1973), spontaneous leukaemia in AKR/J mice (Randawa et al 1974) and to have antitumour activity against Friend leukaemia virus (Sidwell et al 1975). In the light of this work, Ribarvirin was given i.p. to VM mice injected i.c. with tumour. There was, however, no effect on tumour development in mice treated with Ribavirin. The dose, 75mg/kg/day, was chosen as the

safest maximum as Ribavirin was toxic to mice at higher does (Sidwell et al 1975). Other dose regimes may, however, be more effective against the VM tumour. Khare et al (1973) suggested that the efficacy of Ribavirin was dependent also on the concentration of influenza virus used to initiate infection, and that there was a maximum dose of virus above which Ribavirin was ineffective. It is possible therefore, that the standard dose of VM tumour overwhelmed any effects of the Ribavirin. The i.p. route of administering Ribavirin may not be suitable in the case of the VM tumour. Ribavirin was most effective when given i.p. against Friend leukaemia virus (Sidwell, et al 1975) but was only effective against encephalitis when given i.c. (Sidwell et al 1973). Ribavirin should be tried again in the VM mice by subcutaneous, oral, i.v. and especially i.c. routes. A simultaneous i.c. injection of VM tumour and Ribavirin might be the best way to test the drug <u>in vivo</u>.

It is interesting that Ribavirin has been shown to have an immunosuppressive effect on humoral antibody response, but not the cell mediated response,

in influenza virus in ferrets (Potter et al 1974). It is possible that such immunosuppression in VM mice could outweigh any therapeutic effects of Ribavirin against the VM tumour.

Combined immunotherapy and chemotherapy has been examined in the ASV - Fischer rat glioma model (Mahaley et al, 1977). It was found that triple therapy with BCG + i.p. injections of sarcoma cells + i.v. administered BCNU prolonged the survival time of rats with virus induced glioma. It is suggested that such treatment be applied to the VM brain tumour model.

CONCLUSIONS

The spontaneous astrocytoma in VM mice has been clinically assessed and passive transfer in the host was standardised. A clear relationship between cell density of the inoculum and tumour incidence and latency period was demonstrated. The VM tumour was maintained in tissue culture and could be transmitted back into the host. Preliminary <u>in vitro</u> drug

trials showed that low concentrations of P113 could delay the onset of the tumour in mice. In vivo treatment with Dexamethasone also delayed the development of tumour. It was possible to transmit the tumour with killed cells and cell fragments, but no tumours were induced by cell free filtrates. The VM tumour was successfully induced in hamsters, gerbils and some other mouse strains. The latter findings suggest that the VM tumour may be transmitted by a membrane associated virus.

The oncogenicity of the passaged VM tumour appears to have increased with successive passages. This is indicated by a decrease in latency period, increase in tumour incidence, and loss of a host age factor described in early passages. (Fraser, 1975). Transmission into other mouse strains and across the species barrier by later passages (> 54th passage) also supports this hypothesis.

In the light of experiments in this work and previous reports on the immunology of human gliomas and artificially induced animal tumours, the immunology of the VM astrocytoma and the possibilities

of immunotherapy and chemotherapy, *in vivo* and *in vitro*, should be extensively examined. The spontaneously arising VM mouse astrocytoma is autochthonous, consistently reproducible and has a short incubation period which makes it an ideal animal model for human glioma study.

