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A Phase 1 trial of the herpes simplex virus HSV1716 in patients with high grade glioma plus an *in vitro* investigation of the interaction between HSV1716 and ionising radiation

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Submitted for Degree of PhD

Submitted to The University of Glasgow

Research conducted in the Division of Cancer Sciences and Molecular Pathology

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I thank my clinical colleagues Professor R Rampling and Mr Papanastassiou for their advice and education in a clinical field in which I had little experience of prior to embarking the project. I also wish to thank Sister Mary Fraser for her advice, knowledge and help in coordinating the clinical trial.

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I wish to acknowledge and note my admiration for the twelve patients who were brave enough to enter into the clinical trial. I hope that the information gained from this body or work will soon result in a new clinical tool that will help patients with this awful disease.

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Gene Therapy (2004) 11, 1648-1658

S Harrow, V Papanastassiou, J Harland, R Mabbs, R Petty, M Fraser, D Hadley, J Patterson, S M Brown, and R Rampling.

Poster Presentation

An in vitro demonstration of synergistic glioma cell kill by the selectively replication competent ICP34.5-null HSV mutant, HSV1716, and ionising radiation.

Sixth meeting of the European Association for NeuroOncology

May 2005

S Harrow, J Harland, A Mace, R Rampling, J Plumb, SM Brown.

Oral Presentation

HSV1716 injection into brain adjacent to tumour following surgical resection of high-grade glioma: safety data and long term survival.

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List of Abbreviations

AA	Anaplastic Astrocytoma
ALL	Acute Lymphocytic Leukaemia
ALT	Alanine Transaminase
APTT	Activated Partial Thromboplastin Time
AST	Aspartate Transaminase
AO	Anaplastic Oligodendroglioma
BBB	Blood Brain Barrier
BCNU	Carmustine
BHK	Baby Hamster Kidney Cells
BS	Barthel Score
CCNU	Lomustine
cdks	Cyclin Dependent Kinases
CNS	Central Nervous System
CSF	Cerebrospinal fluid.
CT	Computed Tomography
CD/5-FC	Cytosine Deaminase/5-Fluorocytosine
DCC	Deleted in Colorectal Cancer
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
EBV	Epstein Barr Virus
ECL	Electrogenerated Chemiluminescence
EGFR	Epidermal Growth Factor Receptor
eIF	Eukaryotic Initiation Factor
ELISA	Enzyme Linked Immunosorbent Assay
FDG	^{18}F-Fluorodeoxyglucose
5-FU	5- Flurouracil
GADD34	Growth Arrest and DNA Damage
GB	Glioblastoma
GCV	Ganciclovir
Gd	Gadolinium
GM-CSF	Granulocyte-macrophage Colony-simulating Factor
GTAC	Gene Therapy Advisory Committee
Gy	Gray
Hb	Haemoglobin
HCMV	Human Cytomegalovirus
HMPAO ^{99m} Tc	Hexamethylpropyleneamine - Technietium 99
HSV	Herpes Simplex Virus
HSV*tk*	Herpes Simplex Virus type-1 Thymidine Kinase
HGG	High Grade Glioma
ICP	Infected Cell Protein
IE	Immediate Early
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INR	International Normalised Ratio
kb	Kilobase
KD	Kilo-daltons
KeV	Kilo-electron Volts
KS	Karnofsky Status

LOH	Loss of Heterozygosity
L	Long
Lb	L-Broth
LAT	Latency Associated Transcripts
M.A.O.I	Monoamine Oxidase Inhibitor
MDM2	Mouse double minute 2
MGMT	O^6-methylguanine-DNA methyltransferase
MMC	Mitomycin C
MOI	Multiplicity of Infection
MRI	Magnetic Resonance Imaging
mRR	Mamalian Ribonucleotide Reductase
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay
MyD116	Myeloid Differentiation protein
MYC	Proto-oncogene
NDV	Newcastle Disease Virus
NICE	National Institute for Clinical Excellence
OD	Optical Density
OS	Overall Survival
OTCD	Ornithine Transcarbamylase Deficiency
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
P.C.V.	Procarbazine, Cisplatinum, Vincristine
PDGF/R	Platelet derived growth factor/receptor
PET	Positron Emission Tomography
p.f.u	Plaque Forming Units
PNS	Peripheral Nervous System
PTEN	Phosphate and Tensin Homology
RB	Retinoblastoma
ROI	Region of Interest
r.p.m	Revolutions per Minute
RR	Ribonucleotide Reductase
RNA	Ribonucleic Acid
S	Short
SSB	Single Strand Break
SCID	Severe Combined Immune Deficiency
tk	Thymidine Kinase
^{201}Tl	Thallium-201
TR_L	Terminal Repeat Long
TR_S	Terminal Repeat Short
TTP	Time to Tumour Progression
SPECT	Single Photon Emission Computed Tomography
U_L	Unique Long
U_S	Unique Short
VEGF	Vascular endothelial growth factor
VE	Virotech Enzyme Units
VSV	Vesicular Stomatitis Virus
v/v	volume/volume (ratio)
WBC	White Blood Count
WHO	World Health Organisation

w/v	weight/volume (ratio)
w/w	weight/weight (ratio)

Summary

HSV1716 is a mutant herpes simplex virus with a deletion in both copies of the RL1 gene, that encodes the protein ICP34.5, a specific determinant of virulence.

Section one presents a phase I study in which it has been demonstrated that herpes simplex virus 1716 (HSV1716) does not generate toxicity following injection into brain adjacent to excised high-grade glioma, in patients who proceeded to receive further immunosuppressive radiotherapy or chemotherapy. The survival and imaging data, in addition to the lack of toxicity, were encouraging.

Section two investigates the possibility of enhanced cell kill when ionising radiation is employed in conjunction with the ICP34.5 null mutant HSV1716 in a tissue culture model and the possible mechanisms responsible for enhanced cell kill. Using the MTS assay, experiments combining HSV1716 and ionising radiation demonstrated additional cell kill in 373 and MOG cells by day six compared to either modality in isolation. Isobologram analysis confirmed a synergistic relationship between ionising radiation and HSV1716 in 373 cells and an additive relationship in the MOG cell line when the cells were irradiated one hour prior to inoculation.

Lastly, stable 3T6 cells expressing ICP34.5 were developed with the aim of elucidating the mechanism of action of the ICP34.5 in the replication cycle of HSV. Functional ICP34.5 and ICP34.5-GFP in the transfected 3T6 cells was detected and able to support the replication of HSV1716. These cells were

analysed with respect to HSV1716 infection and were seen to support replication with the appearance of characteristic plaque morphology.

Declaration

I declare that the work contained within this thesis is my own work. The surgery was undertaken by Mr Papanastassiou and the imaging was reviewed by Professor Hadley. The analysis of blood samples from patients enrolled in the phase I study was assisted by Mr Mabbs. This work has not been previously submitted for a higher degree.

Chapter 1

Introduction

1 Cancer in the United Kingdom

Each year 200,000 people are diagnosed with cancer in England and Wales. This equates to one in three people developing cancer during their lifetime. One in four people still die from cancer. The survival rates for patients with cancer in the United Kingdom, and in particular Scotland, fall below the European average for many types of cancer. The government of today has made the development of cancer services a priority (The NHS Cancer Plan).

2 Cancer of the Nervous System

Tumours of the nervous system can arise in the peripheral nervous system (PNS) or the central nervous system (CNS). Tumours can either be benign or malignant and if malignant, they may be primary disease or a manifestation of a metastatic process. Clinically, tumours of the nervous system manifest in different ways. The treatments available and response, which can influence the prognosis and overall survival, are determined by the histological classification of the tumour. Consequently, accurate pathological diagnosis is crucial, prior to commencing management.

The sub-speciality of Neuro- Oncology has developed over the last decade as advances have been made in the accurate diagnosis and treatment of CNS tumours. The management of patients with primary malignant tumours of the nervous system still provides a huge challenge for the medical and nursing professions.

2.1 Classification of Tumours of the Nervous System

In 1993 the World Health Organisation (WHO) developed a classification system of brain tumours that is now used internationally. This classifies brain tumours on the basis of the cell of origin (Kleihues P *et al.*, 1993). The WHO classification system has now superseded other classification systems such as Kernohan and Ringertz (Kernohan JW *et al.*, 1949; Ringertz N, 1950). In addition to the WHO classification system the St Anne/Mayo grading system, based on four histological criteria, has been used in astrocytomas, and has been shown to be predictive of survival (Daumas-Duport C *et al.*, 1988; Kim T *et al.*, 1991).

2.2 Prevalence, Incidence and Epidemiology of Brain Tumours

Although primary high grade glioma (HGG) is a relatively rare malignant disease compared to cancer of the lung, breast and bowel, it is still a significant cause of morbidity and mortality in the Western world.

There is an increasing incidence of primary malignant brain tumours, most marked in the elderly population (Inskip PD *et al.*, 1995). The treatment of primary malignant brain tumours has however not advanced significantly over the past three decades. It is important therefore to try and elucidate the causation, distribution and disease pathways that control these tumours.

The difference in health care practices in different countries makes the interpretation of primary HGG data difficult. Incidence rates appear to be higher in developed countries compared to developing countries. This however, may represent a discrepancy in the accurate diagnosis and reporting in developing countries, rather than any real difference. When compared to the incidence and prevalence rates of other tumour types between countries, the variation of incidence and prevalence in primary HGG between nations is small (Inskip PD *et al.*, 1995; Sant M *et al.*, 1998).

The most recent information available on the incidence of brain tumours is available from the GLOBOCAN 2000 database. The prevalence amongst the fifteen member states of the European Community in 2000 was 29,133. The incidence per 10,000 head of population was calculated for each of the fifteen member states using population figures from the United Nations Population Division (total population = 375,276,000). The incidence of all CNS tumours per 10,000 head of population was calculated to be 0.78 (Globocan data base). Currently there is no specific data indicating the prevalence or incidence rates of primary malignant glioma within the UK or Europe.

Epidemiological studies have only identified a few potential risk factors for the development of primary HGG (Surawicz T *et al.*, 1999). It appears that the development of brain tumours may be linked with the inheritance of certain genes. One such disorder Li-Fraumeni syndrome, is a autosomal dominant, familial condition, associated with a high incidence of germline p53 mutations. The disorder is characterised by the early development of HGGs as well as sarcomas, breast carcinomas, leukaemias and adrenocortical carcinomas (Inskip PD *et al.*, 1995).

Exposure to ionising radiation has been demonstrated as a cause of primary HGG, however few people are exposed to large enough doses to lead to an appreciable rise in risk. No link has yet been made with doses of radiation used in diagnostic imaging. Concern has been raised regarding non-ionising electromagnetic fields such as power lines and electrical appliances however this has not been confirmed. In recent years the use of mobile phones has risen markedly and concern has been expressed as to this being a possible risk factor. Recent studies have failed to demonstrate a causal relationship between the development of brain tumours and use of mobile phones, however research continues in this area (Inskip PD *et al.*, 2001; Muscat J *et al.*, 2000).

No link has been made between HGG in humans and exposure to chemicals or infectious agents in the environment or in food. In addition, research into occupational exposure to chemicals has also failed to highlight any potential carcinogen (Wrensch M *et al.*, 1993, Wrensch M *et al.*, 2002).

Due to the aggressive nature of HGG and its effect on cognitive function and memory, the gathering of accurate information for epidemiological studies through recall is a problem. In view of this it is necessary, where possible, that information be obtained from patients as soon after diagnosis when they are potentially in optimal health.

Modification of the risk factors for HGG is not possible since they have yet to be fully identified. Consequently efforts are being channelled into developing new diagnostic and management strategies for this devastating and universally fatal disease.

2.3 Malignant Tumours of the Central Nervous System

Primary malignant brain tumours can arise from any of the cell types making up the CNS. Discussion of all primary brain tumours is beyond the scope of this introduction. Importance is therefore given to the most malignant sub-types of neuro-epithelial tumours, in particular those from the oligodendroglial and astrocytic cell lineage.

Tumours of Neuro-epithelial origin	
Oligodendroglial tumours	Oligodendroglioma
	Anaplastic Oligodendroglioma (AO)
Astrocytic tumour	Diffusely infiltrating astrocytoma
	Anaplastic Astrocytoma (AA)
	Glioblastoma (GB)

Table 2.3; *Tumours of Neuro-epithelial origin*

2.3.1 Oligodendroglial Tumours

Oligodendrogliomas, WHO grade II and III, are relatively uncommon tumours accounting for approximately 5-7% of all intracranial gliomas. In the United

States the annual incidence of all oligodendrogliomas is 0.3 per 100,000 population. Figures for the exact incidence and prevalence of anaplastic oligodendrogliomas are difficult to acquire as many studies fail to differentiate between the less malignant WHO grade II oligodendroglioma and the grade III anaplastic oligodendroglioma. It has been noted by a number of investigators that the histological classification of oligodendroglial tumours has a bearing on the overall survival. It appears that in a number of cases grade II oligodendroglial tumours are wrongly classified, and are in fact the more malignant grade III anaplastic astrocytomas. The management of an anaplastic astrocytoma is different from that of a less aggressive oligodendreglioma and is associated with a worse prognosis (Nijjar T *et al.*, 1993; Celli P *et al.*, 1994).

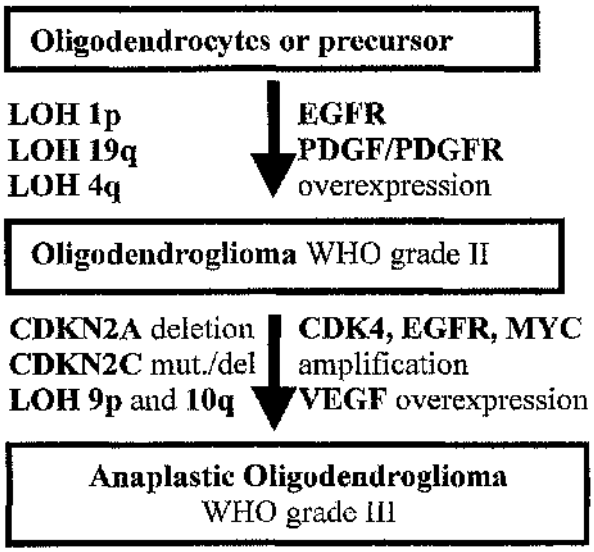
2.3.1.1 Anaplastic Oligodendroglioma

This type of malignant tumour has a slight male predominance, with patients being slightly older (48.7 years) compared to the age of patients diagnosed with WHO grade II oligodendrogliomas (Ludwig C *et al.*, 1986; Kros J *et al.*, 1994).

As with the other types of primary HGG there are no specific symptoms associated with this specific type of HGG. The site of the tumour often determines the symptoms experienced by the patient. The clinical history is often short, although a longer clinical history may be suggestive of an initial low-grade intracranial lesion, that has transformed into an anaplastic oligodendroglioma.

Anaplastic oligodendrogliomas are predominantly located in the frontal lobes, or less commonly in the temporal lobes, although they can appear in any part of the CNS. Macroscopically anaplastic oligodendrogliomas appear as well defined, soft greyish-pink masses. Calcification is frequently present in the periphery of the tumour and in the adjacent cortex. Histopathologically these tumours comprise oligodendroglial cells with focal or diffuse features of increased cellularity, marked cytological atypia, and high mitotic activity, indicative of a more malignant phenotype. Micro vascular proliferation and necrosis may be present (Reifenberger G *et al.*, 2000).

Malignant progression from the less malignant grade II oligodendroglioma is thought to be associated with the acquisition of an increasing number of genetic abnormalities. The genetic development of these tumours in highlighted in the figure below.



CDKN: cyclin D kinase
EGFR: epidermal growth factor receptor
VEGF: vascular endothelial growth factor
PDGF/R: platelet derived growth factor/receptor
LOH: loss of heterozygosity
MYC: proto-oncogene
(Reifenberger G *et al.*, 2000)

***Figure2.3.1.1**; Genetic development of Anaplastic Oligodendroglioma*

Deletions on chromosomes 1p and 19q in anaplastic oligodendrogliomas can be a positive predictor of response to combination chemotherapy, and is associated with prolonged survival post chemotherapy (Cairncross JG *et al.*, 1998). A median survival time from the diagnosis of anaplastic oligodendroglioma of 3.9 years has been reported, with 5 and 10 year survival rates of 41% and 20% respectively (Shaw E *et al.*, 1992).

2.3.2 Diffusely Infiltrating Astrocytoma

This is the most frequently occurring intracranial neoplasm accounting for approximately 60% of all primary brain tumours. The incidence is approximately 5-7 new cases per 100,000 population per year (Cavenee W K *et al.*, 2000).

This tumour is most common in adults and most frequently is located in the cerebral hemispheres although can occur in any site within the CNS. Irrespective of histological grade, astrocytomas diffusely infiltrate the surrounding brain. They have a wide range of histopathological features and biological behaviour. Diffusely infiltrating astrocytomas have an inherent tendency for malignant transformation, with glioblastoma being the most malignant endpoint phenotype.

The most malignant phenotypes within this group are the Anaplastic Astrocytoma and Glioblastoma.

WHO		**St.Anne /Mayo Classification**	
Grade	*Designation*	*Designation*	*Histological criteria*
III	Anaplastic Astrocytoma	Astrocytoma Grade 3	Two criteria; nuclear atypia and mitotic activity
IV	Glioblastoma	Astrocytoma Grade 4	Three criteria; nuclear atypia, mitoses, endothelial proliferation and/or necrosis

***Table 2.3.2**; Classification of Anaplastic Astrocytoma and Glioblastoma*

2.3.2.1 Anaplastic Astrocytoma

Anaplastic Astrocytoma can arise from a low-grade astrocytoma, although in some instances there is no prior indication of a less malignant precursor. The average age of diagnosis is approximately 41 years with males more frequently

affected than females. Anaplastic astrocytomas have the potential to progress to the more malignant and aggressive glioblastoma (Kleihues P *et al.*, 1999).

Clinical suspicion is often raised by non-specific symptoms of neurological deficit, seizures and symptoms of raised intracranial pressure. The symptoms the patient experiences as a result of the intracranial lesion largely depend on the site of the tumour within the CNS. The presence on imaging of an ill-defined low-density mass, located in the cerebral hemispheres supports the clinical suspicion of an AA.

It is not possible to confirm an anaplastic astrocytoma on macroscopic analysis alone. The histopathological features are similar, but more marked, than those of a low-grade astrocytoma. They consist of neoplastic astrocytes with hypercellularity, distinct nuclear atypia and increased mitotic activity. Histological analysis must not reveal any necrosis as this would classify the tumour as a grade IV, glioblastoma.

Progression to glioblastoma is a key prognostic factor. The interval between diagnosis and progression has a mean length of 2 years with a mean overall survival from diagnosis of 3 years (Donahue B *et al.*, 1997). Prognosis is improved in younger age groups with a good preoperative clinical condition and in those who undergo gross total tumour resection (Kleihues P *et al.*, 1999).

2.3.2.2 Glioblastoma

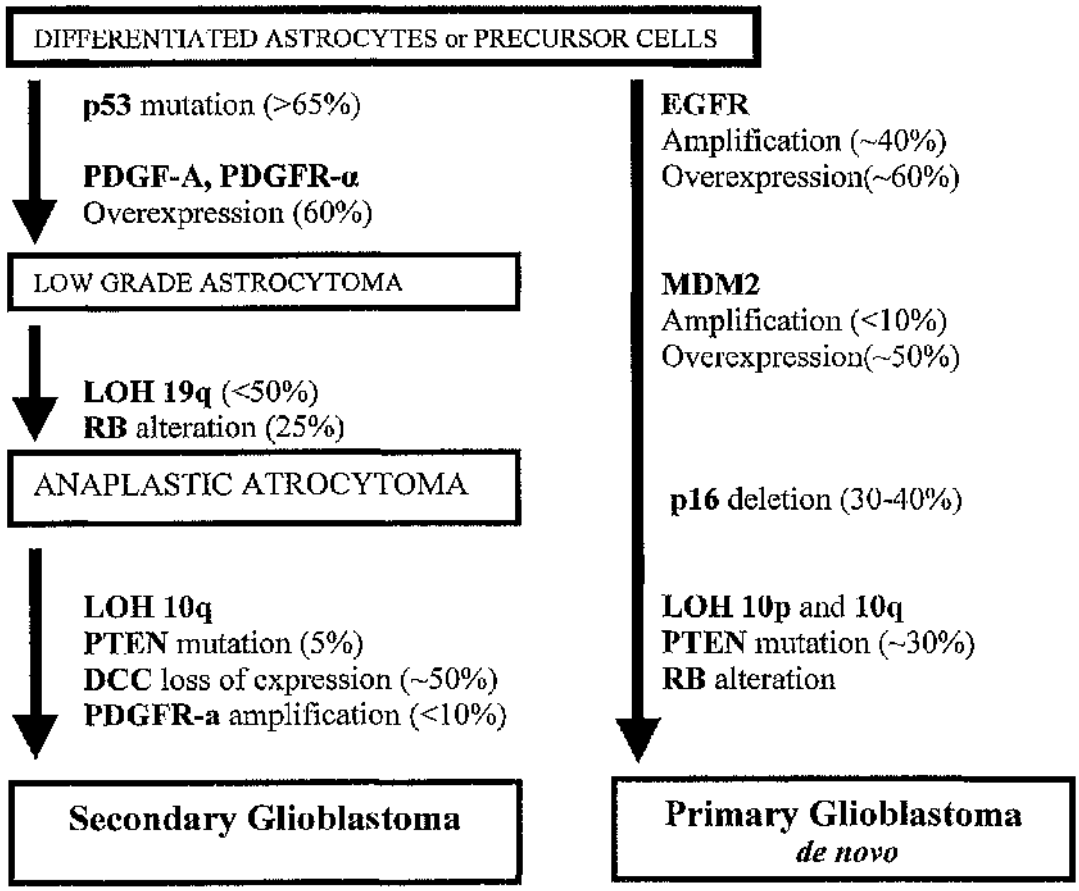
Glioblastomas account for approximately 15% of all intracranial neoplasms, and comprise 50-60% of astrocytic tumours. It is the most prevalent primary malignant brain tumour and has the most malignant phenotype. (Kleihues P *et al.*, 1999).