APPENDIX

1. Tissue Culture Media

Dissecting fluid

Tanks balanced salt solution (BSS)	(Gibco Biocult Ltd)	100ml
Penicillin Streptomycin		100 units/ml
Kanamycin		100 µg/ml
Amphotericin (Fungizone ®)		2.5µg/ml

Culture Medium (Hams F12 and antibiotics)

F12 (10 x)	(Flow)		45 ml
Nonessential amino acids	(Gibco)		5 ml
Herpes buffer (M)	"		9 ml
$NaHCO_3$ 7.5%			2.5ml
Glutamine	"		5 ml
Na OH (IN)		to pH	7.2
Foetal calf serum	"	to	20%
Water			400 ml
Penicillin Streptomycin			50u/ml
Kanamycin			100µg/ml

PBS/1mMEDTA

0.9% Saline	900ml
Phosphate buffer (0.2M NaH_2PO_4 .$2H_2O$: Na_2HPO_4) pH 7.2	100ml
EDTA	1 millimole

2. Weight monitor for 26 normal VM ♀ mice

Age Days	$\bar{x}$ grams	SD
21	6.1	± 1.12
28	7.95	1. 7
31	8.9	1.24
35	10.98	1.95
40	12.59	2.64
42	14.5	1.51
47	15.8	1.23
50	16.05	0.99
55	16.25	0.8
58	16.59	1.11
63	16.97	1.33
70	17.69	1.56
77	18.83	2.73
84	20.72	2.7

3. Weight monitor of 26 normal VM ♂ mice

Age Days	$\bar{x}$ grams	SD
21	6.2	± .99
28	8.52	1.38
31	10.38	1.55
35	12.63	1.55
40	15.23	1.7
42	16.45	1.58
47	18.1	1.28
50	18.36	1.45
55	19.72	0.94
58	19.35	1.07
63	19.71	1.32
70	20.25	0.93
84	21.86	1.38

4. Weight monitor for VM passage mice injected at 21 days

Age (days)	No. & sex	$\bar{x}$ grams	S.D.
21	23 ♀	5.43 ±	0.42
26		7.34	0.81
31		10.42	1.35
35		11.52	1.68
40		10.22	1.94
43		9.81	1.02
46		8.3	0.98
21	25 ♂	6.56	1.08
26		8.56	0.9
31		10.56	1.02
35		12.2	1.36
40		11.06	1.84
43		9.56	0.94
46		9.12	0.83

5. Weight monitor of VM passage mice injected at 33 days

Age (days)	No. & sex	$\bar{x}$ grams	S.D.
35	15 ♀	9.71	± 0.92
41		12.3	1.61
45		12.94	1.72
50		12.50	2.6
54		12.25	2.86
56		10.79	2.36
35	15 ♂	10.57	1.13
41		13.21	2.32
45		14.86	3.04
47		16.92	2.59
50		15.72	3.33
54		14.94	1.92
56		12.44	2.93

6. Weight monitor of VM passage mice injected Day 28 & 40

Age (days)	No. & sex	$\bar{x}$ grams	S.D.
TIC 28 days	6 ♀		
28		9.53	± 1.34
35		12.42	0.66
42		13.3	0.93
50		11.42	1.88
52		9.66	1.08
TIC 40 days	5 ♂		
40		11	1.0
45		12.8	0.84
52		14.8	0.84
57		13.8	1.6
59		12.6	2.6
61		10.4	1.95
40	7 ♀	11	0.82
45		11.5	0.82
52		12.5	0.82
57		11.28	1.49
59		10.14	2.48
61		9.21	2.23

7. Dose Response for VM Tumour cell Suspension frozen to $-70^{\circ}C$ in 10% DMSO at 10^7 cells/ml TCF

Dose no.cells/20µl	Mean Incubation Period	S.D. Standard Deviation	Tumour Incidence
2.10^5	18.8	± 1.3	16/16
1.10^5	26.1	± 1.9	9/9
1.10^4	39.3	± 8.0	6/6
4.10^3	43	± 12.2	7/8
2.10^3	43.9	± 12.1	12/13
1.10^3	58.2	± 14.8 *	2/5
500	60	± 15.6 *	1/3

(* for the purpose of this calculation it was assumed that all survivors succumbed on day 69 (end of experiment)).