Though glioblastoma may occur at any age it is most prevalent in adults, with a peak incidence between 45 and 70 years. As with AA there is a higher incidence in males than females.

Glioblastomas occur most commonly in the cerebral hemispheres and are most often found to be unilateral. The most frequently affected site is the temporal lobe followed by the parietal lobe then the frontal lobe and lastly the occipital lobe (Kleihues P *et al.*, 1999). Glioblastomas in other sites within the CNS are rare. Multi-focal glioblastomas have been reported, although it has been noted that in some cases there were interconnecting strands between what were considered discrete lesions.

Glioblastomas can be sub-divided into two groups, primary and secondary on the basis of clinical and genetic differences. There is increasing evidence that these two groups constitute distinct disease entities arising through different genetic pathways that affect patients at different ages (Kleihues P *et al* .,1999; Watanabe K *et al.*, 1996).

The diagram below indicates the known genetic alterations in the development of primary and secondary glioblastoma.



PDGF-A: platelet derived growth factor
EGFR: epidermal growth factor receptor
PDGFR: platelet derived growth factor receptor
PTEN: phosphatase & tensin homolog
RB: retinoblastoma
DCC: deleted in colorectal cancer gene
LOH: loss of heterozygosity
MDM: Mouse double minute 2 (human homolog of p53-binding protein)
(Kleihues P *et al*, 1999)

***Figure 2.3.2.2**; Genetic development of primary and secondary Glioblastoma*

Primary glioblastomas account for the majority and occur in older age groups. They always, by definition manifest themselves *de novo*, usually following a short clinical history without evidence of a pre-existing, less malignant precursor. Secondary glioblastomas arise from a low-grade astrocytic lesion or from the less malignant anaplastic astrocytoma. These tumours are prevalent in a younger age group, approximately 45 years of age. The time for progression from a low grade lesion to a glioblastoma is approximately 4-5 years (Vertosick F *et al*., 1991; McCormack B *et al*., 1992). It remains unclear as to whether there is a survival difference between primary *de novo* glioblastoma and secondary glioblastoma (Winger M *et al*., 1989; Dropcho E and Soong S, 1996).

In most cases a glioblastoma is depicted on MRI or CT as a peripheral, contrast enhancing, ring lesion. The outer enhancing margin of the glioblastoma does not represent the outer border of the lesion and tumour cells can be identified 2 or more centimetres beyond the perceived edge of the tumour (Burger P *et al*., 1988; Nagashima G *et al*., 1999).

Macroscopically most glioblastomas are intraparenchymal with the epicenter in the white matter. They often have marked haemorrhage on gross dissection. Despite the short duration of symptoms the tumour can be extensive often occupying much of the cerebral lobe. Central necrosis may occupy as much as 80% of the tumour mass. These tumours are notoriously highly invasive and extend into surrounding brain. Metastases are rarely apparent elsewhere in the CNS or systemically as the tumour cells seem unable to invade the subarachnoid spaces or a vessel lumen. Histopathologically the tumour is composed of highly

cellular, anaplastic glial cells with marked nuclear atypia and a high mitotic rate. Microvascular proliferation and necrosis are prerequisites for the diagnosis of a glioblastoma (Kleihues P *et al.*, 1999).

Despite meta-analysis studies it is still not possible to predict patients with a favourable prognosis. Glioblastomas are among the most malignant tumours known to occur in man with a mean length of survival of less than 1 year, despite optimal treatment.

2.4 Diagnostic Imaging

To support the clinical suspicion of a lesion within the brain, imaging seeks to locate the area of abnormality. Following localisation of the lesion the clinician may be able to obtain a histological diagnosis that will influence the subsequent management. Computed Tomography (CT) was initially used to image intracranial lesions, however with the emergence of Magnetic Resonance Imaging (MRI) over the past 20 years it has proven to be more sensitive than CT in the detection and evaluation of tumour extent. MRI is now the imaging modality of choice.

One weakness of MRI is the lack of specificity. Although a tumour is correctly identified, it is not possible to interpret with accuracy the histological type and grade. The introduction of the contrast agent gadopentetate dimeglumine (gadolinium), has sought to overcome this problem and define the growing active areas of the tumour. As for many tumours the growth of HGG is associated with angiogenesis (i.e. the development of abnormal capillaries). This process is responsible for extravasation of gadolinium, through non-tight junctions, into the surrounding extracellular space identified on MRI as enhancement. Higher-grade lesions tend to allow more enhancement than lower grade lesions, though this association is not exclusive (Kondziolka D *et al.*, 1993).

The area of enhancement on MRI acts as a marker of bulk cell division and as a locus for the surgeon from which to obtain a diagnostic tissue biopsy or to resect the tumour. It is important to note that the outer rim of tumour enhancement does

not delineate the outer margin of the tumour. This has been noted particularly with glioblastomas, where biopsies have indicated tumour extension down white matter tracts, which show oedema on T2 weighted images, and were not detected by contrast enhanced MRI (Kelly PJ *et al.*, 1987).

The aim of non-invasively predicting tumour grade has led to the investigation of functional imaging. In particular Single Photon Emission Computed Tomography (SPECT) using the tracer Thallium-201has been studied. Thallium-201 decays by electron capture to Hg-201 which emits characteristic x-rays in the 60-80KeV range. In the late 1980s it was demonstrated with 89% accuracy that there was correlation between Thallium-201 uptake and tumour grade (Kim KT *et al.*, 1990). Although investigation with Thallium-201 SPECT has not replaced MRI, it is useful in discriminating between tumour grade and recurrence as opposed to necrosis or scarring. In addition Thallium-201 SPECT can differentiate between radiation necrosis and tumour recurrence. Unfortunately it cannot yet be used as the sole non-invasive diagnostic or prognostic tool (Benard F *et al.*, 2003).

Positron Emission Tomography (PET) using ^{18}F-fluorodeoxyglucose (FDG) has been shown effective in detecting brain tumours. PET-FDG imaging exploits the observation that tumour cells have an increased capacity for glucose transport compared to normal cells and therefore demonstrate increased FDG uptake. PET-FDG has been shown effective in determining the degree of malignancy and can identify malignant change in a low grade glioma. In addition it has proven effective in distinguishing tumour growth from radiation necrosis (Benard F *et al.*, 2003).

2.5 Treatment of High Grade Glioma

High grade glioma remains a devastating disease and its effective management provides the oncologist with a huge hurdle. As yet there is no treatment available that can offer the possibility of cure. Any improvement in survival obtained by surgery, radiotherapy or chemotherapy is minimal. Compared to the advances made in the management of other malignancies over the last few decades, the

management of brain tumours has not improved to the same degree. Despite on-going research into primary HGGs, the prognosis of patients with primary HGG remains dire. Clinicians must optimise the treatments that are available, and strive to investigate and develop new treatment strategies for these patients.

2.5.1 Surgery

The primary objective of surgery is to provide a histological diagnosis. More extensive surgery may provide symptomatic benefit and could contribute to a delay to tumour progression and improve the overall survival time.

A tissue sample obtained from a biopsy of the suspicious lesion will in most cases be sufficient to provide the clinician with a histological diagnosis. An intracranial biopsy can be performed either as an open procedure or via a burr hole using stereo-tactic localisation. The stereo-tactic biopsy procedure is associated with a low morbidity and mortality rate and has a diagnostic yield of 95% (Kreiger M *et al.*, 1998).

Maximal surgical resection of a HGG is the preferred surgical intervention where possible. When the suspicion of a HGG is strong, some surgeons proceed to cytoreductive surgery at the outset. The malignant nature of the lesion is confirmed from analysis of a smear preparation intra-operatively (Jackson R *et al.*, 2001) There is continual debate concerning the role of cytoreductive surgery in the management of patients with primary HGG. Cytoreductive surgery may seem a logical management plan, however there are no prospective studies that have investigated the benefit of this strategy to the patient. Retrospective analysis of patients with glioblastoma has shown that the extent of tumour resection and amount of residual tumour identified on post operative images, has a significant bearing on time to tumour progression (TTP) and overall survival (OS) (Keles G *et al.*, 1999).

The concern regarding such extensive surgery, given the modest improvement in overall survival, is the possibility of causing more harm than good. This is not supported by the literature. Ciric *et al* reported that following maximal resection

for HGG, 55% of patients demonstrated no deterioration compared to pre-operative neurological function and 41% of patients experienced neurological improvement (Ciric I *et al.*, 1987). With the development of intra-operative cortical stimulation mapping techniques and image guided computer assistance, extensive tumour resections should be more accurate with a decreased chance of compromising patient's quality of life (Keles G *et al.*, 1999; Barnett GH, 1999; Matz P *et al.*, 1999).

The benefit of repeat surgery for tumour recurrence following the failure of first line therapy remains unclear. For certain patients with symptoms associated with raised intracranial pressure repeat surgery may offer symptomatic relief. With regard to any possible associated survival advantage from repeat surgery it is believed that younger patients, with a good performance status, who have had stable disease for longer than 6 months following the initial operation will benefit most (Young B *et al.*, 1981; Salcman M *et al.*, 1982; Barker F *et al.*, 1998; Brandes AA *et al.*, 1999).

Apart from tumour cytoreduction, surgery also has a role to play in the insertion of single direction catheters. These catheters drain excess fluid that accumulates in the ventricles of the brain that can cause symptoms of increased intracranial pressure.

2.5.2 Radiotherapy

The aim of radiotherapy is to deliver a tumouricidal dose of radiation to a defined target volume whilst sparing the surrounding normal tissue, thereby achieving an optimal therapeutic ratio. Ionising radiation aims to sterilize tumour cells and remove their proliferative ability through a series of intracellular molecular events, the most important of which is double strand breaks of genomic DNA.

Post-operative conventional radiotherapy using x-rays has been proven effective in improving overall survival in prospective studies although it is not given with curative intent (Walker MD *et al.*, 1978). Patients who receive post operative radiotherapy survive longer than those treated with either supportive care or

chemotherapy alone (Leibel S *et al.*,1994). The optimum regime for patients with HGG is the delivery of 60 Gray (Gy) in individual daily fractions of between 1.8 and 2.0Gy for 5 days per week over the duration of 6 weeks (Leibel and Sheline., 1987). Less radical regimes, delivering a lower total dose of radiation can be given to patients who have a low performance status and would not be able to tolerate a 6 week regime of radiotherapy (Bauman G *et al.*, 1994).

The treatment of HGG by radiotherapy is limited due to the inherent radioresistance of this tumour tissue and the radiosensitivity of the surrounding normal brain tissue. Partial brain field irradiation is preferred over whole brain irradiation due to the risk of long-term side effects associated with irradiating normal brain tissue seen with the latter regime (Levin V *et al.*, 1995). High grade glioma recurs in 80% of cases within 2cm of the perceived outer enhancing edge on imaging (Garden AS *et al.*, 1991). In view of this observation, standard radiotherapy practice delivers radiation to a field that encompasses an additional 2-3 centimetre margin around the enhancing outer edge of the tumour visualised on the pre-surgery imaging (Leibel S *et al.*, 1991).

Conformational therapy enables the conformation of the radiation beam to the tumour alone, with the aim of sparing radiation to normal tissue outside of the intended treatment volume. This may allow the possibility of dose escalation. Results of ongoing studies using this method are awaited. Radiosensitisers and hyperfractionation regimes have been trialed but offer no benefit over conventional radiotherapy.

Stereotactic radiosurgery can be used to increase the actual dose of radiation to the tumour (Larson D *et al.*, 1990). This method of delivery ensures that a dose of radiation is delivered as a single fraction to a single intracranial target thereby limiting the amount of radiation to the adjacent normal brain. To maintain a steep dose gradient at the periphery of the tumour it must be of limited size, ideally less than 4 cm in diameter. As a result stereotactic radiosurgery has limited use as the main radiotherapy tool in the treatment of malignant glioma as the size of a malignant tumour at the time of presentation is often in excess of four centimeters (Wilson CB *et al.*, 1992). The use of a focal radiotherapy boost following surgery

and conventional radiotherapy has suggested a modest survival advantage in a subset of patients below 40 years of age (Shrieve D *et al.*, 1999). Stereotactic radiosurgery is not used routinely in the management of primary HGG, the results of further investigations are awaited.

Another approach that can deliver a higher dose of radiation to the tumour whilst sparing the surrounding brain tissue is interstitial brachytherapy. In most cases gamma ray emitting 125-Iodine and 192-Iridium sources have either been inserted into the cavity following tumour resection or inserted as temporary catheters into a tumour which is later resected (Leibel S *et al.*, 1989). Initially this method was trialed in patients with recurrent HGG who had relapsed following initial surgery and radiotherapy. The results from these studies were encouraging, although the quality of evidence is now in doubt due to apparent patient selection bias. Interstitial brachytherapy as a boost following external beam radiotherapy has been trialed in patients with *de novo* HGG in a randomised trial. A prior study had detailed encouraging pathological data indicative of improved tumour control (Siddiqi S *et al.*, 1997) however this has not translated into clinical practice in terms of statistically significant improved overall survival (Laperriere N *et al.*, 1998). The likely reason for the failure to improve overall survival is the probability that tumour cells were present out with the volume that received the high radiation dose. From these studies it appears unlikely that just increasing the radiation dose is going to be able to offer greater tumour control.

2.5.3 Chemotherapy

Chemotherapy has a limited role to play in the treatment of HGGs. It can either be given in the neo-adjuvant, adjuvant or palliative setting. Several key issues complicate the use of chemotherapy in the management of HGG. Firstly HGGs are inherently chemo-resistant, secondly the delivery of the agent to the brain is hampered by the blood brain barrier and finally the concurrent use of anticonvulsants, often required in this group of patients, is known to induce the p450 cytochrome that can result in a sub-therapeutic dose of chemotherapy reaching the tumour.

2.5.3(i) Chemoresistance in High Grade Glioma

A number of mechanisms have been identified that induce chemoresistance in malignant gliomas. The P glycoprotein (Pgp), a transmembrane efflux pump protein, is present on the luminal surface of endothelial cells of the blood brain barrier. It acts to maintain the integrity of the CNS environment by actively excreting toxic substances (Cordon-Cardo C *et al.*, 1989). Pgps and other types of efflux protein pumps are over expressed on the surface of cancer cells. Another mechanism that diminishes the effectiveness of chemotherapy agents is the presence in cancer cells of the DNA repair protein 0^6-alkylguanine-DNA-Akyltransferase. This enzyme removes any potential alkylating sites on tumour cell DNA and this could be responsible for the poor clinical response seen with alkylating agents such as the Nitrosoureas and Temozolomide, at least in the majority (Gerson SL *et al.*, 1992; D'Atri S *et al.*, 1995).

2.5.3(ii) Blood Brain Barrier

The Blood Brain Barrier (BBB) is a single layer of vascular endothelial cells that maintain the integrity of the brain environment by preventing toxic agents entering the brain parenchyma. These specialized endothelial cells lack fenestrations and pinocytic vesicles, have extended tight junctions and express specific transport mediators (Rapoport S and Robinson P, 1986). In the main only small lipophilic compounds (< 180 daltons) penetrate the BBB hence many chemotherapeutic agents are excluded. In primary HGGs the BBB is disrupted and this, in theory, should create an avenue for drug delivery. However the disruption appears to be variable. Large sections of the endothelium may possess an intact BBB preventing the transfer of chemotherapy to those sections of the tumour. Furthermore, the BBB of the brain adjacent to tumour, where 80% of tumour recurrence is known to occur, is likely to be intact and therefore impervious to many chemo-therapeutic agents. As a result, it seems likely that tumour cells both in the main mass and those in distant locations, may evade the effects of chemotherapy thereby diminishing its effectiveness.

2.5.4 Role of Chemotherapy in High Grade Glioma

Currently the role of chemotherapy in the adjuvant setting is unclear. A modest improvement in median survival was detected in a meta-analysis study in patients treated with radiation and adjuvant chemotherapy compared to radiotherapy alone (Fine H *et al.*, 1993). A recent meta-analysis conducted by the Glioma Meta-analysis Trialists group reported that there was clear evidence that patients benefited from adjuvant chemotherapy in terms of an increase in overall survival of 2 months. However, they concluded that no assessment was made as to the tolerability of the treatment and quality of life, which were considered important concerns for patients with such a bleak prognosis (Stewart LA, 2002). Within the United Kingdom the consensus has been that any benefit offered from adjuvant chemotherapy is extremely limited and therefore is not routinely offered to patients at present (Thomas D, 1998). Recently however Stupp *et al* published data demonstrating a survival advantage in patients with Glioblastoma who received Temozolomide chemotherapy concurrent with IR followed by 6 cycles of adjuvant Temozolomide (Stupp R *et al.*, 2005). This is the first study in this patient population that has demonstrated a survival advantage with adjuvant chemotherapy and the reported schedule may soon be recommended as the standard of care in the United Kingdom.

Advances in molecular biology have identified that patients with anaplastic oligodendroglioma tumours have a more favourable response to chemotherapy in the adjuvant setting compared to other malignant gliomas. It had been demonstrated that tumours with a Loss of Hetero-zygosity resulting from allele deletions on chromosomes 1p and 19q are more chemo-responsive. In light of these findings, patients with anaplastic oligodendroglioma now receive combination chemotherapy (P.C.V.) following surgery and radiotherapy (Cairncross G *et al.*, 1998; Ino Y *et al.*, 2001).

Chemotherapy for HGG is given primarily in the palliative setting, following tumour regrowth after first line treatment with surgery and radiotherapy. For astrocytomas, of any grade, the role of chemotherapy has never been defined by randomised controlled trials. Rather its role is based on historical data obtained

from trials conducted prior to CT imaging, and in some cases from studies when chemotherapy was given in the adjuvant setting. A modest improvement in median survival has been detected in studies investigating single agent (Nitrosourea or Temozolomide) or combination (P.C.V.) chemotherapy (Yung W *et al.*, 2000; Chang C *et al.*, 1983; Green S *et al.*, 1983; Shapiro W, 1986).The role of chemotherapy in HGG is however at best limited. The response rate to chemotherapy in patients with recurrent disease varies between 10 - 40%. Those with good prognostic factors after first relapse respond better, albeit temporarily, with a disease free survival of only a few months (Burton E and Prados M, 1999).

The following chemotherapeutic agents are used currently in routine clinical practice for patients with recurrent malignant glioma in the United Kingdom.

2.5.4(i) Nitrosoureas

- Carmustine - BCNU
- Lomustine – CCNU

Nitrosoureas are small, highly lipid soluble, non-ionised drugs that rapidly cross the blood brain barrier (Warren and Fine, 2002). They are cell cycle non-specific agents, that degrade rapidly into two reactive compounds, one with a carbamoylation activity, and the other acts as an alkylating agent. Carbamoylation of amino groups inhibits DNA repair and disrupts the synthesis of RNA. DNA alkylation leads to crosslinking of the DNA and ultimately cellular instability (Warren and Fine, 2002). The value of nitrosourea based chemotherapy in patients with relapsed malignant glioma has been poorly assessed. Occasionally it has a role to play as a single agent in the first line management of anaplastic astrocytomas and primary glioblastomas, at the time of first relapse (Stewart LA, 2002). Current practice however, is to use nitrosoureas in combination with other types of chemotherapy in the management of HGGs. Nitrosoureas are relatively well tolerated; the main side effects being delayed myelosupression and lung fibrosis (Rampling R *et al.*, 1994).