8. Transmission of VM Astrocytoma into Mongolian Gerbils

Age at i.c. injection	Tumour incidence	Latency (days)	Pathological grading of tumour
6 months	0/7	-	
6 months immuno-suppressed	0/8	-	
3 weeks	0/4	-	
10 days	0/7	-	
neonatal	1/4 *	28	+ +
neonatal	0/4	-	
neonatal	0/4	-	
neonatal	0/2	-	
neonatal	4/5	44	+
neonatal	0/4	-	
neonatal	0/3	-	

* 3 siblings were found dead and cannibalism made histological examination impossible.

9. Transmission of VM Astrocytoma into neonatal Syrian Hamsters

Tumour incidence	Latancy (days)	Pathological grade of tumour
0/6	-	
0/5	-	
0/4	-	
0/3	-	
1/3	27	+ +
0/2	-	
0/4	-	
1/2	54	+ + +
1/2	32	+ +
2/2	19	+
	30	+ +
0/2	-	
0/1	-	
0/5	-	

10 Dexamethasone - weight monitor

Days post i.c. injection	No. of mice	$\bar{x}$ grams	S.D.
0	6 controls	12.08	± 1.93
5		12.0	± 1.3
10		16.25	± 1.54
14		16.0	± 2.56
19		15.12	± 1.88
21		13.0	± 1.41
0	10 Dexamethasone	11.14	± 1.9
5		10.46	1.36
10		13.33	1.29
14		15.24	1.33
19		13.37	2.13
24	5 Dexamethasone (from day 0)	12.6	1.82
28		11.0	1.41

11. Weight monitor for mice treated *in vivo* with Ribavirin

Days post i.c. injection	No. of VM mice (n)	Weight (g) ($\bar{x}$)	S.D.
0	20	8.95	± 2.09
6	20	10.38	± 2.09
12	20	12.21	± 2. 0
16	20	12.88	± 2.03
20	20	13.32	± 2.87
25	17	11.57	± 2.08
29	17	10.32	± 1.73
35	17	9.7	± 0.95
38	14	9.17	± 1.93
CONTROLS			
0	11		± 1.03
6			± 0.82
12			± 0.97
16			± 1.25
20			± 1.76
25			± 1.09
29			± 1.15
35			± 1.04

12. Weight monitor for mice treated with Levamisole

Days post i.c. injection	No. of VM mice (n)	Weight (g) ($\bar{x}$)	S.D.
0	24	13.7	± 1.13
7		13.65	± 1.34
11		14.07	± 0.95
14		14.6	± 1.41
18		15.02	± 1.73
21		13.79	± 0.7
24		11.63	± 1.04

REFERENCES

Adams, D.A., Gilbert, R.W. & Bigner, D.D. Cellular immunity in rats bearing primary brain tumours: specific inhibition of macrophage migration by soluble extracts of ASV-induced tumours. Journal of the National Cancer Institute 56 p.1119 1976.

Bigner, D.D., Odom, G.L., Mahaley, M.S. & Day, E.D. Brain tumours induced in dogs by the Schmidt-Ruppin strain of Rous Sarcoma virus. Journal of Neuropathology and Experimental Neurology 28 p.648-680 1969.

Bigner, D.D. and Pegram C.N. Virus Induced Experimental Brain tumours and putative associations with human brain tumours: A review. Advances in Neurology 15 p.57-83 1976 (editors Thomson and Green Raven Press.)

Bigner, D.D., Self, D.J., Frey, J., Ishizaki, R., Langlois, A.J., & Swenberg, J.A. Refinement of the Avian oncornavirus - induced primary rat brain tumour model for therapeutic screening. Recent Results in Cancer Research 51 p.20-34 1975.

Byfield, J.E., Zerbavel R., Fonkalsrud, E.W. Murine neuroblastoma cured *in vivo* by an antibody - dependent cellular cytoxicity reaction. Nature 264 p. 783-5 1976.

Duffel, D., Hinz, R., & Nelson, E. Neoplasms in Hamsters induced by simian virus 40 - Light and electron microscope observations.
American Journal of Pathology 45 p.59 1964.

Druckey, H., Ivanovick, S. & Preussman, R. Selektive Erzeugung bon hirntumoren bie ratten durch methylnitroso harnstoff.
Naturwissenshafter 51 p.44 1964. (Cited Swenberg Adv. Neurol. 15 p.85 1976).

Durr, F.E. & Lindh H.F. Efficacy of Ribavirin against influenza virus in tissue culture and in mice.
Annals of the New York Academy of Sciences 225 p. 366-371 1975.

Fraser, H. Astrocytomas in an inbred mouse strain.
Journal of Pathology 103 p. 266-270.

Fraser, H. A viable cell transfer of spontaneous astrocytoma: Differences in susceptibility between mouse strains.
Journal of Neuropathology and Experimental Neurology 37 p. 614 1978.

Fraser, H. & McConnell, I. Experimental Brain Tumours.
The Lancet January 4th p. 44 1975.

Fraser, H. Spontaneous astrocytomas in inbred mice: serial transmissions with intact cells.
Proceedings of the VII International Congress of Neuropathology (Budapest) 491 1975.

Gurcay, O., Wilson, C.B., & Barker M. Corticosteroid effect on transplantable rat glioma. Archives of Neurology 24 p. 266-269 1971

Gurcay, O., Wilson, C.B., & Eliason, J. The effect of methyl-Prednisolone acetate on transplantable rat glioma. Surgical Forum 21 p. 437-439 1970.

Fraser, H. & Dickinson, A.G. The sequential development of brain lesions of scrapie in three strains of mice. Journal of Comparative Pathology 78 p.301 1968.

Gerber, P. & Kirchstein, R.L. SV-40 induced ependymomas in newborn hamsters 1. virus - tumour relationships. Virology 18 p. 582-8 1962.

Greene, H.S.N. The transplantation of tumours to the brains of heterologous species. Cancer Research II p. 529 1951.

Huffman, J.H., Sidwell, R.W., Khare, G.P. Witowski, J.T., Allen, L.B. & Robins, R.K. In vitro effect of 1-B-D-Ribofuranosyl -1,2,4 - Triazole -3-Carboxamide (Virazole, ICN 1229) of Influenza Virus-Induced Infections in mice. Antimicrobian Agents and Chemotherapy 3 p.235-241 1973.

Ikuta, F & Kumanishi, T. Experimental virus induced brain tumours. Progress in Neuropathology 2 p. 253 1973 (editor Zimmerman, H.M., Grune and Stratton).

Ivanovick, S. & Druckey, H. Transplacentare Erzeugung maligner Tumoren des Nervensystems I Äthyl-nitroso harnstoff (ANH) und BD 1x-ratten Zeitschrist Krebsforch 71 p.320 1968

Joshi, S.R., Shenefelt, R.E., & Rice, J.N. Transplacental Carcinogenicity of DMBA in the rat. Proceedings of the American Association for Cancer Research 15 p.27 1974

Kjellin, K., Miller, R. & Astrom, K.E. The occurrence of brain tumours in several members of a family. Journal of Neuropathology and Experimental Neurology 19 p. 528 1960.

Khare, G.P., Sidwell, R.W., Witowski, J.T., Simon, L.N. & Robins, R.K. Suppression by 1-B-D- Ribofuranosyl -1,2,4 - Triazole -3- Carboxamide (Virazole ICN 1229) of influenza virus induced in mice. Antimicrobial Agents and Chemotherapy 3 p.517-522 1973.

Mahaley, M.S., Gentry, R.E., & Bigner, D.D. Immunobiology of primary intracranial tumours. Journal of Neurosurgery 47 p.35-43 1977.

Mancina, L.O., Yates, V.J., Jasty, V., & Anderson, J. Ependymomas in hamsters inoculated with -1 avian adenovirus (CELO) Nature 222 p. 190-191 1969.