2.5.4(ii) Combination Chemotherapy

Within the United Kingdom a combination of Procarbazine (Oral), CCNU (oral) and Vincristine (intravenous bolus) - P.C.V is given every six weeks. The cycle is repeated up to a maximum of six times provided the side effect profile is tolerated. Previously, combination chemotherapy was reserved for the management of HGG following relapse after treatment with single agent Nitrosourea. Combination chemotherapy in now used first line, following recurrence after surgery and radiotherapy of anaplastic astrocytoma or glioblastoma. It remains that no randomized studies have been done to compare single agent chemotherapy and combination chemotherapy in these patients.

Procarbazine

Procarbazine (*Natulan*) is taken orally for the first ten days of a cycle of combination chemotherapy. After absorption it is activated in the liver. The active metabolite is then converted by cytochrome p450 into two active metabolites. The active metabolites act by inhibiting DNA, RNA and protein synthesis. In addition it has weak monoamine oxidase inhibitor (M.A.O.I.) properties (Newton H *et al.*, 1999). Procarbazine is generally well tolerated with the main side effects being nausea, vomiting, fatigue, rash, myelosupression and food intolerance. (Newton H *et al.*, 1990)

Vincristine

Vincristine (*Oncovin*) is a derivative of the periwinkle plant and is water-soluble. It therefore penetrates the BBB poorly. It is administered intravenously and undergoes extensive metabolism in the liver (Newton H *et al.*, 1999). By binding to tubulin in S phase, causing metaphase arrest, it disrupts the cell cycle of tumour cells. Its use is limited by peripheral neuropathy (Newton H *et al.*, 1999).

CCNU

As detailed in section 2.5.4(i).

2.5.4(iii) Temozolomide

Temozolomide is a small molecule that can penetrate the BBB. It has excellent oral bioavailability and functions as an alkylating agent. (Galanis E and Bucker J, 2000; Clark AS *et al.*, 1995). Alkylating agents bind covalently via alkyl groups to DNA. Through cross linking they cause the cell cycle to be arrested in the G1-S transition. This is either followed by DNA repair or apoptosis.

In the United Kingdom, Temozolomide (*Temodal*) is reserved for second line (or third line if a nitrosourea was used as a single agent) chemotherapy treatment for patients with relapsed HGG. In a phase II study, 35% of patients had a clinically meaningful objective response and 26% had disease stabilisation when Temozolomide was used first line in patients with relapsed AA or AO. Temozolomide demonstrated a good safety profile, however 10% experienced myelosupression (Yung W *et al.*, 1999). Temozolomide was evaluated in a randomised phase II study using Procarbazine as a reference agent, in patients with relapsed glioblastoma who had failed conventional first line treatment. The primary end point, progression free survival at 6 months, was significantly higher in the Temozolomide group than the Procarbazine group. Unfortunately, although there was an increase in the overall survival detected with Temozolomide it was not statistically significant (Yung W *et al.*, 2000). The study did however demonstrate that patients who received Temozolomide had a better quality of life whilst on treatment and experienced fewer side effects (Osoba D *et al.*, 2000). A study is currently under way to compare Temozolomide with PCV in patients with recurrent HGG. In England and Wales the National Institute for Clinical Excellence (NICE) has sanctioned the use of Temozolomide as second line chemotherapy for patients with recurrent malignant glioma (www.nice.org.uk/article).

Temozolomide has been investigated in the adjuvant setting in a randomised trial involving 573 patients with glioblastoma (Stupp R *et al.*, 2005). The primary end point was overall survival. Patients with histologically proven newly diagnosed disease were randomly assigned to receive radiotherapy alone or radiotherapy plus concomitant Temozolomide followed by six cycles of adjuvant Temozolomide.

The data recently published has demonstrated a median survival benefit of 2.5 months and a 2 year survival rate of 26.5% in patients who received Temozolomide and IR compared with a 2 year survival rate 10.4% with radiotherapy alone. Temozolomide was well tolerated with only 7% of patients experiencing grade 3 or 4 haematological toxic effects.

The clinical trial was designed with the intention of delivering chemotherapy early in the course of the disease. It was not intended however to determine the benefit of administering Temozolomide concurrently with IR versus adjuvant treatment with Temozolomide. A companion translational study suggested that the silencing of the O^6-methylguanine-DNA methyltransferase (MGMT) promoter may, by methylation, benefit patients treated with Temozolomide and IR (Hegi M *et al.*, 2005). Methylation of the MGMT promoter results in loss of MGMT expression and consequently diminished DNA repair. MGMT promoter methylation was demonstrated to be an independent favourable prognositic factor in patients treated with IR and Temozolomide.

Ionising radiation may induce MGMT, which in turn may result in repair of the damaged DNA. Alkylating agents however have been shown to deplete MGMT. The concurrent administration of Temozolomide and IR may result in an overall reduction in MGMT expression and enhanced tumour cell kill. A synergistic relationship between Temozolomide and IR has been demonstrated *in vitro* and this may explain the observed clinical trial data (Wedge SR *et al.*, 1997; Van Run J *et al.*, 2000).

This clinical trial data is beginning to alter current clinical practice. It is expected that Temozolomide administered concurrently with IR followed by adjuvant Temozolomide will soon be adopted as the standard of care for the majority of patients with newly diagnosed glioblastoma.

2.5.5 Non-Chemotherapeutic Medication

Corticosteroids

Corticosteroids, in particular Dexamethasone, are routinely used in the management of patients with HGG. Peri-tumoural oedema is a cause of elevated intracranial pressure that can induce symptoms such as headache, nausea, vomiting, confusion and limb weakness. Through mechanisms that remain unclear corticosteroids can reduce the build up of peri-tumoural oedema and control these disabling symptoms. Due to the side effects associated with corticosteroids the lowest dose of steroid that controls the patients symptoms is used (Newton HB, 1994).

Anticonvulstants

Seizure activity is a common symptom either at the time of presentation, or it can be indicative of tumour progression. Anticonvulsants are not prescribed to patients unless a seizure has occurred due to the side effect profile associated with these drugs (Glatz M *et al.*, 1996). Monotherapy is the goal in treatment, using additional anticonvulsants only when absolutely necessary (Newton HB, 1994).

2.5.6 General Support and Rehabilitation

Patients with a diagnosis of HGG often require physical, emotional and social support. This is often best coordinated by a specialist nurse who is dedicated to the care of the patients and their families. Due to the relentless debilitating nature of the disease a multidisciplinary approach is often necessary to manage these patients effectively. Physiotherapists, occupational therapists and social workers all have a role to play in the care of patients with malignant glioma. Towards the latter stages of a patient's illness it is often necessary to involve the palliative care team and hospice services.

3 Cancer Gene-Therapy

In 2003 the human genome was fully described, fifty years after the discovery of the double helix DNA structure. There are approximately 30-35,000 genes within the human genome that encode the transcription of the proteins that allow the human cell to function normally (Collins FS *et al.*, 2003). Gene mutations may cause the encoded protein to be structurally altered or be produced in abnormal quantities and consequently it may not function normally. As a result genetic disorders may ensue and become clinically manifest.

Normal tissues within the human body are proliferating, differentiating or in a state of growth arrest. In addition a process of programmed cell death, apoptosis, selectively removes certain mature cells. Cell survival within the human body is reliant on the accurate transfer of genetic material from parent cell to daughter cells. In addition, DNA needs to be protected from spontaneous and induced damage. Through complicated processes, DNA repair and cell cycle progression are co-ordinated.

The cell cycle comprises four phases (figure 3). DNA synthesis takes place during synthesis (S) phase and is followed by a gap period (G_2). Following G_2 mitosis (M) phase occurs after which the cells enter G_1. Non-proliferating cells become growth arrested in a phase of the cell cycle between mitosis and DNA synthesis referred to as G_0. Cells may be stimulated to undergo proliferation in either G_0 or G_1. It appears that between different cell lines the main difference in the rate of cell division is in the length of time each cell line is in G_1. All other phases of the cell cycle are roughly similar. This is an important concept, as the sensitivity of a cell to ionising radiation appears dependent on the phase of the cell cycle it is in (Tannock and Hill).

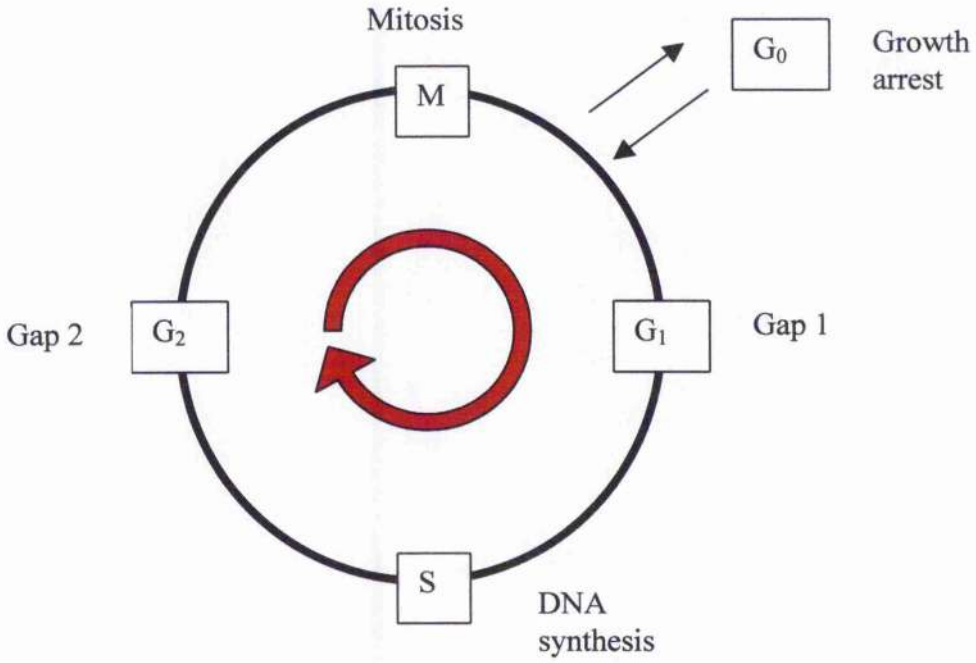


***Figure 3**; Cell cycle comprising Mitosis (M), Gap1 (G_1), DNA synthesis (S), Gap 2 (G_2) and growth arrest (G_0).*

Controlling the cell cycle are a number of interconnected molecular events governed by cyclin dependent kinases (cdks). Cdks regulate the molecular pathways and check-points that coordinate the cell cycle. Cyclin D-associated kinases are involved in the phosphorylation of the retinoblastoma protein (pRB). Phosphorylaytion of pRB is required for the progression of the cell cycle from G_1 to S phase (Sherr C and McCormick F, 2002). Cdks are positively regulated by cyclins and negatively regulated by cdk inhibitors. It has been suggested that cdks, cyclins and cdk inhibitors can be genetically altered or influenced by other oncogenic events. Disruption of these regulatory pathways can result in the progression of tumours.

The RB gene has been identified as a tumour suppressor gene. Mutations in the RB gene may predispose the cell to malignant transformation. Dephosphorylated RB protein binds to the transcription complex involved in cell cycle regulation, E2F, thereby deactivating it. Phosphorylation of the RB gene product prior to S phase enables E2F to promote cell cycle progression. Loss of RB gene function,

noted in glioblastoma development may allow uncontrolled cell cycle progression hence uncontrolled cell division and tumour development.

p53 is another well characterised tumour suppressor gene. It is noted that the p53 gene is mutated in numerous tumours including over 65% of Secondary Glioblastomas. p53 plays an important role in the response to cellular DNA damage. Following DNA damage p53 is involved in either promoting transient cell cycle arrest in G_1 to allow DNA repair, or apoptotic cell death. p53 function is lost through point mutation or deletion, thus allowing the cell DNA to accumulate genetic damage and genetic instability. Functional p53 is thought to have a protective effect against tumourigenesis (Cox L, 1997).

The natural history of diseases can be altered by using gene therapy to correct the defect in the genetic code. There are many ways in which defective genes can be manipulated, depending on the genetic problem. In some instances a vector can be used to introduce a gene into a target cell to restore the normal transcription of the defective gene. Other less common approaches aim to restore the normal function of a gene by repairing the abnormality through homologous recombination. Malfunctioning genes can be up regulated or down regulated to increase, normalise or reduce protein production. In addition, through a process of selective reverse mutation an abnormal gene can be repaired to restore its function to normal.

The process of gene transfer is fraught with obstacles and potential problems. Most diseases that affect humans are multi-factorial and are the result of a number of genetic alterations. As a result they are difficult to correct by gene therapy alone. DNA that is introduced into a target cell must be stable and may be required to function for a prolonged period of time. With this strategy there is a risk that the transfer of foreign material into a target cell may be detected by the immune system. This could result in toxicity experienced by the patient as well as potential damage to the exogenous DNA and/or vector. Methods of delivering the genetic material, either viral or non-viral, provide additional problems for scientists and clinicians. Available vectors are relatively imprecise and inefficient in their ability to target the correct tissue and/or gene locus. In addition they may

stimulate the immune system resulting in toxicity symptoms experienced by the patient. Some viruses used in gene therapy trials may generate additional morbidity due to their intrinsic pathological properties and in extreme cases prove fatal.

Gene therapy based research was dealt a blow when a patient with ornithine transcarbamylase deficiency (OTCD), a non-life threatening in-born error of urea synthesis, died from multi-organ failure four days after participating in a gene therapy trial. The aim of the phase 1 trial was to correct the single gene and restore ornithine transcarbamylase enzyme activity. Adenoviral vector designed to mediate human ornithine transcarbamylase cDNA production, was infused via the hepatic artery, to the liver (Raper S *et al.*, 2002). The fatality was the result of a severe immune response to the adenovirus vector. The study was immediately discontinued as a result of concerns over the safety of the adenovirus vector.

Some successes with gene therapy strategies have been reported. In 2000, Cavazzano-Calvo *et al* reported the correction of the x-linked severe combined immunodeficiency (SCID) syndrome in two children. Infusion of *ex vivo* CD 34^{+} enriched bone marrow cells led to the correction of the T-cell immunodeficiency, sufficient to prevent the clinical manifestations of the illness (Cavazzano-Calvo M *et al.*, 2000). This strategy successfully corrected the deficiency in three children who remain well three and a half years after treatment (Fischer A *et al.*, 2002). Unfortunately, towards the end of 2002 it was reported that two other children treated in the same way, had developed a monoclonal lymphocytosis consistent with acute lymphocytic leukaemia (ALL). This is presumed the result of an insertional mutagenesis associated with retroviral mediated gene transfer. The retrovirus vector had inserted an intron of the LMO-2 gene, which is associated with ALL, into chromosome 11 (Hacein-Bey-Abina, S *et al.*, 2003). As a result recruitment into the study has terminated and retroviral gene therapy has been suspended in America. In the United Kingdom, however, similar studies are still underway albeit with caution (Gene Therapy Advisory Committee (GTAC) Report 2001-2002).

Despite the recent setbacks, the proof of principle of gene therapy has been demonstrated. Consequently continued groundbreaking gene therapy research could offer the possibility of break-through in the treatment of serious illnesses and, in particular, cancer.

Traditionally, pharmaceutical companies have supported the development of 'small molecules' in the treatment of cancer. These are designed to interrupt specific targets in the cell cycle. Despite recent success stories such as STI-571 (Gleevec) in the treatment of chronic myeloid leukaemia (Druker BJ et al., 1996) this method of combating cancer has not proven to be particularly effective.

Cancer manifests itself clinically following the mutation of gene(s) involved in the normal growth and repair of cells. It therefore seems logical to combat this condition through gene therapy techniques. Improved understanding of the human genome and cancer biology has identified a number of new potential genetic targets amenable to genetic manipulation. Sixty two percent of gene therapy trials proposed in America have been directed towards combating cancer. More than half of these have involved one of five tissue types: melanoma, prostate, ovary, squamous carcinoma of the head and neck and leukaemia (Gottesman M, 2003).

Cancer gene therapy strategies are divided into those that are targeted against the tumour cells themselves, and those that target the host's ability to control the development and progression of the disease.

Gene therapy targeting of tumour cells	*Gene therapy targeting of host cells*
Tumour suppressor genes	Tumour angiogenesis
Dominant-negative genes	Chemo/radiotherapy protection
Apoptosis induction	Immunomodulation
Oncolytic viruses	
Tumour-specific gene expression	
Sensitisation to radio/chemotherapy	

***Table 3**; Gene therapy stategies*

In addition to developing gene therapy targets, there has been extensive research into developing safe and effective viral and non-viral vectors and delivery mechanisms.

3.1 Gene Therapy in the Management of High Grade Glioma

The management of malignant gliomas has been a target for gene therapy strategies for some time. The relentless progression of the disease coupled with the lack of effective treatment options have made clinicians investigate gene therapy strategies that may augment available therapies or prove to be independently successful. A number of potential gene therapy strategies, translatable to different cancer types, have been investigated in malignant glioma.

Gene therapy in the management of malignant glioma is logistically difficult. Despite the identification of potential gene targets, the ability to correct the defect efficiently remains elusive. Transfection in experimental brain tumours is rarely able to transfer genes to more than 5% of the tumour mass due to the lack of effective delivery mechanisms or vectors (Lam P and Breakefield X, 2001). As a result, the vast majority of the tumour will not benefit directly from the gene therapy strategy. For gene therapy to affect the tumour as a whole there must be an additional mechanism by which the transduced cells can exert an indirect effect on the non-transduced cells i.e. the 'bystander effect'. The bystander effect may result from either direct contact with transduced dying cells (Frank DK *et al.*, 1998), intercellular transfer of toxic products (Moolten F, 1994), inhibition of

angiogenesis or by harnessing the immune system to attack the tumour as a whole (Bouvet M *et al.*,1998).

However, even improving the transduction efficiency, in addition to maximising the bystander effect, may still be insufficient to control malignant gliomas. The cells that comprise this tumour have inherent motility resulting in their dissemination down the white matter tracts generating recurrence distal to the site of the primary disease.

Gene therapy strategies investigated in malignant glioma are discussed according to their mechanism of action.

3.1.1 Tumour Suppressor Gene / Oncogenes

This type of gene therapy in the management of malignant glioma has mainly concentrated on exploiting the mutation or deletion of the p53 tumour suppressor gene, which occurs early in the development and progression of anaplastic astrocytoma and glioblastoma. The primary aim of this strategy is to restore wild-type p53 production to promote growth arrest of the tumour (Gomez-Manzano C *et al.*, 1996; Cirielli C *et al.*, 1999). In addition restoration of p53 would enhance the cytotoxic effect of chemo and radiotherapy via the p53 mediated apoptosis pathway (Biroccio A *et al.*,1999; Broaddus WC *et al.*,1999).

Adenovirus vector mediated gene transfer of p53 in patients with malignant glioma resulted in the transduction of tumour cells to a limited distance from the site of injection. The patients experienced minimal toxicity and there was anecdotal evidence suggestive of efficacy. Further clinical studies using this strategy are under development (Lang F *et al.*, 2003).

The p16 / RB / E2F-1 pathway involved in driving the cell through the G_1 check point to S phase is also being investigated as a possible gene therapy target. Mutations in the RB gene and p16 gene are frequently seen in anaplastic astrocytoma and glioblastoma. Restoration of the RB or p16 gene to the glioma

cell results in a cytostatic effect. The E2F-1 protein has a dual action both as an oncogene driving cell cycle progression as well as a tumour suppressor gene. In the role of tumour suppressor gene, the E2F-1 protein promotes apoptosis in glioma cells independent of the RB, p16 or p53 status of the cell line (Gomez-Manzano C *et al.*,1999).

3.1.2 Antiangiogenesis

Through the process of angiogenesis a malignant tumour manages to maintain an adequate blood supply to support its continued growth (Folkman J, 2003). In human malignant glioma the more aggressive pathological phenotypes are characterised by increased neovascularisation. Anti-angiogenic gene therapy targets the protein mediators of the neovascularisation process. One such protein is the vascular endothelial growth factor which is up-regulated in hypoxic conditions and high levels are correlated with tumour progression – a process which occurs in malignant glioma (Plate KH *et al.*, 1992). Transfection of anti-sense VEGF cDNA in mice down-regulates the endogenous VEGF and reduces glioma formation. *In vivo*, adenovirus mediated transfer of antisense VEGF has been shown to reduce subcutaneous glioma growth in mice (Im S *et al.*, 1999). This work proposed a vascular target in the treatment of human malignant glioma and research continues in this field.

3.1.3 Immunomodulation

It has been demonstrated that perivascular lymphocyte infiltration of primary brain tumours is a favourable prognostic sign (Brooks WH *et al.*, 1978). This observation suggests that harnessing the immune system may be an alternative treatment strategy for these malignancies. Several groups have shown prolonged survival in animals harbouring intra-cranial malignant glioma xenographs by using antigen presenting dendritic cells as a vaccine (Siesjo P *et al.*, 1996; Liau LM *et al.*, 1999). Phase I studies in humans using autologous dentritic cells, either fused with glioma cells or pulsed with peptides eluted from the surface of autologous glioma cells, have demonstrated safety with some evidence to support the stimulation of the immune system (Kikuchi T *et al.*, 2001; Yu J *et al.*, 2001).

Cells or vectors have been injected, subcutaneously or intracranially, with the aim of stimulating the immune system by either encoding tumour antigens or cytokines (IL-2, IL-4, IL-12, GM-CSF, TNF-alpha and INF gamma). This approach has also been shown to be safe and effective *in vitro* and *in vivo* (Yu JS *et al.*, 1993; Sampson JH *et al.*, 1997; Liu Y *et al.*, 2002). Clinical trials using these methods have demonstrated immune responses but have yet to show improvement in survival (Schneider T *et al.*, 2001).

3.1.4 Pro-drug Activation (Suicide Gene Therapy)

Gene therapy techniques have been used to sensitise tumour cells to toxins or chemotherapeutic agents. A 'suicide gene' is introduced into tumour cells where it can encode a specific enzyme. A clinically inactive pro-drug is then delivered which is converted into a clinically active derivative by the expressed transfected enzyme. Although, due to the inefficiency of the system not every cell is transfected, clinical benefit is enhanced by the 'bystander effect'. As discussed previously this term is used to describe the process by which cells neighbouring cells are killed, though they themselves remain not transfected.

Various pro-drug activating systems are under investigation, however the most widely studied is the Herpes Simplex Virus type-1 Thymidine Kinase enzyme (HSV*tk*) with ganciclovir (GCV) as the pro-drug (Moolten F., 1994). GCV is phosphorylated by HSV*tk*, and is then incorporated into replicating DNA resulting in cell death (Cheng Y *et al.*, 1983). The bystander effect occurs as the phosphorylated GCV can pass through tumour cell gap junctions (Touraine RL *et al.*, 1998).

Phase I/II clinical trials in patients with glioblastomas investigated intratumoural injection of HSV*tk* - retroviral mediated transduced vector producing cells followed by GCV. These early studies demonstrated the procedure to be safe, with efficacy suggested by a few long-term survivors (Shand N *et al.*, 1999; Klatzmann D *et al.*, 1998).

These trials led to the largest randomised gene therapy trial in cancer being undertaken. In a phase III study involving 248 newly diagnosed patients with glioblastoma, retroviral mediated transduced glioblastoma cells were injected into the resection cavity wall following surgical resection of the malignant glioma. The patients were then treated with GCV. The study failed to demonstrate any significant increase in time to tumour progression or overall survival. A poor transfection rate, as well as intra-operative technical difficulties, were cited as reasons for failure (Rainov NG, 2000). Other systems to deliver HSV-*tk* to tumour cells with increased efficiency are under investigation.

Another system with an effective bystander effect is *Escherichia coli* cytosine deaminase/5-fluorocytosine (CD/5-FC). 5-FC is non-toxic to mammalian cells as they lack the enzyme cytosine deaminase(CD). Certain bacteria, such as E-coli and fungi do possess CD and are able to convert 5-FC into 5- Flurouracil (5-FU). Introduction of the gene to mammalian cells by transfection or via retroviral vectors results in the production of toxic 5-FU, leading to cell death through inhibition of DNA and RNA synthesis. A bystander effect is achieved through diffusion of 5-FU across cell membranes (Mullen C *et al.*, 1992).

Cyclophosphamide, which normally cannot cross the blood brain barrier, is converted by cytochrome p450 2B1 to active phosphoramide mustard. When cytochrome p450 2B1 is locally transfected within the brain tumour, the active drug has the potential to kill the brain tumour cells (Wei MX *et al.*, 1994).

4 Oncolytic Viral Vectors for Gene Therapy

All of the viruses described above are replication incompetent. That is, they can infect a cell and express virally encoded proteins but are not capable of going through a full replication cycle to release progeny virus particles.

Given the poor transfection rates achieved with replication incompetent viral vectors, researchers have tried to improve efficiency by using viruses that can replicate in tumour cells, resulting in oncolysis and wider dissemination of gene expression (Russell SJ, 1994).

To be effective in killing tumour cells, the virus must be able to infect the target cell, replicate, transcribe any additional therapeutic genes and ultimately destroy the tumour cell. Ideally, the virus should be capable of infecting all the cells of the tumour, including non-cycling cells, thereby exerting maximum impact on the tumour. After several rounds of replication the number of potentially oncolytic viral particles may be many logs higher than the input dose.

Oncolytic viruses may kill the tumour cell directly through cell lysis, however additional cell kill may be achieved through the delivery of exogenous cytotoxic therapeutic genes and as a result of the bystander effect.

The use of replication competent viruses in the treatment of cancer was first proposed in the late 1950's when adenovirus was investigated as a treatment for cervical cancer (Southam C.M, 1960). The understanding of virus and cancer cell biology has improved greatly, enabling the manipulation of viruses to kill cancer cells more effectively. In 1991 Martuza *et al* engineered a thymidine kinase deleted Herpes Simplex Virus -1, *(dl*sptk*)* capable of replicating only in dividing cells. Subsequently there has been extensive pre-clinical and clinical investigation into the use of viruses as oncolytic agents (Martuza RL *et al.*, 1991).

4.1 Adenovirus

Adenoviruses are non-enveloped and contain double stranded DNA. In humans wild type virus infection results in non-life-threatening, non-specific, flu-like illness. Wild type adenovirus expresses the protein E1B-55kD, which in conjunction with other viral proteins, binds to and destroys host cell p53 thereby precluding the host cell anti-viral, p53 mediated, apoptosis defence mechanism. Consequently, wild type adenovirus creates a cell state conducive to viral replication.

The adenovirus mutant, E1B-55kD (*dl*1520 or ONYX-015) has a deletion in the EIB-55kD locus. It was hypothesised that *dl*1520 as a result of this deletion would only be able to replicate in cells that did not produce p53 (Biscoff J *et al.*, 1996). It is known that many cancer cells have limited or no p53 function and therefore it

was postulated that *dl*1520 would replicate preferentially in tumours with this genetic defect. However, it has become apparent, that other viral proteins influence p53 function. In addition, the E1B protein exerts additional influences, as well as p53 modulation, on the viral replication cycle. From *in vitro* and *in vivo* data the selectivity and effect of *dl*1520 is unclear and seems dependent on the cell type.

The adenovirus mutant, *dl*1520 was the first replication competent virus to be used in a phase I human clinical trial. Twenty-two patients with recurrent squamous cell carcinomas of the head and neck, refractory to radio- or chemotherapy, received a single intratumoural injection of *dl*1520. The results of the study failed to demonstrate toxicity other than mild flu-like symptoms up to a maximum dose of $1x10^{11}$ plaque forming units (p.f.u.). The maximum tolerated dose was not achieved. The majority of the 22 patients mounted an immune response and there was evidence of viral replication and tumour necrosis in some of the injected tumours (Ganly I *et al.*, 2000). Phase II studies, in patients with squamous cell carcinoma of the head and neck demonstrated disappointing efficacy with less than 15% demonstrating response to single agent *dl*1520 (Nemunitis J *et al.*, 2000).

Phase I and I/II clinical trials in tumour types other than head and neck squamous carcinoma using various delivery mechanisms, have demonstrated single agent *dl*1520 to be relatively well tolerated. However, there is scant evidence to suggest efficacy when used alone (Nemunaitis J *et al.*, 2001; Hamid O *et al.*, 2003).

In vitro and *in vivo* data have suggested a potentially additive, possibly synergistic effect when *dl*1520 is combined with Cisplatin-based chemotherapy (Bischoff JR *et al.*, 1996). A controlled clinical trial of patients with squamous cell carcinoma of head and neck investigated intratumoural injection of *dl*1520 and adjuvant 5-FU and Cisplatin chemotherapy. The combination demonstrated improved response rates and delayed time to tumour progression when compared to virus or chemotherapy in isolation. There was no evidence of an increase in the toxicity profile when the treatments were combined (Khuri F *et al.*, 2000).

In patients with progressive colorectal hepatic metastasis, partial response and in some instances disease stabilisation, were achieved following the infusion of *dl*1520 via the hepatic artery with adjuvant 5-Fluorouracil and Folinic acid chemotherapy. Interestingly these patients had experienced tumour progression whilst receiving the same chemotherapy as used in the trial with *dl*1520 (Reid T *et al.*, 2002). A synergistic, as opposed to an additive relationship between *dl*1520 and chemotherapy is not confirmed by either of the data sets.

Another adenovirus mutant has been developed by deletion of the E1A region of the viral genome resulting in the mutant, *dl*922/947. Wild type viral E1A protein binds to and inactivates the retinoblastoma protein (pRB), a protein involved in the regulation of normal cell proliferation. The inactivation of RB by E1A, results in the cell continuing to replicate, thereby supporting adenoviral replication. *dl*922/947 has been demonstrated to replicate selectively in human glioma cells that have abnormalities of the RB pathway (Fueyo J *et al.*, 2000).

A method of ensuring selective oncolytic replication of adenoviral infection is to have the E1A protein expression under a tumour specific promoter. Prostate specific antigen, and alpha-fetoprotein promoters have been used to introduce tumour specificity and limit viral replication to prostate and hepatocellular tumours respectively (Rodriguez R *et al.*, 1997; Hallenbeck PL *et al.*, 1999). Prostate specific adenovirus, CN706, has undergone phase 1 clinical trial and was seen to be safe with evidence of intra-prostatic replication detected. Further studies are underway (DeWeese TL *et al.*, 2001).

4.2 Vaccinia Virus

Vaccinia virus is an enveloped, double-stranded DNA virus capable of lysing tumour cells as a result of viral replication in addition to inducing an anti-tumour immune response. Wild-type vaccinia virus is relatively safe in humans. It has a large genome (200kb) and is able to accommodate the insertion of transgenes into the region of the *tk* gene to make it selective for dividing cells. Phase I clinical trials of oncolytic vaccinia virus expressing tumour associated antigens interleukin 2 or GM-CSF, which stimulate the immune system, have been well

tolerated. In patients with malignant melanoma, vaccinia virus expressing GM-CSF demonstrated evidence of local anti-tumour effects. Partial responses were noted in a few patients and with one patient experiencing complete remission (Conroy RM *et al.*, 1999; Mukherjee S *et al.*, 2000; Mastrangelo MJ *et al.*, 1999).

4.3 Newcastle Disease Virus (NDV)

NDV a paramyxovirus, is a negative stranded RNA virus that in humans may generate a mild conjunctivitis. Recently it was demonstrated to inhibit the growth of tumour xenographs in mice following intratumoural injection (Phuangsab A *et al.*, 2001). In a recent phase I trial, intravenous injection of NDV (PV701) in patients with solid tumours was well tolerated with clinical and radiological reports of response (Pecora AL *et al.*, 2002).

4.4 Reovirus

Reoviruses are double stranded RNA viruses which are naturally occurring oncolytic viruses and therefore do not require any engineering. Their selectivity for tumour cells is dependent on activation of the RAS signal-transduction pathway that is upregulated in many tumours. Intratumoural injection of reovirus into tumour xenographs in the flanks of immunodeficient mice has demonstrated regression (Coffey M *et al.*, 1998). Intratumoural and intravenous injection of reovirus into colonic, ovarian and glioma cancer cell xenographs has resulted in tumour regression and improved survival in immunocompromised mice (Hirasawa K *et al.*, 2002; Wilcox ME *et al.*, 2001). Phase I clinical trials are awaited.

4.5 Poliovirus

Poliovirus is a positive stranded RNA virus that is capable of causing severe morbidity and mortality in humans with the clinical condition termed poliomyelitis. Through genetic manipulation, the neurotropic sequence has been deleted which has climinated its neurovirulence. *In vitro* data have demonstrated the ability of the virus to replicate in tumour cells and inhibit tumour growth *in* vivo (Gromeier M *et al.*, 2000). The mechanism of the resultant cytotoxicity is not understood. Phase I clinical trials are awaited.

4.6 Vesicular Stomatitis Virus (VSV)

VSV is a negative-sense RNA virus that is relatively asymptomatic in humans. It has been shown to replicate in tumour cells and inhibit the growth of tumour xenographs in immunodeficient mice. Although still not understood, the oncolytic capability of VSV appears to be enhanced by the presence of interferon (Stojdl DF *et al.*, 2000).

4.7 Herpes Simplex Virus (HSV)

Herpes simplex viral therapy is the most studied oncolytic treatment for brain tumours and represents the subject of this thesis. It is described in detail in the subsequent chapter.

5 Herpes Simplex Virus

5.1 Classification and Structure

Eight human herpesviruses have been identified, herpes simplex 1 (HSV1), herpes simplex 2 (HSV2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein Barr virus (EBV) and human herpes viruses 6,7 and 8. HSV-1 has a similar structure to the other human herpes viruses. It is neurotropic and establishes a latent infection in the peripheral nervous system, which on reactivation causes cutaneous cold sore lesions. Infection of the central nervous

system results in uncontrolled viral replication leading to potentially fatal encephalitis. The HSV-1 particle is approximately 150-200nm in size and comprises four key structures; core, capsid, tegument and lipoprotein envelope.

Core – contains the dsDNA viral genome (approximately 153kb in size).

Capsid – approximately 100nm in size and icosahedral in shape.

Tegument – an electron dense proteinaceous matrix located between the capsid and the envelope. The proteins contained within the tegument appear to be involved in the induction of viral gene expression and the process of host cell protein synthesis shut off, immediately post infection.

Envelope – the outermost structure of the virus that is formed in the most part of lipids derived from the host cell membrane as the virus exits the cell. It consists of a trilaminar membrane, within which are contained approximately 11 virally encoded glycoproteins which enable viral invasion into a potential host cell.

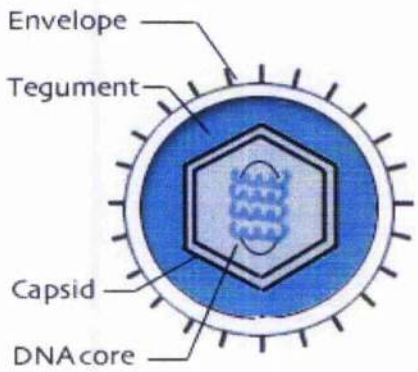


Figure5.1; Structure of HSV

5.2 HSV Genome

The 153kb genome is a linear double stranded DNA structure composed of two covalently joined segments; the long (L) and short (S) regions. Each region has a unique sequence of DNA (U_L and U_S) flanked by inverted terminal and internal repeat regions (TR_L, IR_L, TR_S and IR_S). The long and short regions can invert relative to one another creating four different isomers. The 'a' sequence is present

as a direct repeat at the genomic termini and as an inverted repeat at the L-S junction (*Figure 5.2*).

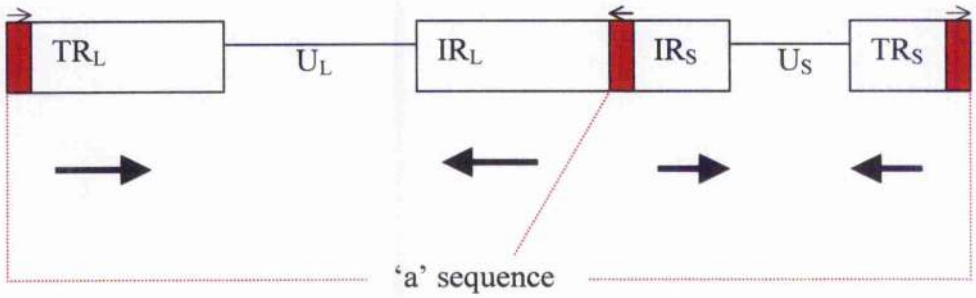


***Figure 5.2**; Organisation of the HSV-1 genome*

5.3 HSV Lytic Replication Cycle

The binding of the viral particle to the host cell is mediated by three of the glycoproteins, gB, gC and gD, which are located on the surface of the virus envelope. The positively charged glycoproteins interact with the negatively charged heparin sulphate (HS) moieties on the cell surface proteoglycans. Following secure attachment, the virus particle fuses with the cell membrane which involves the viral glycoproteins; gB, gD, gH, gL and gK. During the process of fusion, the viral membrane is lost and the virion contents enter the cytoplasm. The majority of the tegument proteins leave the capsid, which is transported with the remaining tegument proteins via microtubules, through the host cell cytosol to the nucleus. On virus entry to cells, the viral genes are expressed in a regulated, temporal sequence. Viral Protein 16 (VP16), present in the tegument of HSV-1, enters the host cell with the nucleocapsid and transactivates promoters that control gene expression and subsequent transcription of the immediate early (IE) proteins, ICP0, ICP4, ICP22, ICP27 and ICP47. Immediate early protein expression leads to the transcription of early (E) genes, which primarily encode enzymes involved in viral DNA replication. HSV DNA replication occurs by a rolling circle mechanism producing linear concatamers of HSV DNA. Finally late genes are expressed that code for structural components of the virion. Late gene expression leads to the assembly in the nucleus of capsids containing one genome equivalent. The process by which the viral particle acquires its trilaminar membrane is unclear. The capsid containing viral DNA,

migrates to the nuclear membrane and is expelled with the nuclear membrane forming its outer membrane. The virions are transported to the cell membrane via the endoplasmic reticulum and egress from the cell into the extracellular matrix. The lytic replication cycle results in the destruction of the cell as hundreds of mature virions with the potential to infect neighbouring cells are released.

5.4 HSV Latency and Reactivation

Herpes simplex virus is a neurotropic virus, which has evolved to cause a latent infection in the peripheral nervous system of its human host. Typically HSV-1 infects the buccal mucosa and travels along the axons of the trigeminal nerves to the trigeminal ganglia. Here it exits the replication cycle and enters a latent state securing life long presence within the host. During latency the viral genome remains as a stable circular episome formed by the joining of the termini (Deshmane SL and Fraser N, 1988). The gene expression, present during lytic replication, is suppressed during latency. Remaining latency regions within the long repeat elements of the viral genome are the only genes expressed and encode RNA molecules called latency associated transcripts (LATs) (Branco F and Fraser N, 2005; Spivack J and Fraser N, 1987; Stevens JG *et al.*, 1987). The precise role of LATs is unclear however, given that they remain stably expressed during latency, it has been proposed that they are required to establish and maintain latency and appear to have a role in preventing apoptosis of the host cell. In addition LATs appear to be involved in HSV-1 reactivation from latency (Stevens JG *et al.*, 1989; Roizman B and Sears A, 1996). The latency period can be interspersed with periods of viral reactivation following a stress response to a stimulus such as, ultraviolet light, hyperthermia, hormonal fluctuations (menstruation) and elevations in cAMP. During reactivation viral proteins are made and infectious particles are manufactured that can result in clinical manifestation. The exact molecular pathways involved in the reactivation process are unknown (Roizman B and Sears A, 1996).

6 Oncolytic Herpes Simplex Viruses

The HSV-1 genome is well characterised with a number of non-essential genes identified as being associated with neurovirulence. Due to the diverse range of cell types that HSV-1 can infect and its inherent lytic capability, a number of HSV mutants lacking certain non-essential virulence associated genes have been investigated as potential cancer therapies.

6.1 Thymidine Kinase Mutation

The first oncolytic HSV described has a deletion in the thymidine kinase gene. HSV-1*dl*sptk causes a lytic infection in glioma cell tissue culture but is avirulent in normal mouse brain. *In vivo* murine studies demonstrated tumour volume reduction in subcutaneous and intracranial glioma xenographs. In some cases there was evidence of prolonged survival and even reports of complete cure (Martuza R *et al.*, 1991). At high doses however HSV-1*dl*sptk was associated with fatal encephalitis. The major concern that halted the progression of HSV-1*dl*sptk into clinical studies was the reduced efficacy of anti-herpes medication such as Acyclovir or Gancyclovir, which are reliant on thymidine kinase for conversion to the active metabolite. Other HSV mutants have been developed that are non-neurovirulent but maintain the inbuilt safety mechanism afforded by the intact thymidine kinase gene.