Myers D.D., Meier, H., Heubner, R.J., Vernon, L., & Walker, J. Cell Free Transmission of Mouse Neuroblastoma. Nature 234 p.100 1971.

Philips, J.P., Sujatarond, M., Murtuza, R.L., Quindlen, E.A., Wood, W.C., Kornblith, P.L. & Dohan F.C. Cytotoxic antibodies in preoperative glioma patients, a diagnostic assay. Acta Neurochirurgica 35 p.43-52 1976.

Potter, C.W., Phair, J.P., Vodenlich, L., Fenton, R. & Jennings, R. Antiviral immunosuppressive and antitumour effects of Ribavirin.
Nature 259 p. 496-497 1976.

Prasad, K.N., Zambernard, J., Ladher, R., & Van Woert, M.H. Transmission of mouse neuroblastoma by cell free extracts.
Nature 228 p. 997 1970

Roszman, T.L., Brooks, W.H., Markesbery, W.R., & Bigner, D.D. General Immunocompetence of rats bearing Avian sarcoma virus induced intracranial tumours.
Cancer Research 38 p. 74-7 1978.

Randhawa 14th ISAAC Abstract 207 1974
(cited Sidwell et al. Proc. Soc. Exp. Biol. and Med. 148 p. 854 1975)

Scheinberg, L.C. & Taylor, J.M. Immunologic aspects of brain tumours.
Progress in Neurological Surgery 2 p.267 (editor Krayenbuhl) 1968

Schelling, P., Fischer, H., Hutchinson, J., & Ganten, D. Influence of Angiotensin II and Angiotensin II antagonist (P113) on in vitro cell growth in 3T3 and virus transformed SV40 3T3 cells.
Abracts of the 16th Spring Meeting Deutsche Pharmakologische Geselleshaft Naunym-Schmiesdeberg's Arch. Pharmacol. 287 Suppl. March 1975.

Schmidek, H.N., Neilsen, S.L., Schiller, A.L.
Morphological studies of rat brain tumours induced by N-nitrosomethylurea.
Journal of Neurosurgery 34 p.335 1971.

Seligman, A.M., Shear, M.J. & Alexander, L. Studies in carcinogenesis (experimental production of brain tumours in mice with methylcholanthrene)
American Journal of Cancer 37 p.364 1939.

Serano, R.D., Pegram, C.N., Fraser, H., Dickinson, A.G., & Bigner, D.D. Established tumorogenic cell lines from a spontaneous murine astrocytoma.
Proceedings of VIII International Congress of Neuropathology. Abst. 325 p.689 1978.

Serano, R.D., Pegram, C.N. & Bigner, D.D. Tumorogenic cell lines from a spontaneous VM/DK murine astrocytoma (SMA)
Acta Neuropathologica 51 p.53-64 1980.

Shapiro, W.R. & Posner, J.B. Corticosteroid hormones: Effect on experimental brain tumours.
Archives of Neurology 30 p.217 1974.

Shein, H.M. Neoplastic transformation of hamster astrocytes
Science 26 p.60 1968

Shein, H.M. Neoplastic transformation of hamster astrocytes and choroid plexus cells in culture by polyoma virus.
Journal of Neuropathology and Experimental Neurology 29 p. 70 1970.

Sidwell, R.W., Khare, G.P., Allen, L.B., Huffman, J.H., Witowski, J.T., Simon, L.N., & Robins, R.K. In vitro and in vivo effect of 1-B-D Ribofuranosyl -1,2,4 - Triazole -3- carboxaminde (Ribavirin) on types 1 and 3 parainfluenza virus infections Chemotherapy 21 p. 205-220 1975.

Sidwell, R.W., Allen, L.B., Khare, G.P., Huffman, J.H., Witowski, J.T., Simon, L.N., & Robins, R.K. Effect of 1-B-D Ribofuranosyl -1,2,4 Triazole- -3- carboxamide (Virazole ICN 1229) on Herpes and Vaccinia keratitis and encephalitis in Laboratory Animals. Antimicrobial Agents and Chemotherapy 3 p. 242-246 1973.