6.2 Ribonucleotide Reductase Mutation

The hrR3 virus contains an insertional mutation of the *E.coli* lacZ cDNA into the ICP6 locus, which results in a ICP6-lacZ fusion protein that has no viral ribonucleotide reductase (RR) activity (Goldstein D and Weller S, 1988). It has markedly reduced neurovirulence and the lacZ expression allows the tracking of the virus in tissue. The RR mutation prevents the virus from replicating in normal tissue but in highly replicating tissue such as occurs in a tumour, the elevated

levels of the host cell ribonucleotide reductase provides the deoxynucleotides necessary as substrates for viral DNA synthesis (Yamada Y *et al.*, 1991). *In vivo* intratumoural inoculation of hrR3 in rats bearing malignant gliosarcomas resulted in improved overall survival. The extension to overall survival was further enhanced if adjuvant gancyclovir was given (Boviatis EJ *et al.*, 1994).

6.3 ICP34.5 Mutation

To date the most neuroattentuated of any HSV mutants are those with deletions of the RL1 gene encoding ICP34.5, a protein thought to have more than one mechanism of action. The RL1 gene is located in the long repeat (R_L) region and is therefore present in two copies per genome.

It has been shown that the 63 amino acid carboxyl terminal region of ICP34.5 shares significant homology with two cell cycle regulation proteins (McGeoch DJ and Barnett BC, 1991). The proteins are mouse myeloid differentiation protein (MyD116), expressed following myeloid differentiation (Lord KA *et al.*, 1990) and hamster growth arrest and DNA damage protein (GADD34), expressed following growth arrest and DNA damage (Fornace AJ Jnr *et al.*, 1989). Although the mechanism of action of each protein is still unclear, it has been suggested that GADD34 and MyD116 may function to prevent cell protein synthesis from ceasing following cellular stress (Lieberman DA and Hoffman B, 1998).

It has been shown that one function of ICP34.5 is to overcome the block in host cell protein synthesis, which occurs in some cells (for example the neuroblastoma cell line SK-N-SH) as a response to viral infection. When these cells are infected with HSV-1 there is activation of interferon-inducible double stranded protein kinase (PKR). This activation results in the phosphorylation of eukaryotic initiation factor (eIF2α) and consequently inhibition of cellular protein synthesis (Gale M and Katze M, 1998). Expression of ICP34.5 allows wild type HSV-1 to overcome this block by interacting with protein phosphatase-1-alpha to dephosphorylate eIF2α thereby negating the effects of the antiviral PKR pathway

(Chou J and Roizman B, 1992; Chou J *et al.*, 1995; He B and Roizman B, 1997). However, although the protein shut-off mechanism has been well characterised it does not fully explain the avirulence phenotype of ICP34.5-null HSV.
In an experiment designed to demonstrate that the PKR pathway is the main mechanism by which ICP34.5 determines the virulence of HSV, Leib *et al* demonstrated restoration of virulence of an ICP34.5 deletion mutant in PKR knockout mice (Leib D *et al.*, 2000). However, the 17termA mutant they used is 10,000 fold less attenuated than other ICP34.5-null mutants (Bolovan C *et al.*,1994) making the claim for the restoration of virulence questionable.

An indication that the PKR pathway mechanism does not determine the avirulence of ICP34.5-null mutants comes from the work of Mohr *et al.* (2001). They isolated an ICP34.5-null mutant with a second-site suppressor mutation that overcomes the host cell protein synthesis block in SK-N-SH cells. The second-site mutation removes the Us11 late gene promoter resulting in the transcription of Us11 being under the control of the immediate early Us12 promoter. Immediate early expression of the Us11 protein abrogates the host cell protein shutoff phenotype. However, the mutant remains as neuroattentuated as other ICP34.5-null mutants following intracerebral inoculation, indicating that the protein synthesis shutoff phenotype is not the main determinant of virulence.

The work of Brown *et al.* has highlighted a different virulence determining function of ICP34.5. They showed that the replication competence of ICP34.5-null mutants is cell type and state dependent and that in the vast majority of cell lines investigated, ICP34.5-null HSV kills the cells by lytic replication and not by host cell protein synthesis shutoff (Brown SM *et al.*, 1994a; Brown SM *et al.*, 1994b). They have demonstrated that in general actively dividing cells are capable of supporting a productive HSV infection in the absence of ICP34.5 but that to produce a lytic infection in non-dividing cells HSV must express ICP34.5.

Brown *et al* have demonstrated that ICP34.5 and homologous region of MyD116 complex with the cellular protein proliferating cell nuclear antigen (PCNA) (Brown SM *et al.*, 1997). PCNA is an accessory protein to DNA polymerase delta (Prelich G *et al.*, 1987) and it is postulated that, regulated by interaction with

other proteins, it acts as a switch between the processes of DNA replication and repair (Cox LS, 1997). Brown *et al* therefore proposed that ICP34.5 interacts with PCNA to prime the cellular replication machinery to allow initiation of viral replication.

It has been shown that a HSV infection results in host cell DNA acquiring single strand breaks (Aranda-Anzaldo A *et al.*, 1992). It has been proposed that following this damage to cellular DNA, PCNA is rapidly recruited to the sites of damage within the nucleus to initiate DNA repair (Harland J *et al.*, 2003). Immuno-fluorescence studies indicate that ICP34.5 co-localises with PCNA in the cell nucleus at early stages following viral infection and that later ICP34.5 accumulates in the cytoplasm. It has been postulated that ICP34.5 switches the function of PCNA from repair to replication mode, which in turn supports viral replication. In non-dividing cells PCNA function is modulated by interacting proteins to allow only DNA repair processes. Therefore in non-dividing cells HSV requires the presence of ICP34.5 to switch the PCNA to DNA replication mode in order to initiate its own DNA replication. ICP34.5 is an absolute requirement for the replication of HSV in terminally differentiated (non-dividing) cells (Bolovan CA *et al.*, 1994). However in cells that are rapidly dividing, such as in a tumour, the quantity of PCNA is elevated and in a state that can support ICP34.5-null mutant replication.

6.3.1 Herpes Simplex Virus 1716 (HSV1716)

HSV1716 is a mutant herpes simplex virus type 1 with a 759 base pair deletion in both copies of the RL1 gene. The RL1 gene is located in the long repeat region of the HSV genome and encodes the protein ICP34.5, a specific determinant of virulence. HSV1716 was derived from a spontaneous HSV1714 in which the RL1 deletion was initially isolated. However, HSV1714 had mutations in *Xba*1 restriction enzyme sites and was *tk* negative. IISV1716 was generated by recombining the region of HSV1714 containing the RL1 deletion into the parent strain HSV 17^{+}, thus ensuring that HSV1716 was solely altered in the RL1 gene (MacLean A *et al.*, 1991). HSV1716 replicates with the same growth kinetics as wild type HSV in most tissue culture cell lines but fails to replicate in central

nervous tissue and does not cause encephalitis (Brown SM *et al.*, 1994a). RL1 deletion mutants are the most neuroattentuated of any of the HSV mutants that have been isolated.

Early studies demonstrated that a deletion or mutation in the region of the RL1 gene resulted in the HSV variants being unable to replicate in the central nervous system. Intracranial injection of less than 10p.f.u of wild type HSV17^{+} caused encephalitis in immune competent Balb/C mice. In contrast HSV1716 at a dose of 10^{7} p.f.u failed to demonstrate evidence of viral replication or encephalitis in the same model (MacLean A *et al.*, 1991). In addition HSV1716 was avirulent following ocular or intracranial inoculation in studies using severe combined immune deficient (SCID) mice. Wild type HSV17^{+}was noted to be extremely virulent in the same model (Valyi-Nagy T *et al.*, 1994).

In vivo studies have demonstrated efficacy with HSV1716 in addition to safety. Melanoma tumour deposits were grown in the brains of immunocompetent C57B mice. Intratumoural HSV1716 inoculation, 5-10 days after melanoma deposits were introduced, resulted in prolonged overall survival and in some cases tumour eradication. None of the mice experienced signs or symptoms suggestive of encephalitis. Immunohistochemical analysis of the tumours indicated that the replication of HSV1716 was limited to the tumour, with no evidence of viral replication in the surrounding brain (Randazzo B *et al.*, 1995). Tumour regression was also noted when intracerebral deposits of human embryonal carcinoma cells (NT2) in nude mice were injected with HSV1716. Once again there was no evidence of HSV1716 out-with the tumour deposit (Kesari S *et al.*, 1995).

Although HSV1716 is neurotropic, experiments have indicated that it can replicate in non-neuronal tissue *in vivo*. Intraperitoneal mesothelioma deposits in SCID mice demonstrated viral replication limited to the tumour. In addition there was a survival advantage seen in the mice that had been injected with HSV1716 (Kucharczuk J *et al.*, 1997).

Lasner *et al* called for caution in the use of ICP34.5 null mutants in severely immunocompromised patients after providing evidence of HSV antigen within the

ependyma and sub-ependyma of nude mice following intraventricular injection of high doses of HSV1716. All of the mice injected with high doses of HSV1716 died prematurely as did many of the mice injected with a slightly reduced dose (Lasner TM *et al.*, 1998). In addition, following injection of HSV1716 into the ventricular system of immuno-competent mice there was evidence of viral replication and destruction of the ependymal lining (Kesari S *et al.*, 1998).

These reports conflicted with the safety demonstrated in previous *in vivo* studies and although they indicate the need for caution in the use of ICP34.5 null mutants the concerns raised were not considered as a reason to halt the therapeutic application of HSV1716. Clinical trials to date have confirmed the safety profile of HSV1716 in humans.

6.3.2 R3616

This is another replication competent HSV mutant that is deleted in the region of the ICP34.5 gene. R3616 is derived from the F strain of HSV-1, unlike HSV1716, which is derived from the parent wild type HSV17^{+} (Markert JM *et al.*, 1993). R3616 is capable of replicating in glioma xenographs in nude mice and remains avirulent with no evidence of encephalitis. Enhanced tumour cell kill when combined with ionising radiation has been demonstrated (discussed in more detail later). *In vitro* analysis of cultured samples of ovarian tumours from patients who were resistant to chemotherapy, demonstrated effective oncolysis with R3616 suggesting that it may have a role in the adjuvant treatment in a number of solid tumours (Coukos G *et al.*, 2000).

6.3.3 R7020 / NV1020

NV1020 is a multi-mutated, non-selected clonal derivative of the virus R7020. It was originally developed as a vaccine against HSV-1 and HSV-2. It has various deletions in the HSV joint region. Only one copy of the ICP34.5 gene is deleted and it has an intact ICP6 gene that encodes ribonucleotide reductase. In addition there is a deletion in the thymidine kinase locus that overlaps the promoter for the UL24 gene thereby preventing its expression. The L/S junction has a fragment of

HSV-2 DNA inserted and an exogenous copy of the thymidine kinase gene under the control of the HSV1 α4 promoter. These deletions severely attenuate the virulence, and has been suggested that it is safer than viruses with only one gene deleted. Its development as a vaccine was unsuccessful, however the *in vitro* and *in vivo* safety data highlighted it as a potential oncolytic viral therapy (Meigner B *et al.*, 1988). In primates known to be sensitive to HSV-1 it proved to be avirulent following inoculation in the brain (Meignier B *et al.*, 1990). Extensive *in vitro* characterisation has demonstrated its lytic replicative ability. *In vivo* analysis has demonstrated safety as well as efficacy in a number of different tumour types by various methods of delivery, namely intraperitoneal delivery to gastric cancer, intravesical treatment of bladder carcinoma, intratumoural injection into prostate cancer, intra tumoural injection of squamous cell carcinoma of head and neck and intrapleural delivery to non-small cell lung cancer (Bennet J *et al.*, 2002; Cozzi P *et al.*, 2001; Cozzi P *et al.*, 2002; Wong R *et al.*, 2001; Ebright M *et al.*,2002).

In some scenarios NV1020 has proven more effective at lytic replication and reducing tumour burden than another HSV mutant G207, which has been investigated in clinical trials by the same research group (see below) (Cozzi P *et al.*, 2002; Bennet J *et al.*, 2002). A phase I clinical trial administering NV1020 via the hepatic artery in patients with intrahepatic colorectal metastasis is reportedly underway (Fong Y *et al.*, 2002).

6.3.4 G207

G207 derived from wild type HSV-1 strain F is deleted in both copies of the ICP34.5 gene in addition to the inactivation of the ICP6 gene by insertion of the E-coli Lac Z gene. ICP6 encodes the large subunit of the enzyme ribonucleotide reductase, required for viral DNA synthesis. This enzyme is particularly important to support efficient viral replication in non-dividing cells such as neurones, but not in rapidly dividing cells. The double mutation was thought to confer added safety by reducing the potential for reversion to the wild type. In addition mutants deficient in ICP6 were discovered to be more sensitive to antiherpetic agents such as Acyclovir and Gancyclovir (Coen DM *et al.*, 1989).

G207 has been demonstrated to be safe following intracerebral inoculation of nude mice and new world monkeys, up to doses of 1 x 10^9 pfu. Neuro-attenuation and efficacy in terms of reduction in tumour volume and prolonged survival have been demonstrated in murine subcutaneous and intracerebral glioma xenograft models (Mineta T *et al.*, 1995). Intracerebral injection of G207 up to a dose of $1x10^9$ pfu, up to 6 log units higher than the lethal dose of wild type HSV-1, was demonstrated into be safe in non-human primates (Hunter W *et al.*, 1999). *In vitro* and *in vivo* studies demonstrated efficacy of G207 in other tumour cell types (Cozzi P *et al.*, 2001).

In a phase 1 clinical trial of 21 patients with non-histologically proven recurrent malignant glioma, G207 was delivered by intra-tumoural injection. During the study the dose of G207 was escalated to a maximum dose of 3 x 10^9 pfu. The maximum tolerated dose was not achieved. None of the patients demonstrated evidence of toxicity that could be attributed to G207. Complications that were identified amongst this cohort were considered not uncommon for such patients. In line with the protocol, tumours were volumetrically analysed. In some patients a decrease in the amount of tumour enhancement at 1month post-injection was detected. In one patient there was a 25% decrease in the enhancing mass between the post procedure scan and 1month scan.

Five of the 21 patients were seronegative for HSV specific antibody, however only one patient seroconverted. Six tumour tissue samples were obtained from patients whilst they were still alive. Two of the tumour samples were positive for HSV-1 and *lac Z* by PCR, indicative of the presence of G207. Post mortem cerebral analysis from five of the patients was unremarkable with no evidence of encephalitis.

The clinical trial confirmed G207 to be safe following intratumoural injection into human malignant glioma (Markert JM *et al.*, 2000). Further clinical studies are reportedly underway.

6.3.5 JS1/ICP34.5-/ICP47-/GM-CSF (Oncovex)

A novel virus strain was derived from a clinical isolate and deleted in both copies of the ICP34.5 gene. In addition ICP47 was deleted and the gene for GM-CSF inserted into the DNA backbone. The deletion of ICP47 changes the temporal expression of US11 to an immediate early gene, as opposed to its normal expression as a late gene, apparently enhancing the mutant's replication in tumour cells. In addition the deletion of the ICP47 gene removes the expressed protein's role of blocking antigen presentation of HSV infected cells to the immune system. It is proposed that this, coupled with the insertion of GM-CSF should maximally stimulate the immune system following the release of tumour antigens generated by lytic viral replication.

In vivo studies have demonstrated the oncolytic properties of Oncovex in addition to enhanced, tumour specific immune response following viral intratumoural injection. Oncovex also appears to have afforded some protection to the mice following re-injection with tumour cells (Liu BL *et al.*, 2003).

Liu *et al* claim that Oncovex is superior to other ICP34.5 deleted oncolytic viruses due to the fact that as a recent clinical isolate it has not been serially passaged over many years. This claim lacks clarity as elite stocks are always held at the lowest possible number of passages, as it is the number of passages undertaken, not the period of time over which they were performed that is important in the potential introduction of deleterious mutations. The claim that other ICP34.5 null mutants cannot infect, replicate and disseminate as well in human tumours as Oncovex is yet to be proved. Clinical trials began in 2002 in a number of tumour groups and the results are awaited.

7 Clinical trials in humans using HSV1716

In vitro and *in vivo* studies have demonstrated that HSV1716 is safe in immunocompetent and immunodeficient animal models. The demonstration of tumour reduction in addition to evidence of viral replication within the tumours supported the move into clinical studies.

7.1 High Grade Glioma

7.1.1 1st Phase I Trial in High Grade Glioma

The strategy of the first phase I study was to demonstrate the safety of HSV1716 following intratumoural injection of malignant gliomas in humans.

Patients enrolled in the study had confirmation of malignant glioma by intratumoural biopsy. They had been treated with surgery and radiotherapy and following relapse had received palliative chemotherapy. HSV1716 was injected via intratumoural stereotactic injection. The initial input dose for first three patients was 10^3pfu, the following three patients were injected with 10^4pfu and the remaining three patients were injected with the maximum dose, 10^5pfu. Patients were observed initially in hospital and were then followed up in the outpatient clinic. The tumours were regularly monitored using MRI and SPECT to assess tumour volume.

The dose of HSV1716 was not escalated to achieve the maximum tolerated dose, as is current practice in conventional phase 1 studies with non-biological agents such as chemotherapy. Given that HSV1716 is a replication competent virus it is proposed that following rounds of replication the final titre within the tumour could be several orders of magnitude higher than the inoculated dose. Extrapolating *in vitro* data, it was proposed that an input titre of 10^5 pfu could produce 2 x 10^6 pfu within 12 hours. Investigators felt that by using a dose of 10^5pfu under optimal conditions, a level of cell kill could be achieved that would be detected in future efficacy studies. Using a novel agent such as HSV1716, which has the potential to produce non-specific and sporadic endpoints, requires a shift from phase I clinical trial design where the maximum tolerated dose is sought (Arbuck SG, 1996).

The study demonstrated that HSV 1716 could be injected intratumourally in immunocompromised patients at a dose of 10^5pfu with no evidence of associated toxicity. One patient was seronegative for antibodies to HSV-1 prior to injection

of HSV1716 and did not sero-convert. The other eight seropositive patients did not show an elevation in IgG or IgM antibody titre following injection. Post inoculation tissue was obtained from five of the patients. Examination of the tumour and surrounding brain did not demonstrate encephalitis or evidence of ongoing virus replication. There was no evidence by immunohistochemical analysis of viral antigen or PCR evidence of viral DNA.

Although the study was not an efficacy study there have been some interesting long-term survivors. The expected survival following primary therapy is approximately 5 months, the continued survival of a number of the patients enrolled in the study is therefore of interest (Rampling R *et al.*, 2000).

Pt	Age	Sex	Diagnosis	Survival post injection (mo)
1	22	M	rGB	94*
2	48	M	rGB	10
3	62	M	rGB	3
4	34	M	rGB	26
5	41	M	rGB	6
6	63	M	rGB	6
7	37	M	AA	87*
8	65	F	rGB	38
9	56	F	rGB	2

Study to October 2005 *patient still alive

M = male rGB = recurrent Glioblastoma

F = female AA = Anaplastic Astrocytoma

***Table 7.1.1**; Patient details 1st Phase 1 Trial*

7.1.2 2nd Phase 1 Trial in High Grade Glioma

The second phase I trial was undertaken as a 'proof of principle' study. Twelve patients with recurrent and newly diagnosed malignant glioma received a single intratumoural injection of HSV1716 at a dose of 10^5 pfu. The lesion was then surgically resected between 4-9 days after the injection. Patients were observed initially in hospital and were then followed up in the outpatient clinic. The tumours were regularly monitored using MRI and SPECT to assess tumour volume.

None of the patients enrolled in the study experienced any toxicity following intratumoural injection of HSV1716. Infectious virus particles were detected in samples taken from the sites of tumour injection in two patients who were both HSV-1 seronegative. In one of the tumour samples more virus was recovered than was injected. Both patients subsequently seroconverted.

PCR analysis of tumour tissue detected HSV DNA in tumour around the injection site from ten patients. In four of the ten patients HSV DNA was detected in tumour tissue distal to the site of injection. Semiquantitative PCR in one tumour tissue sample provided evidence that virus replication had taken place. In two patients (one seronegative and one seropositive), immunohistochemical analysis of the resected tumour samples detected HSV antigen at the injection site. The evidence suggests that the presence of antibodies against HSV within the blood stream did not prevent HSV1716 from replicating within tumour tissue.

The conclusions from this study were that HSV1716 could replicate in malignant glioma *in situ* without causing toxicity. The clinical course of the patients enrolled in the study was unremarkable. None of the patients enrolled in the study is still alive (Papanastassiou V *et al.*, 2002).

Pt	Age	Sex	Diagnosis	Survival post injection (mo)
1	61	M	rGB	9
2	49	F	GB	23
3	57	F	AA	10
4	40	M	rGB	6
5	51	M	GB	26
6	40	F	GB	18
7	40	M	GB	11
8	38	M	GB	1
9	54	F	GB	13
10	64	F	rGB	13
11	51	M	rGB	4
12	47	M	GB	1

M = male rGB = recurrent Glioblastoma

F=female GB = newly diagnosed Glioblastoma

AA = Anaplastic Astrocytoma

***Table7.1.2**; Patient details 2nd Phase 1 Trial*

7.2 Phase I Trial in Melanoma

As melanocytes are derived from the neural crest during foetal development, neurotropic HSV1716 was considered a potential new treatment strategy.

Five patients, with stage 4 metastatic melanoma with peripheral tissue lesions were enrolled. In two patients the tumour nodules were injected once with 10^3 pfu. The nodules in two further patients were injected with 10^3pfu on two separate occasions. The fifth patient had two separate tumour nodules injected on four occasions with 10^3pfu.

None of the patients experienced any toxicity associated with the intra-tumoural injection of HSV1716. Clinical examination revealed flattening of both tumour nodules in the fifth patient who had received four injections of HSV1716 into two

tumour nodules. There was no evidence of morphological change in any of the other four tumour nodules from the preceding patients. All of the patients were seropositive for HSV prior to the HSV1716 injection and there was no substantial change in their IgG or IgM titres during the trial.

On pathological analysis the tumour nodules of the three patients who received more than one injection were noted to have microscopic evidence of tumour necrosis. There was no evidence of necrosis in the adjacent normal tissue.

Immunohistochemical analysis of all the tumour nodules demonstrated positive staining for HSV antigen confined to the tumour. There was no staining for HSV antigen seen in the surrounding normal tissue. None of the samples demonstrated evidence of live virus.

This study demonstrated that HSV1716 could replicate safely in melanoma cells whilst leaving the surrounding normal skin cells unharmed. The extent of the immunohistochemical staining suggested that the flattening of the tumour nodules was due to HSV1716 induced tumour necrosis (Mackie R *et al.*, 2001).

7.3 Phase I Trial in Head and Neck (Squamous Cell) carcinoma

In a study of twenty patients with oral squamous cell carcinoma, HSV1716 was injected directly into the lesion. The tumours of the first five patients were injected with $1x10^5$pfu and resected at 24 hours. The next five patients tumours were injected with $5x10^5$ pfu and then resected at 24 hours. The remaining ten patients all received an intratumoural dose of $5x10^5$ pfu with five tumours resected at 72 hours and the remaining five resected 2 weeks after the initial injection.

The procedure was well tolerated and there was no suggestion of toxicity associated with the intratumoural injection of HSV1716. Two of the patients were seronegative and the other 18 were seropositive. The two seronegative patients

seroconverted between the 4^{th} and 5^{th} week post intratumoural injection of $5x10^5$ pfu.

PCR analysis detected HSV DNA in 2 tumours at the injection site and in one HSV DNA was also detected distal to the site of injection. Tumour samples analysed by either immunohistochemistry or *in situ* hybridisation failed to detect any evidence of HSV.

It has been demonstrated that HSV1716 is safe following intratumoural injection in patients with oral squamous cell carcinoma. The complete results of the trial are awaited (Mace A, personal communication).

8 Ionising Radiation

Ionising radiation has been used in the treatment of cancer for over a century. X and γ rays constitute part of the electromagnetic spectrum. Quanta of energy are known as photons. Ionisation occurs when a photon of radiation has sufficient energy to eject one or more orbital electron from the atom or molecule. The photon of energy transferred to the molecule or atom may not displace an electron but rather excite it to a different energy level in a process called excitation.

X and γ rays transfer their energy to the attenuating material and subsequently generate biological damage. In direct action the radiation is absorbed by the target DNA and disrupted. More frequently the damage is caused by indirect action.

In indirect radiation damage, photon energies used for example in the treatment of cancer produced by a cobalt-60 unit or linear accelerator, generate Compton scattering. This is where the incident energy is transferred to an electron as kinetic energy. The electrons produced are usually from H_20 molecules and result in the production of radical ions.

$$H_2O \longrightarrow H_2O^+ + e^-$$

This charged radical ion is very short lived and decays to an uncharged free radical.

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + OH^*$$

The incident photon proceeds with reduced energy in an altered direction to generate more reactions. The production of large numbers of electrons within the absorbed material generates many more free radicals through various different reactions. The production of free radicals, in particular H_2O_2 and HO_2^* generates the damage to nuclear DNA (Hall E, 2000).

DNA is the main target of radiation to produce a biological effect (Warters RL *et al.*, 1997 Warters RL *et al.*, 1992). A break of only one DNA strand, a single strand break (SSB), appears to be readily repaired and of little biological significance. Less common yet more biologically significant is when both strands are broken, a double strand break (DSB). The repair of a DSB is complicated and can either occur by homologous recombination or non-homologous recombination. The predominant process in mammalian cells is non-homologous recombination, which is prone to erroneous repair. Homologous recombination is known to be involved in cell signalling and is also regulated by the cell cycle (Valerie K and Povirk L, 2003).

The type of nuclear damage as well as the cellular repair process determines the response to ionising radiation. Cell death can occur via necrosis, apoptosis or mitotic death. Necrosis is a passive process in contrast to the programmed cascade of events identified in apoptotic cell death. Mitotic death occurs, when due to damaged chromosomes, mitosis fails to be successful (Cohen-Johnathan E *et al*, 1999).

The sensitivity to radiation is influenced by the phase and progression of the cell cycle. If the damage exerted by the radiation can be repaired, the cell replication cycle will halt until repair is complete and then the cell cycle can continue. Ionising radiation can induce arrests in G_1, S and G_2 phases of the cycle to enable repair process to occur. The G_1 check point prevents the replication of damaged

DNA before entry into S phase. The G_2 check point (which is divided into early and late G_2/M) (Xu B *et al.*, 2002) prevents the segregation of aberrant chromosomes during M phase (Kaufman WK and Paules R, 1996) If the damage is beyond repair then the apoptosis pathway machinery will be activated.

Ionising radiation induces the protein kinase activity of the ATM gene, which then phosphorylates many down stream targets such as p53, MDM2, CHK2 and BRCA1, which are involved in controlling cell cycle progression (Samuel T *et al.*, 2002). Through the ATM gene the activation of p53 has been shown to induce apoptosis in addition to regulating the cell cycle to enable DNA repair. The induction of either pathway by p53 is a complicated matter and appears to be related to the quantity of the protein present at the time of DNA damage. If the cell is committed to apoptosis, then p53 activates the pro-apoptotic BAX protein. BAX protein in turn initiates the release of factors such as cytochrome C from the mitochondria. Cytochrome C then activates the caspase cascade, which leads ultimately to cell death (Mihara M *et al.*, 2003).

p53 induced cell cycle arrest is mediated through downstream activators such as growth arrest and DNA damage (GADD45) and p21, a Cip/Kip family of Cdk inhibitors. p21 protein binds to the cyclin-E-CDK2 complex and by hypophosphorylating the complex, arrests the cell cycle at the G_1/S transition (Samuel T *et al.*, 2002). In addition p53 appears to be required to sustain G_2/M arrest after DNA damage (Bunz F *et al.*, 1998). The ATM-CHK pathway is involved in the initiation of G_2 arrest. These various check points allow repair of DNA to occur.

For decades it has been thought that the response to radiation may be related to the phase of the cell cycle. Several methods have been used to synchronise cells and these studies have identified the phases of the cell cycle that differ in their response to radiation. Maximal response to radiation has been identified during mitosis and G_2, less response during G_1 and least response during the later stages of S phase. Cells that are in a resting state, G_0, are found to be radio-resistant (Sinclair W *et al.*, 1968). It has been considered that using chemotherapeutic agents to synchronise cells into sensitive phases of the cell cycle could enhance

the effect of ionising radiation (Minarik L and Hall EJ, 1994). Although, *in vitro* and *in vivo* data has been interesting there has been little clinical success (Lawrence TS., 2003). Ionising radiation itself causes cells to accumulate in the G_2 phase of the cell cycle preventing further mitotic division. This accumulation in G_2 appears to be dose related. In addition, low dose radiation has also been shown to induce hypersensitivity in various cell lines (Bartkowiak D *et al.*, 2001). Fractionation of radiation has been shown to synchronise previously asynchronous cells into more sensitive phases of the cell cycle resulting in the cell population being more at risk (Geldolf AA *et al.*, 2003). Although the phase of the cell cycle clearly plays a role in the cell population response to radiation it appears that the different cell lines have different inherent radio-sensitivities (Deschavanne PJ and Fertil B., 1996).

Radiotherapy remains the mainstay of clinical management in the majority of tumours and in particular has shown to be effective in treating high grade gliomas. Consequently we have sought to combine ionising radiation with oncolytic viruses *in vitro* and *in vivo*.

9 Combination of Oncolytic Viruses with Conventional Therapy

Despite multi-modality treatment, no combination of surgery, radiotherapy or chemotherapy has proven effective for the treatment of HGG. Toxicity to normal tissue associated with the delivery of high doses of ionising radiation and chemotherapy limits the doses that can be used. Phase I studies using HSV mutants have failed to demonstrate any associated toxicity and combining the modalities should not, in theory, prove more toxic to the patient. It is noteworthy that unlike chemotherapy, which declines over time as the drug is excreted, the influence of a single dose of oncolytic virus should, under optimal conditions, increase over time as viral replication proceeds and progeny virus are produced.

9.1 Chemotherapy

Toyoizumi *et al* investigated the interaction between HSV1716 and four different chemotherapies in five different lung cancer cell lines. *In vitro* cytotoxicity assays demonstrated that HSV1716 exerted oncolytic activity in all the cell lines. In one of the cell lines, a synergistic relationship was detected when HSV1716 was combined with the chemotherapeutic agent, Mitomycin C (MMC). An additive relationship was demonstrated between HSV1716 and the other three chemotherapies. None of the chemotherapies was deleterious to HSV1716. The synergy demonstrated between MMC and HSV1716 was investigated in four different cell lines. In one cell line there was a synergistic relationship and an additive relationship with the other three. An *in vivo* murine xenograft model demonstrated an additive oncolytic effect between HSV1716 and MMC (Toyoizumi T *et al.*, 1999).

G207 has demonstrated enhanced cell killing of rhabdomyosarcoma cell lines *in vitro* and *in vivo* when combined with Vincristine chemotherapy. The ability of G207 to infect and replicate was not hampered by the presence of Vincristine (Cinatl J *et al.*, 2003).

The combination of G207 and Cisplatin demonstrated additive cell kill in murine xenografts of squamous cell carcinoma cells known to be sensitive to Cisplatin. Another squamous cell carcinoma cell line known to be weakly sensitive to Cisplatin demonstrated no increased cell kill over G207 alone. This observation suggested that G207 and chemotherapy were acting independently. There was no additional toxicity when the two modalities were combined prompting the authors to propose further research with other combination therapies (Chahlavi A *et al.*, 1999).

Recent *in vitro* studies have demonstrated that HSV1716 is able to undergo lytic replication in a number of squamous cell carcinomas cell lines derived from the head and neck. In addition, isobologram analysis has shown an additive relationship in terms of cell kill between HSV1716 and Cisplatin chemotherapy in these cell lines (Mace A, personal comunication).

The observation of additive and on occasion supra-additive cell kill when modalities commonly used in the treatment of cancer are combined with oncolytic agents is encouraging.

9.2 Ionising Radiation

Radiotherapy is the most effective treatment currently available in the management of malignant glioma. HSV null mutants, including HSV1716, have been shown to infect, replicate and lyse malignant glioma cells *in vitro* and *in vivo*. The combination of the two modalities, killing tumour cells through different mechanisms may improve the outlook for patients with this disease without additional toxicity. Ideally if the two modalities were to have a synergistic relationship with regard to cell kill this would offer the patient the best chance of an improvement in overall survival.

The combination of large doses of external beam radiation and R3616 in nude mice glioma xenografts demonstrated enhanced tumour kill when compared to either modality in isolation. Human U-87 malignant glioma xenografts in mice

were inoculated with $2x10^7$ pfu of the HSV mutant R3616, and after 24 or 48 hours were exposed to ionising radiation. The dose of radiation used was 45Gy, delivered over two fractions of 20Gy and then 25Gy. In another cohort of mice xenografts were injected with $2x10^7$ pfu on three consecutive days. The tumours were irradiated four hours after the second and third doses of virus. The doses that were used were far in excess of dose fractions used in clinical practice, which are in the order of 1.8-2Gy. The authors argued however, that doses of this magnitude were currently used in stereotactic radio-surgery. Each day the volume of the tumours was measured and was considered to have regressed if there was a 10% reduction in volume. Total regression was seen in 22 out of 33 treated mice. The results demonstrated a significant interaction between R3616 and ionising radiation in terms of tumour regression. It was shown that tumour reduction was greater when the HSV mutant and radiation were combined than the sum of either treatment in isolation leading the authors to conclude that the observed relationship was synergistic. In addition R3616 grew to a higher titre in irradiated tumours than in non-irradiated tumours. It was proposed that the enhanced effect following the combination was due to increased viral replication and dissemination of R3616 within the tumour (Advani SJ *et al.*, 1998). When the mutant R7020 was used the virus yield obtained from irradiated glioma cells was significantly higher than obtained with R3616. This is probably a reflection of the fact that R3616 is more attenuated than R7020.

Another study using a mouse xenograft model investigated the less attenuated HSV mutant R7020. In this study a different radiation schedule was adopted that more closely reflects the application of radiation in clinical practice. The xenografts were irradiated using 4Gy on Monday, Tuesday, Thursday and Friday over two weeks to a total of 32Gy. The first radiation dose was given 6 hours after inoculation with $2x10^7$pfu of R7020. By using a combination of R7020 and ionising radiation the same group demonstrated a three fold volume reduction in chemo- and radio-resistant human epidermoid cell tumours compared to using virus or radiation alone (Advani SJ *et al.*, 1999).

Bradley *et al.* combined ionising radiation with R3616 in a hind limb and intracranial glioma xenograft model. The hind limbs of the mice were irradiated

using the same schedule as detailed by Advani *et al.* (1999) up to a dose of 40Gy. The intracranial gliomas were irradiated three times a week for two weeks, to give 6 fractions of 5Gy totalling 30Gy. The first radiation dose was delivered 6 hours after the virus inoculation. In this series of experiments the authors were again able to demonstrate enhanced tumour growth delay with the combination of radiation and R3616 compared to either modality given in isolation. Statistical analysis of the intra cranial xenograft model indicated that the interaction was synergistic. Immunohistochemical analysis demonstrated R3616 in both irradiated and non- irradiated tumours one day following virus inoculation. By three days the unirradiated tumours did not stain for R3616. In contrast at 12 days post virus injection the irradiated tumours demonstrated the presence of virus antigen (Bradley J *et al.*, 1999).

In contrast to the above studies, there was no additive or synergistic enhanced kill when G207 (delivered either intratumourally or intravenously) was combined with radiation in prostatic carcinoma xenografts in athymic mice. Prostate carcinoma xenografts in mice treated with intravenous G207 alone regressed slower than when treated with intravenous G207 followed by fractionated radiation. By the end of the experimental period there was however no difference in the final tumour volumes or recurrence rates demonstrated between the virus alone, radiation alone and combination cohorts (Jorgensen TJ *et al.*, 2001).

The scheduling of virus and radiation in this study was not identical to the studies where synergy had been observed. The authors did not feel however that the minor temporal differences between protocols could explain the failure to demonstrate a synergistic relationship between G207 and ionising radiation. They proposed that the observed differences were explained by differences in the tumour biology of different tumour cell types. In this study although radiation did not enhance the effects that G207 had on the tumour, it was not demonstrated to be deleterious. The authors noted that the xenografts remained sensitive to the effects of G207 following irradiation and from this observation they concluded that viral oncolytic therapy may best be reserved for clinical use after the failure of first line therapy such as radiation (Jorgensen T *et al.*, 2001).

Spear *et al.* (2000) investigated the interaction with respect to cell kill between radiation and the HSV mutant hrR3 or wild type HSV strain KOS, in pancreatic carcinoma, glioblastoma and cervical carcinoma cell lines using an *in vitro* cytotoxicity assay. Cell survival was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. In their *in vitro* experiments the cells were irradiated 2 hours prior to the addition of virus. Radiation, of various doses, combined with the various MOI of virus resulted in increased cell kill compared to either modality used in isolation. The observed relationship was however described as additive and not synergistic. A single cycle growth curve demonstrated an increase in viral yield from irradiated pancreatic carcinoma cells compared to non-irradiated cells.

These results were in conflict with the *in vivo* observations made by Advani *et al.* (1999) and Bradley *et al.* (1999). A number of reasons were proposed to explain this dichotomy. In the first instance they suggested that the *in vitro* experimental conditions and the assays used to demonstrate the additive relationship were not appropriate to detect a synergistic relationship between a replication competent virus and radiation. Secondly, hrR3, unlike R3616, is not dependent on cellular mammalian ribonucleotide reductase (mRR) for replication, therefore the induced elevation of cellular mRR following irradiation thought to enhance the replication and growth of R3616 was not necessary for hrR3 replication. Spear *et al* also proposed that the hrR3 may already have been replicating at full efficiency, thus the induction of cellular proteins due to ionising radiation may not have influenced the replication of hrR3.

In response to the observed synergistic relationship demonstrated with R3616 and ionising radiation Advani *et al* theorised that ionising radiation resulted in an elevated level of the cellular protein GADD34. GADD34 is known to complement the HSV replication viral protein ICP34.5 and may have supported the growth and replication of R3616, which is deficient in ICP34.5. As hrR3 is not mutated in ICP34.5, the induction of GADD34 following radiation would probably not enhance its lytic replication.

Spear *et al.* (2000) also suggested that the absence of a synergistic relationship between hrR3 and radiation was perhaps due to the inability to translate the relationship detected with other HSV mutants from an *in vivo* model to an *in vitro* model. The nude mice used in these experiments had some residual immune function. Irradiating the nude mice may have caused additional local immune suppression secondary to leukocyte or other effector cell toxicity enough to enabled the attenuated virus to replicate more efficiently and cause a more wide spread lytic infection. Alternatively the high doses of radiation employed by Advani *et al*, in the 1998 study may have caused the tumour xenografts to be sterilised. If there were static tumour volumes amongst the irradiated, non-virus infected, xenografts, combination with oncolytic virus might therefore be able to demonstrate tumour regression. It is also possible that cellular replication of the non-irradiated xenografts outpaced the rate of viral lytic replication thereby masking the effect of viral oncolysis when R3616 was administered in isolation.

HSV mutants, G207, R3616 and R7020 demonstrated cell kill of cervical cancer cells *in vitro*. The HSV mutants all generated cell kill through lytic replication. G207 was investigated *in vivo* and generated a significant reduction in tumour volume of mouse xenografts, which was further enhanced by additional injections of virus (Blank S *et al.*, 2002). Using a cytotoxicity assay, supra-additive cell kill was achieved when G207 or R3616 was administered one hour after low dose radiation (1.5Gy or 3Gy) in cervical carcinoma cell cultures. An additive relationship was demonstrated when the same cell line was irradiated with the low dose radiation one hour prior to infection with R7020. Ionising radiation was also noted to enhance the replication of G207. A single cycle growth experiment with G207 in pre-irradiated cells resulted in a 4.45 fold increase in burst size compared to non-irradiated cells (Blank S *et al.*, 2002). The data to support this were not presented and the authors omitted to indicate in which cell line this had been seen. Furthermore the increased burst size was noted after 48 hours, which in fact represents multi-cycle growth. *In vivo* data demonstrated a significant reduction of cervical carcinoma xenografts in nude mice following treatment with low dose radiation (3Gy) one hour prior to G207 inoculation.

Stanziale *et al* were the first group to demonstrate a relationship between G207 and radiation in cell culture using a cytotoxicity assay (Stanziale S *et al.*, 2002). In HCT-8 human colorectal cancer cells the cell kill generated by a combination of ionising radiation and G207 was greater than the calculated additive effect of each modality delivered individually. In this study, the tissue culture cells were irradiated immediately prior to virus infection. Using the same assay, it was also demonstrated that R3616 caused significant cell kill on its own. The authors stated that when R3616 and radiation were combined there was additional cell kill compared to the virus alone, however they did not specify if there was infra-additive, additive or supra-additive cell kill compared to the cell kill produced by radiation alone. The cell kill achieved with G207 on its own was significantly less than with R3616. When G207 was combined with radiation, the additional cell kill was more significant than with R3616 as G207 is clearly more attenuated than R3616.