Sidwell, R.W., Huffman, J.H., Khare, G.P., Allen, L.B., Witowski, J.T., & Robins, R.K. Broad spectrum antiviral activity of virazole 1-B-D - Ribofuranosyl -1,2,4 Triazole -3- corboxaminde. Sciences 177 p. 705-6 1972.

Sidwell, R.W., Allen, L.B., Huffman, J.H., Witowski, J.T., & Simon, L.N. Effect of 1-B-D Ribofuranosyl -1,2,4 Triazole -3- carboxamide (Ribavirin) on Friend Leukaemia virus in mice. Proceedings of the Society for Experimental Biology and Medicine 148 p. 854-858 1975.

Smith, C.M., Fontanelle, L.J., Muzik, H., Paterson, A.R.P., Unger, H., Brox, L.W. & Henderson, J.F. Inhibitors of Inosinate dehydrogenase activity in Erlich ascites tumour cells in vitro. Biochemical Pharmacology 23 p. 2727-2735 1974.

Streeter, D.G., Witowski, J.T., Khare, G.P., Sidwell, R.W., Bauer, R.J., Robins, R.K. & Simon, L.N. Mechanism of 1-B-D Ribofuranosyl -1,2,4 Triazole -3- carboxamide (Virazole), a new broad spectrum antiviral agent. Proceedings of the National Academy of Sciences of U.S.A. 70 p. 1174-78 1973. (cited Oxford J.S. J. General Virology 28 p. 409 1975)

Strooband, G. & Brucher, J.M. Etude des tumeurs nervenses obtenues par l'administration de methylnitrosuree au rat. Neurochirurgerie Paris 14 p. 515 1968

Swenberg, J.A. Chemical Induction of brain tumours. Advances in Neurology 15 p. 85-99 1976

Swenberg, J.A., Koestner, A., & Wechsler, W. The induction of tumours of the nervous system with intravenous methylnitrosourea. Laboratory Investigations 26 p. 74 1972.

Thomas, D.G.T., Lannigan, C.B. & Behan, P.O. Impaired cell-mediated immunity in human brain tumours. Lancet 21 p.1389 1975.

Walker, D.L., Padgett, B.L., & Zurhein, G.M. Human papovirus (J.C.) : Induction of brain tumour in hamsters. Sciences 181 p. 674-6 1973.

Wilfgong, R.F., Bigner, D.D., Self, D.J., & Wechsler, W. Brain tumour types induced by Schmidt-Ruppin strain of rous sarcoma virus in inbred Fischer rats.
Acta Neuropathologica (Berlin) 25 p.196-206 1973.

Witowski, J.T., Robins, R.K., Sidwell, R.W. & Simon L.N. The design, synthesis and broad spectrum activity of 1-B-D Ribofuranosyl - 1,2,4 - triazole -3- carboxamide and related nucleosides
Journal of Medicinal Chemistry 15 p.1150-54 1972.

Zimmerman H.M. & Arnold, M. Experimental brain tumour (tumours produced with methylcholanthrene)
Cancer Research 1 p.919 1941.

Zimmerman, H.M. The nature of glioma as revealed by experimentation.
American Journal of Pathology 31 p.1 1955.

BIBLIOGRAPHY

Bigner, D.D. & Swenberg, J.A. (editors)
Experimental brain tumours induced with viruses
in Experimental Tumours of the Nervous System 1975
(Upjohn Press, Kalamazoo.)

Fraser, H. Spontaneous astrocytoma in mice and its
transmission with viable cells.
chapter 31 p. 393 - 404
in Animal Models of Human Neurological Diseases
Editors Rose, F.C. and Behan, P.O. Pitman Press
London (in press).

Russel, S.R. and Rubinstein, L.J. Pathology of
Tumours of the Nervous System.
(4th Edition, Arnold, 1977)