A number of reasons for the increased cytotoxicty from combining ionising radiation and viral treatments were proposed. In the first instance a higher titre of G207 was achieved from a multicycle growth curve using irradiated HCT-8 cells than from un-irradiated HCT-8 cells. In addition the group demonstrated that the activity of cellular RR, necessary for G207 DNA synthesis, was demonstrated to be elevated 12 hours after exposure to radiation, but returned to pre-radiation levels by 24 hours. They also showed that following a radiation dose of 5Gy the expression of GADD34 was elevated, even 72 hours later. These results led the authors to postulate that the elevated levels of GADD34 induced by the radiation may, as a result of its homology with ICP34.5, prevent host cell synthesis shut off, thereby ensuring continued production of cellular proteins able to support a G207 lytic replication (Stanziale S *et al.*, 2002).

Ionising radiation was seen to enhance the replication of the HSV mutant R7020 in hepatoma xenografts (Chung S-M *et al.*, 2002). Tumour bearing flanks were injected with 2×10^7pfu of R7020 and irradiated 6 hours after infection. At various intervals the tumours were excised and the viral titres determined. The viral titres were higher in the irradiated tumours of two different tumour cell types, Hep3B and Huh7 cells, however the difference was only statistically

significant in the Hep3B cell type. Additionally, only Hep3B cells showed a greater reduction in tumour volume with a combination of R7020 and radiation than with either in isolation. Enhanced cell kill when the two modalities were combined was considered to be cell type and cell state dependent. The authors proposed that the lack of response in the Huh7 xenografts was due to the growth rate of the cells in this tumour outstripping the rate of viral lysis and lack of efficient spread of the virus from the injection site (Chung S-M *et al.*, 2002).

The mechanism of action for the documented synergistic association between ionising radiation and various replication competent HSV mutants is unknown. Stanziale *et al* demonstrated a rise in the activity of cellular ribonucleotide reductase in cells after exposure to radiation. They also demonstrated that by antagonising RR with hydroxyurea, viral production was inhibited (Stanziale S *et al.*, 2002). This adds weight to the proposition by those investigating RR mutants that an increase in cellular RR following ionising radiation can complement the defect in the virus thereby facilitating viral replication and the production of progeny virus to continue the infection further. The premise that radiation induces cellular genes encoding proteins that complement the defect in the virus is an attractive theory, although it appears that the association may be cell type specific.

At the outset of the research project, towards the end of 2000, no association between HSV1716 lytic replication and ionising radiation had been demonstrated. It was considered important to investigate this potential interaction, especially as HSV1716 was the replication competent HSV mutant in the most advanced clinical trials. Furthermore, there was no published data assessing the relationship between replication competent viruses and ionising radiation in an *in vitro* assay. The establishment of a reproducible *in vitro* assay that could determine the relationship between the replication competent ICP34.5 null mutant HSV1716 and ionising radiation in terms of cell kill formed the basis of the initial portion of the laboratory based research project.

The results presented in this thesis are separated into three sections detailing the three different strands that comprise the research that was undertaken.

The aims of this thesis are separated into three sections detailing the three different strands that comprise the research that was undertaken.

1. The aim of the first section was to investigate the safety of HSV1716 when injected into the brain adjacent to tumour following resection of high grade glioma in patients who then proceeded to receive further immunosuppressive therapy.
2. The aim of the second body of work was to develop a tissue culture system to investigate the possibility of enhanced cell kill when ionising radiation is employed in conjunction with the ICP34.5 null mutant HSV1716 and if possible to elucidate the mechanisms responsible for the enhanced cell kill.
3. The third section of research was undertaken to investigate the reasons behind the selective replication phenotype of HSV 34.5 null mutants in different cell types and states. The strategy involved the development of stable cell lines expressing ICP34.5 with the aim of elucidating the mechanism of action of the ICP34.5 in the replication cycle of HSV.

Each body of work will be discussed individually.

Chapter 2

Methods and Materials

10 Clinical Trial Methods

10.1 Recruitment and Selection

The Institute of Neurological Sciences, sited at the Southern General Hospital in the South of Glasgow, is a tertiary neurosurgical referral centre serving the West of Scotland. The neurosurgical department receives approximately 150 new referrals each year of patients with a diagnosis suggestive of intracranial malignancy. Further investigation, imaging and surgical intervention, will reveal that approximately 75 of these new referrals will be primary high-grade glioma.

The majority of these patients are referred to the Beatson Oncology Centre in Glasgow for consideration of further treatment; radiotherapy or chemotherapy, under the care of Professor R. Rampling (Professor of Neuro-Oncology). The Beatson Oncology Centre clinicians liase closely with the Neuro-Oncology surgeons and in particular Mr. V Papanastassiou. It is from this managed clinical network, that the ten of patients were selected.

Two patients were recruited from other national centres either as a result of direct clinician to clinician referral or direct patient contact with one of the principal investigators.

10.2 Inclusion criteria

1. Patients with histologically confirmed high grade glioma previously treated with surgery and radiotherapy and who were scheduled to undergo tumour decompression for recurrence.

2. New patients with a clinical and radiological diagnosis of high grade glioma based on an appropriate clinical history, examination and imaging consisting of contrast enhanced CT or MRI. These images were interpreted by an independent neuroradiologist as showing a solitary malignant intracranial lesion suggestive of high-grade glioma.
3. Patients previously treated for low-grade glioma considered to have tumour recurrence suggestive of transformation to a high-grade lesion based on clinical history, examination and imaging. These patients were included in the study as newly diagnosed patients as they had no prior diagnosis or treatment for high grade malignancy.
4. Patients suitable for surgical resection.
5. Tumour bed amenable to injection with HSV1716 without risk of intraventricular injection.
6. Supratentorial tumour.
7. Age between 18 and 70 years.
8. Karnofsky status (KS) > 60.
9. Life expectancy > 8 weeks.
10. Able to give informed consent to take part in the research study.
11. Haematology

    Hb > 10 gm/dl

    WBC > 3×10^9 (neutrophils > 1.0×10^9)

    Platelets > 100×10^9/L
12. Renal function:

    Creatinine: < 1.5 mg/dl
13. Hepatic function:

    Bilirubin < 1.5 x upper limit of normal

    AST; ALT; < 2 x upper limit of normal
14. Coagulation function

    PT/ INR; APTT normal

10.3 Exclusion Criteria

1. Active acute infection
2. Other severe systemic disease
3. Pregnancy. (All fertile patients were using a reliable method of contraception throughout the duration of the trial.)
4. Previous treatment with viral therapy of any kind.

10.4 Pre-operative Investigations

10.4.1 Within Two Weeks of Operation

The following clinical details were compiled within 2 weeks of the proposed surgery and injection of HSV1716:

- Clinical history;
  - Details of presenting complaint
  - Past medical history
  - Details of current medication
  - General systemic enquiry
  - Details of social circumstances
- Clinical examination;
  - General systemic examination
  - Detailed neurological examination
- Laboratory investigations;
  - Haematology - Hb; WBC and differential; Platelets
  - Biochemistry - Urea; Creatinine; Na; K; Cl; $HC0_3$
  - Hepatic function - Bilirubin; AST; ALT; Alkaline Phosphatase; Protein
  - Clotting function - PT/INR; APTT
  - Serum antibody to herpes virus - Quantitative serum gamma globulin levels
  - Urinalysis
  - Whole blood phenotype

- Non-laboratory based investigations; Electrocardiogram
- Non- neurological radiological imaging; Chest x-ray

10.4.2 One Week Prior to Operation

The following investigations were performed within one week of proposed virus injection:

Neuro-radiological imaging;

- Magnetic Resonance Imaging (MRI) / Computed Tomography (CT)
  Contrast enhanced MRI was the preferred imaging modality however some patients had a contrast enhanced CT scan as they were not able to tolerate the MRI due to noise or feelings of claustrophobia.

- ^{99m}Tc-HMPAO SPECT
  Each patient underwent a routine ^{99m}Tc-HMPAO SPECT scan. This scan was used as baseline to allow identification of any areas of hyperperfusion in subsequent scans that would suggest herpes simplex encephalitis. Thus, in the clinical situation if encephalitis was suspected post HSV1716 administration a ^{99m}Tc-HMPAO SPECT scan was performed. In a wild type herpes simplex encephalitis, the characteristic acute appearance is temporal and frontal lobe hyperperfusion.

10.4.3 Twenty-four Hours Prior to Operation

The following investigations were performed after admission to the ward within 24 hours before the procedure:

- Clinical history and examination (as detailed above)
- Laboratory investigations;
  Haematology - as detailed above
  Biochemistry - as detailed above
  Hepatic function - as detailed above
  Viral shedding assay - blood and buccal mucosa samples detailed below

10.5 Informed Consent

Each patient was reviewed by an independent clinician to ensure that they understood the trial fully and were competent to give informed consent. This clinician was a neurologist who was not associated with the study in any other way. Consent was only obtained from the patient when the independent clinician was confident that the patient was competent to fully understand the proposed procedure and its potential complications. Initial contact was made between one of the principal investigators and the patient. Written information was left with the patient and only after a minimum period of 24 hours was written consent obtained. Consent was obtained for entry into the study and additional consent was obtained for the operative procedure.

10.6 Operative Procedure

On completion of the pre-operative investigations, provided the patient was still eligible, he or she was reviewed by an anaesthetist to ensure that they were suitable to undergo general anaesthesia. At the time of operation, anaesthesia was induced. The head was shaved and a skin flap incision made. A craniotomy was performed followed by opening of the dura. The tumour was identified macroscopically. If this was not possible, then the tumour was identified with the aid of the Brain Lab Image Guided Surgery System. Access to the tumour was obtained by selective cerebrotomy with limited brain retraction. On visualising the tumour at operation, a biopsy was sent to neuropathology for intra-operative confirmation of high grade glioma by smear examination. The tumour was decompressed by central evacuation of the tumour core by suction. On removal of the central bulk of the lesion, the lining of the tumour cavity was debulked with the aid of the operating microscope and a cavitron ultrasonic aspirator. Following the removal of the tumour bulk, a resection cavity remained.

Immediately prior to the completion of the tumour resection the vial containing the virus was removed from storage and thawed rapidly to room temperature. The vial was inverted until the contents were mixed. A total volume of 1ml containing

$1x10^5$ pfu HSV1716 was drawn into a 1ml Becton and Dickinson Micro-Fine insulin syringe. The 1ml aliquot of HSV1716 was then injected in 0.1- 0.2 ml aliquots at a depth of 5-10mm in approximately 8-10 sites around the resection cavity wall.

Any evident haemorrhage was controlled prior to closing the craniotomy wound. The anaesthetic was reversed and the patient recovered in theatre recovery area. Vital observations and neurological function were recorded at 15-minute intervals for the first hour. Once the patient was considered clinically stable they were transferred to the neurosurgical ward where they were nursed in relative isolation for 7 days.

All operative intervention was undertaken by the principle investigating surgeon, Mr Papanastassiou. The aim of this was to ensure that the surgical technique was as consistent as possible.

10.7 Post-operative Assessment

The patients were monitored on the ward and recordings of pulse, blood pressure, temperature and neurological status were recorded 4 hourly. A general and neurological examination was performed twice daily. Blood samples were drawn for haematology and biochemistry analysis, a virus shedding assay and IgG and IgM quantification to detect any evidence of HSV1716 activity.

MRI, or CT (when MRI was not tolerated by the patient) with and without contrast was performed within the first three post-operative days. Thallium-201 and $^{99m}Tc^-$ HMPAO SPECT scans were performed no later than the sixth post-operative day to detect any evidence of virus activity at the site of injection.

Provided that the patient was well and the investigations were satisfactory, he or she was discharged on the seventh post-operative day either to home or for further respite care.

10.8 Long Term Follow Up

In line with the protocol, the patients were reviewed weekly for four weeks following discharge. In the case of two patients, this was not possible as they lived out with an acceptable distance to travel for review. In these cases, the clinical condition was sought by telephone directly from the patient and verified with their general practitioner. At each clinic visit, a clinical history and general examination including a neurological assessment was performed, as were routine laboratory investigations (detailed previously). In addition, blood samples were taken to detect HSV DNA, and immunoglobulins as well as to perform a virus shedding assay.

Patients with recurrent disease (N=6) were offered chemotherapy. Patients with newly diagnosed disease (N=6) received cranial radiotherapy. Patients were additionally reviewed during adjuvant treatment in line with local clinical guidelines. These clinical assessments were designed to detect any possible interaction between the injection of HSV1716 and conventional treatment modalities.

After the first four weekly review period patients underwent a comprehensive clinical review, routine laboratory investigations and analysis to assess virus activity. This was repeated monthly for a further two months. MRI or CT (with and without contrast), Thallium-201 and $^{99m}Tc^{-}$ HMPAO SPECT scans were performed between six weeks and two months following surgical resection and HSV1716 injection.

Patients then underwent review every two months for one year, three monthly for the next six months and six monthly thereafter. At any time between reviews dictated by protocol, patients were reviewed when clinically indicated and appropriate investigations were performed. Review with imaging was kept in line with protocol until either evidence of progression clinically and/or radiologically, or death until February 2003.

10.9 Toxicity Assessment

This study was primarily a phase I toxicity study. Each patient was assessed for evidence of toxicity associated with the administration of HSV1716. Assessment for evidence of adverse events commenced immediately following the injection of HSV1716. Any adverse event experienced by a patient was recorded.

Each adverse event was categorised by the clinical team to be related to;

- the administration of HSV1716.
- disease process.
- adjuvant therapy (Radiotherapy / Chemotherapy).
- unknown, where no cause could be conclusively identified.

Toxicity was assessed and recorded in relation to the Common Toxicity Criteria issued by the National Cancer Institute.

Serious adverse events (SAE), which constituted a toxicity of grade 3 or higher according to the NCI Common toxicities criteria, were reported to the Local Regional Ethics Committee (LREC) and to Gene Therapy Advisory Committee (GTAC).

10.10 Markers of Clinical Performance

At each clinical assessment the patient's quality of life was assessed. The tests were designed to be simple and to generate the minimum amount of inconvenience for the patient. Detailed and time consuming tests were thought to be inappropriate given the burden of time already imposed on the patient.

As part of the neurological examination a mini mental state examination was performed to detect any change in cognitive function (appendix 1). In addition an overall level of function was assessed at each visit and scored according to the Karnofsky Rating Scale (Karnofsky D *et al.*, 1948), which is used extensively in the assessment of patients with chronic conditions and malignant disease

(appendix 2). The Barthel Score was used to assess the patient's functional abilities at each clinical assessment (appendix 3).

10.11 Assessment of Response

Although this was primarily a Phase 1 toxicity assessment it was possible to monitor patients for evidence of response. Time to tumour progression and overall survival were recorded and compared to expected survival times for such a cohort.

Patients were monitored throughout the study with regular imaging. All routine imaging was undertaken in the clinical neuroradiological department at the Institute of Neurological Sciences. When clinical circumstances dictated, imaging was undertaken at other centres. All images taken out-with the Institute of Neurological Sciences were reviewed by the neuroradiologist involved in the clinical trial.

Imaging involved MRI (or CT if the patient could not tolerate MRI due to claustrophobia), Thallium-201 SPECT and ^{99m}TcHMPAO SPECT. The MRI (or CT) and Thallium-201 SPECT scans were used to detect any evidence of tumour recurrence.

10.11.1 MRI / CT

MRI is the preferred modality to CT for a number of reasons. Firstly it is more sensitive in detecting blood brain barrier break down indicative of tumour growth, secondly T2-weighted sequences allow better identification of infiltrated brain and lastly MRI is not subject to the bone induced artefact which affects CT.

Pre and post gadolinium (Gd) enhanced 3D T1-weighted MRI volume collection was made. The volume of the tumour was determined using a slice by slice 'Region of Interest' (ROI) area measurement technique where;

$$\text{Volume} = (\text{area} \times \text{slice spacing})$$

Tumour edge detection has a crucial influence on the measurement of volume and was therefore only defined by an experienced imaging technician. Actual tumour edge is difficult to measure, the fluctuating margin associated with neovascularity induced by the tumour was taken as the surrogate marker of bulk cell division border. Tumour volume included all areas of Gd enhancement within the brain, including all separate "islands".

Each patient underwent imaging pre and post-operatively. Thereafter they underwent detailed imaging at two monthly intervals for the first year and six monthly intervals thereafter. When clinically indicated, patients underwent imaging out with protocol guidelines.

The main aim of the imaging was to assess time to progression. Progression was confirmed when two INS neuro-radiologists agreed that the MRI or CT films unequivocally demonstrated one of the following;

1. 25% increase in the volume of contrast enhancing tumour
2. a new mass lesion was identified.

10.11.2 Thallium-201 SPECT

In addition to MRI / CT, patients had Thallium-201 SPECT imaging. When progression as determined by MRI / CT was equivocal, Thallium-201 SPECT was used to confirm or refute the finding. Thallium-201 SPECT is not affected by non-neoplastic processes and can more specifically identify high-grade tumour recurrence.

Thallium-201 SPECT provides a semi-quantitative measurement of tumour volume using the same slice by slice ROI area measurement techniques used with MRI. The tumour edge was defined using a threshold technique as follows;

1. Normal brain uptake was assessed in the contralateral hemisphere in at least three slices at the same anatomical level as the tumour.
2. All the areas in the brain were outlined when the uptake exceeded the threshold level (determined as being 1.7 x normal).

3. The total area of the brain above threshold was calculated for each slice (excluding areas considered to represent normal uptake e.g. in the choroid plexus or the extracerebral soft tissue).

10.11.3 ^{99m}Tc-HMPAO SPECT

Each patient underwent a routine ^{99m}Tc-HMPAO SPECT scan. This scan was used to identify any areas of hyperperfusion that might be associated with herpes simplex encephalitis. Thus when encephalitis was suspected post HSV1716 administration, a ^{99m}Tc-HMPAO SPECT scan was performed. In a wild type herpes simplex encephalitis, the characteristic appearance is temporal and frontal lobe hyperperfusion. Similar patterns were sought on reviewing the images.

10.12 Serum Extraction

The blood samples were spun at 2000 revolutions per minute (rpm) in a bench-top Beckman-type centrifuge for five minutes at room temperature. The upper serum layer was removed and stored at –70 ^{0}C. The remaining whole blood was stored at –20 ^{0}C.

10.13 Enzyme Linked Immunosorbent Assay (ELISA)

Serum obtained by the method above was assayed using the Virotech kit 'Herpes simplex virus type-1 ELISA for the detection of human IgG and IgM antibodies'. Ten microlitres (µl) of serum was diluted in 1ml of dilution buffer. 100µl aliquots of diluted serum were pipetted into antigen coated microtitre wells. The wells were incubated at 37^{0}C for 30 minutes to enable immune complex formation. The wells were then washed four times with washing solution to remove any unbound immunoglobulins. 100µl of either antihuman IgG or antihuman IgM conjugated with sheep-horseradish-peroxidase was added to each well and incubated for 30 minutes at 37^{0}C to allow the conjugate to attach to the complex. The wells were then washed four times to remove the unbound conjugate. 100µl of chromogenic substrate solution (3, 3',5 ,5'-tetramethylbenzidine) was added to each well. The

wells were incubated in the dark for 30 minutes at 37^0C. A substrate reaction ensued producing a blue colour which was terminated by adding 50µl of acidic citrate stopping solution, resulting in the development of a yellow colour. Using a photometer the optical density (OD) was measured at 450/630nm. The results were compared to six diluted sera controls provided with the kit [IgG (negative, positive and cut-off) and IgM (negative, positive and cut-off)]. A previously tested sample from the same patient was run at the same time to allow normalisation of the results.

To calculate the Ig levels (in Virotech Enzyme units) the following formula was used;

VE = 10 x {OD (sample) / OD (cut-off control) }

A VE value of < 9.0 indicates a negative result; VE > 11.0 indicates a positive result.

10.14 DNA Extraction From Blood

DNA was extracted using the Nucleon Biosciences ST (soft tissue) Genomic DNA Extraction Kit (ST kit). Four millilitres (ml) of buffer (10mM Tris-HCL; 320mM sucrose; 5mM $MgCl_2$; 1% Triton X-100; pH 8.0) was added to 1 ml of blood. The tube was then shaken for four minutes at room temperature. This was spun in a Beckman bench top centrifuge at 2500 rpm (1300g) for 5 minutes at 4^0C. The supernatant was discarded and the resultant pellet resuspended in 0.5ml of reagent B to lyse the cells. This was vortexed briefly to resuspend the pellet. The suspension was transferred to a 1.5ml microfuge tube. 150 µl of 5 Molar sodium perchlorate was added to the suspension to deproteinise. The tube was inverted several times to mix. To extract the DNA, 0.5ml of Chloroform was added to the tube which was again inverted several times. To complete the extraction, 150µl of Nucleon resin was added to the mix. The resulting suspension was spun in a microfuge at 2000 rpm for one minute to pellet the Nucleon resin. The upper phase was removed to another 1.5ml microfuge tube to which 2 volumes of absolute ethanol was added and the DNA precipitated by gently

inverting the tube several times. The precipitate was centrifuged at 13,000 rpm to pellet the DNA and the supernatant was discarded. The DNA pellet was washed by adding 1 ml of 70% ethanol and inverting the tube several times. The suspension was spun and the supernatant discarded. The pellet was air dried to ensure that all the ethanol was removed and then resuspended in 50µl of nuclease free water. At this stage the DNA was ready to be used as a template for Polymerase Chain Reaction (PCR).

10.15 DNA Extraction From Tissue

DNA was extracted from tissue using a Nucleon Biosciences ST (soft tissue) Genomic Extraction Kit. The tissue was homogenised in 2.5ml of reagent A and centrifuged at 1500rpm. The supernatant was discarded without disturbing the pellet. To extract the DNA from the tissue the same method as indicated above was used.

10.16 Polymerase Chain Reaction

The procedure was set up in the ultra violet cabinet to minimize DNA contamination. Firstly the primers were made up as a 1nM/µl stock in sterile deionised water. The primers used were HS13 (ACG ACG ACG TCC GAC GGC GA) and HS14 (GTG CTG GTG CTG GAC GAC AC). These primers detect the UL42 locus of HSV (Puchhammer-Stockl *et al.*, 1990). The primers were added to 1.5µl 10mM dNTPs; 5 µl [10x] Gibco buffer; 0.5µl Gibco Platinum Pfx Polymerase (Invitrogen); 1µl 50mM magnesium sulphate; 1µl 10-fold dilution of HS13; 1µl 10-fold dilution of HS14 and 1µl template DNA. This was made up to 50 µl total volume in sterile, deionised water. When running a PCR reaction the template used was one of the following, a positive control (1µl of 1716 DNA), a negative control (1µl of sterile deionised water) or the sample DNA in which case 1µl of neat suspension was required. The PCR was run in a Techne Genius machine. The protocol involved running the PCR at 94^0C for 2 minutes followed by 32 repeat cycles of 94^0C for 15 seconds, 72^0C for 1minute then 72^0C for a further minute. After the 32 cycles the PCR ran for a further 2 minutes at 72^0C. It

was cooled to 4^0C and stored. On completion of the PCR, 25µl of the samples were run on a 1% agarose gel along with size markers. A 278bp band indicative of HSV DNA was sought.

Semi-quantitative PCR assay was performed to indicate the number of viral genome copies present in the samples assayed. These results were normalized to the volume in which the extracted DNA was resuspended for the assay.
After PCR, 20µl of each reaction was electrophoresed on a 1% agarose gel and observed for a 278-bp band indicative of HSV. 100bp and 1Kb DNA markers of known concentration were run alongside the DNA samples to enable confirmation of the fragment size.

10.17 Infectious Virus Assay for Serum Samples

Serum was obtained in the manner indicated above. A suspension of BHK cells was plated onto 60mm diameter culture dishes at 3 x 10^6 cells in 5ml of growth medium. They were incubated overnight at 37^0C in 5% CO_2 to give a confluent monolayer of cells the following day. The medium was removed and two hundred micro-litres of the serum samples added and incubated for 1 hour at 37^0C to enable any virus present to adsorb. The plates were overlayed with 5ml of growth medium containing methylcellulose and incubated at 31^0C, 5% CO_2 for 3 days. After 3 days the medium was discarded and the plates were stained by adding 5ml of Giemsa. The Giemsa stain was left on for 30 minutes at room temperature and then washed off in cold water. Under the microscope the viral plaques were counted and a titre obtained.

10.18 Infectious Virus Assay for Swab Samples

Swab samples were obtained from buccal mucosa or skin lesions. The swab was transferred to the laboratory in a sterile Falcon tube. The swab was added to 5ml of ETC10 and treated in an ultrasonic bath for 5 minutes. The ECT10 was then filtered through a 0.2µl Acrodise to remove any contaminating micro-organisms. BHK cells were plated out at 3 x 10^6 onto 60mm diameter culture dishes in 5ml of

medium. They were incubated over-night at 37^{0}C in 5% CO_2. The following day the plates had a confluent monolayer. The growth-medium was removed and 5ml of the filtered solution was plated onto the BHK plates, incubated at 37^{0}C, 5% CO_2 for 1 hour and then transferred to the 31^{0}C 5% incubator for 3 days. The plates were then stained with Giemsa and the viral plaques counted.

10.19 Infectious Virus Assay for Biopsy Material

The tissue for analysis was ground up using a glass homogeniser. The homogenised tissue was suspended in 1ml of ETC10. This was treated in an ultrasonic bath for 5 minutes to disrupt the cells and release any infectious viral particles. After spinning the sonicated cell solution in a Beckman-type bench top centrifuge at 2000rpm for 5 minutes to pellet the cell debris, the supernatant which potentially contained virus, was decanted to be used to infect BHK cells. 3 x 10^6 BHK cells were plated out, onto 60mm diameter culture dishes in 5ml of medium. They were incubated overnight at 37^{0}C in 5% CO_2. The following day the plates had a confluent monolayer. The growth medium was removed and 500µl of the neat supernatant and a 1 in 10 dilution of the neat supernatant was added to separate 60mm plates containing BHK monolayers. The plates were incubated for 1 hour at 37^{0}C to enable any virus present to adsorb. The plates were then overlayed with 4.5ml growth medium containing methylcellulose and incubated at 31^{0}C, 5% CO_2 for 3 days. After 3 days the medium was discarded and the plates were stained with Giemsa. A microscope was used to count viral plaques and derive a titre.

10.20 Titration of Paired Vial

Prior to the injection of HSV1716 into a patient an aliquot of virus paired to the clinical vial was assayed to obtain an accurate titre. Sixty millimetre plates were seeded with 3 x 10^6 BHK cells in 5ml of ETC10 and were incubated overnight in a 37^{0}C incubator in an atmosphere with 5% CO_2. The following day the monolayers were confluent. Serial 10-fold dilutions of the paired HSV1716 aliquot were made in PBS/calf serum. The medium covering the BHK plates was discarded and 100µl of the serially diluted virus stocks were plated out onto the

BHK monolayers. The plates were gently rocked to ensure coverage of the virus and were incubated at 37^0C, 5%CO_2 for 1hour to enable virus adsorption. 5ml of ETMC 10% was then added to each plate. The plates were transferred to a 31^0C, 5%CO_2 incubator for 72 hours. Following this period the medium was discarded and 2-3ml of Giemsa stain added. The stain was left for 30 minutes and then washed off with water. The plaques were counted using a plate microscope.

The number of plaques corresponds to the titre in the following way;
For example;
50 plaques on a 10^{-6} plate = 5×10^7 in the 100µl innoculum
As the titre is calculated in pfu/ml then the titre would be - 5×10^8 pfu /ml

10.21 HSV Multicycle Growth Experiments in Tumour Samples

Tumour samples taken from all patients at the time of surgery, were dissociated using a scalpel and cultured in DMEM with 10% foetal calf serum and antibiotics at 37^0C and 5% CO_2. Depending on the size of the tumour sample, the cells were seeded into either one or two T175 flasks. The convention used was that the passage number increased by one, every time the cells were dissociated from the plastic ware with the trypsin and were re-plated. Cells from two T175 flasks were used to set up the virus growth experiments. Therefore in cases where only one flask was seeded initially, the cells underwent a 1:2 split (one passage) whereas those seeded into two flasks did not require to be passaged prior to seeding onto plates for the virus growth experiment.

To set up plates for the virus growth experiment, the tumour cells from two confluent T175 flasks were treated with trypsin, re-suspended in growth medium and counted. The suspension was diluted to a concentration of 1×10^6 cells per millilitre, 2ml plated of the suspension was plated onto 35mm tissue culture dishes and the plates incubated overnight at 37^0C in 5% CO_2. For the growth experiment, the assumption was made that on the following day all (2×10^6) of the seeded cells would have settled in the 35mm dish giving a confluent monolayer. However, on occasion these primary culture cells did not settle as well

as established cell lines would be expected to do and consequently the cell numbers for the experiment were lower than calculated. The growth medium was removed from the plates and the cells infected with 100µl of either HSV17^{+} or HSV1716 at a multiplicity of infection of 0.1pfu/cell (assuming 2 x 10^6 cells). The virus was adsorbed for 1 hour at 37^{0}C after which the inoculum was washed off with 2ml of growth medium and the plates overlaid with a further 2ml. One plate was mock infected. The plates were incubated at 37^{0}C and harvested at 0, 6, 24, 48 and 72 hours post infection. The samples were harvested by scraping the cells into the growth medium, transferring the whole sample to a plastic bijou bottle and freezing at –70^{0}C until titration.

Prior to virus titration, the contents of the bijou bottles were thawed quickly in a water bath and then stored on ice. The samples were treated in a soni-bath to disrupt any intact cells and release the virus. The samples were serially diluted (10-fold) in PBS/5% calf serum, and 100µl samples plated onto 60mm dishes of confluent BHK cells from which the growth medium had been removed. After incubating the BHK plates for 1 hour at 37^{0}C they were overlaid with 5ml of growth medium containing methylcellulose and transferred to an incubator at 31^{0}C and 5% CO_2. After 3 days, the medium was discarded, the plates treated with 2-4ml of Giemsa stain for >30minutes at room temperature and washed under cold water to remove the excess stain. The viral plaques were counted on a plaque-counting microscope and the titre calculated.

11 Laboratory Project Methods

11.1 Growth of Cells

11.1.1 BHKC12/13

BHK21/C13 were grown in 850 cm^2 tissue culture roller bottles or T175 flasks containing 125 ml or 70ml respectively of ETC10 medium at 37^{0}C for 3 days in an atmosphere of 95% air and 5%CO_2. The yield following harvesting from a confluent roller bottle is approximately 1 x 10^8 cells and from a T175 flask was 4 x 10^7cells.

11.1.2 3T6, 373, MOG Cells

3T6, 373, MOG and UVW cells were grown in appropriate media at 37^0C in an atmosphere of 95% air and 5% CO_2. Cells were passaged as described in section 11.2. Cells obtained from a T-175 flask were seeded in a 1 in 10 dilution in 70ml of appropriate medium.

11.2 Passaging of Cells

T-175 flasks or Roller bottles with 80-100% confluent monolayers were opened in a category 2 hood. Trypsin, stored in 2.5ml aliquots, was thawed from –20^0C and mixed with 22.5ml Hanks Balanced Salt Solution (HBSS) to yield 10% (v/v) trypsin/HBSS solution in 25ml. The medium was decanted. 25ml or 12.5ml of Trypsin/Hanks Balanced Salt Solution (HBSS), was poured over each monolayer in the roller bottles or T175 flasks (respectively). It was then decanted after 30 seconds. To encourage the cells to detach the roller bottle or flask was incubated at 37^0C. The cells were resuspended in 10-20ml of the appropriate growth medium and used to seed further flasks or roller bottles.

11.3 Cryopreservation of Cells

Confluent cell monolayers were harvested as described above. The cell suspension was pipetted into a 10ml Falcon tube and spun at approximately 1000rpm (Beckman centrifuge) for 10min at 4^0C. The supernatant was discarded and the cell pellet resuspended in the appropriate growth medium containing 10% DMSO. Aliquots of 1ml were pipetted into 1.5ml cryo-vials. These were packed in a well insulated container to ensure slow freezing. They were placed in a –70^0C freezer overnight and were then moved to vapour phase liquid nitrogen for long term storage. If the cells were for example, BHKs from a roller bottle (~2 x 10^8), they were resuspended in 10mls ETC10% +10% DMSO. One 1ml aliquot of the resulting suspension at (2 x 10^7 cells/ml) was sufficient to seed 1 roller bottle at a 1:10 split. However, if the cells were only capable of undergoing a 1:4 split, the resuspension volume was adjusted accordingly. In this case the cells from, for example a T175 flask, would be resuspended in 4ml of growth medium + 10%

DMSO, so that upon recovery a 1ml aliquot would be sufficient to seed one T175 flask.

11.4 Titration of Virus Stocks

Virus stocks were serially diluted 10-fold in ETC10. 100µl aliquots were added to confluent monolayers of BHK21/C13 cells on 60mm petri dishes, from which the medium had been removed. The plates were incubated at 37^0C for 1 hour, to allow adsorption of the virus onto the cells. The plates were washed twice with PBS, before overlaying with 5ml ETMC 10% and incubated at 37^0C for 2 days or 31^0C for 3 days. Monolayers were fixed and stained with Giemsa stain at room temperature for >30mins. After washing, plaques were counted on a plaque counting microscope and virus titres calculated as p.f.u/ml.

11.5 Multicycle Growth Experiments

35mm plates were seeded with 2 x 10^6 cells/plate in 2ml of growth medium at 37^0C with 5% CO_2. The growth medium was then poured off and 100µl of virus was added. The plates were returned to 37^0C and 5% CO_2 for 1 hour. The plates were then washed with 2ml of growth medium and overlaid with a further 2ml. Plates were incubated at 37^0C and harvested at the designated time points (0, 6, 24, 48 and 72 hours) by scraping the cell monolayer into the medium and transferring the suspension into a sterile bijou bottle. The samples were stored at –70^0C until the experiment was complete. The samples were then thawed, sonicated and titrated.

11.6 MTS Cytotoxicity Assay

In vitro cytotoxicity was determined using a Celltitre 96® Aqueous One Solution Cell Proliferation Assay (Promega) in accordance with the manufacturers instructions. This solution contains an MTS tetrazolium compound, an analogue of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], which is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium. The production of formazan is proportional to the number of

living cells; therefore the intensity of the produced colour indicates the viability of the cells.

Cell suspensions were prepared from 80% confluent T-175 flasks as described in section 11.2. Cells were seeded in 96-well plates at various cell densities depending on the cell type, in 100µl volumes. The perimeter wells were filled with sterile water to aid humidification during the experiments. After overnight incubation at 37^0C and 5% CO_2, the medium was removed from all wells and when indicated HSV1716 or HSV17$^+$ of a certain MOI was added in 100µl volumes.

If the experiment involved the investigation of radiation the wells were replaced with medium or medium containing virus. To irradiate the cells, the 96 well plates were placed on a tray under the head of the Cobalt source and a 0.5mm plastic sheet was placed on top in order to aid build-up of the dose to the cell culture at the base of the wells. The time of exposure to the ionising radiation was calculated from a pre-calculated dose rate. For each experiment a 96 well plate containing cells and medium alone acted as a control.

At various time points following incubation at 37^0C and 5% CO_2, the medium from six wells of the plates under investigation was aspirated dry and 100µl of fresh medium added. Six control wells were also prepared with 100µl of medium alone. 10µl of MTS was then added to each well. Optical density readings were taken at 480nm after 4 hours incubation at 37^0C and 5% CO_2.

The average value obtained from six wells with the 'medium alone' wells was subtracted from the average value obtained from six experimental wells. This value was used to calculate the number of viable cells in the experimental wells.

11.7 Restriction Enzyme Digestion

Plasmid DNA was digested using restriction enzymes in experimental conditions consistent with the manufacturer's guidelines. Linearised DNA was separated

using agarose gel electrophoresis. The DNA was identified under ultraviolet light then excised and purified.

11.8 Agarose Gel Electrophoresis

To identify the fragments of DNA obtained following restriction enzyme digestion, the linearised DNA was separated by an electrophoresis gel, containing Ethidium Bromide. 10µl of x6 blue/orange load dye was added to the elute obtained from the above digests and mixed by pipetting. Reservoirs in the gel were filled with the elutions obtained from the enzyme digests. In another reservoir, a 100bp DNA ladder was added. The gel was run at 60MeV for 90 minutes. The gel was then viewed under an UV light and sections corresponding to the DNA fragment sizes were identified and excised from the gel.

11.9 Purification of DNA from Solution Obtained from Restriction Enzyme Digestion

Linearised DNA in solution was purified using the GFX PCR DNA and Gel Band Purification Kit (AMERSHAM). 500µl of Capture buffer was added to a GFX column. The DNA solution obtained following the first digest was added to a GFX column and mixed by pipetting. The GFX column and collection tube was spun in a microcentrifuge for 30 seconds at maximum speed. The flow-through in the collection tube was discarded and the GFX column was replaced into the collection tube. 500µl of buffer-wash was added to the GFX column and centrifuged at maximum speed for 30 seconds. The flow-through was discarded and the GFX column was transferred to a new collection tube. 45µl of elution buffer (TE) was added to each column and incubated in a 37^0C water bath for 1 minute. The GFX column and collection tube was then spun at maximum speed for 1 minute. This last step was repeated. The contents were then tranferred to a fresh mirocentrifuge tube.

11.10 Elution of Restriction Enzyme Fragments from Agarose Gel

Linearised DNA was purified from the gel fragments using the GFX PCR and Gel Band Purification Kit. The gel fragments were dissected into small pieces and transferred to a microcentrifuge tube that had been weighed. The microcentrifuge tube was then reweighed to calculate the exact weight of the gel. 10µl of capture buffer for each 10mg of gel was added to the microcentrifuge tube which was then vortexed for 2 minutes. The micorcentifuge tube was then incubated in a 60^0C water bath until the agarose was dissolved. The dissolved gel was transferred into a GFX column in a collection tube and rested for 1 minute. It was then spun for 20seconds at maximum speed. The flow-through was discarded. 500µl of wash buffer was added to the GFX column and this was centrifuged for 30seconds at maximum speed. The flow-through was discarded. The GFX column was transferred to a new collection tube and 20µl of elution buffer was added. This was incubated for 1 minute at room temperature. To recover the purified DNA the GFX tube was centrifuged at maximum speed for 1 minute.

11.11 Ligation Reactions

Linearised DNA inserts obtained from the purified gel were incorporated into the plasmid DNA through ligation reactions. In a 1.5ml microcentrifuge tube 12µl of the insert was added to 2µl of plamid DNA, 2µl ligase and 4µl x5 buffer. The solution is incubated overnight at 16^0C.

11.12 Transformation of Bacterial Cells

E-coli cells are recovered from -70^0C. Two hundred micolitres were added to 10µl of the ligation reaction mixtures in 1.5ml microcentrifuge tube. The contents were put on ice for 20 minutes and then rewarmed at 37^0C for 30seconds. The tubes were again cooled on ice for a further 2 minutes. Eight hundred microlitres of L-broth growth medium was added to each microcentrifuge tube. The contents were warmed at 37^0C for 45 minutes. The contents were spun in microcentrifuge at 6500 rpms for 3 minutes. Nine hundred microlitres of medium was removed. The cell pellet was resuspended in the remaining 100µl of medium mixed. The

cell suspension was plated onto agar plates containing 100µl of Ampicillin /1ml agar. The plates are left upside down at room temperature for 20 minutes before being placed inside a 37^0C air bacteria incubator overnight.

11.13 Extraction and Purification of Plasmid from *E.coli*

Colonies that were able to grow on the ampicillin/agar plates were selected and individually transferred to a Falcon tube that contained 3ml of Lb and 0.01% Ampicillin. The Falcon tubes were put in a shaker at 37^0C overnight. The following day the DNA was purified from the 3ml of culture using the GFX *Micro* Plasmid Prep Kit.

One millilitre of culture was transferred to a 1.5ml micro-centrifuge tube and spun in a centrifuge at full speed for 30 seconds to pellet the cells. The supernatant was carefully removed. This was repeated to give 2-3ml total volume of culture. The pellet was resuspended in 300µl of solution I with vigorous vortexing. 300µl of solution II was added and mixed by inverting the tube 10-15 times. 600µl of solution III was added and mixed by inverting the tube until a flocculent precipitate appeared. Inversion was repeated until the precipitate disappeared. The micro-centrifuge tube was spun at full speed at room temperature for 5 minutes to pellet the cell debris. The supernatant was extracted carefully and added to a GFX column within a micro-centrifuge tube and incubated at room temperature for 1 minute. The micro-centrifuge tube was spun at full speed for 30 seconds. The 'flow through' was discarded. 400µl of wash buffer was added to the GFX column and centrifuged at 60 seconds to dry the matrix prior to elution. The GFX column was added to a fresh micro-centrifuge tube and 100µl of TE buffer was added. This was incubated at room temperature for 1 minute and centrifuged at full speed for 1 minute to obtain the purified DNA.

11.14 Transfection of Tissue Culture Cells

Tissue culture cells were added to the well of a six well plate and incubated at 37^0C and 5% CO_2 for 24 hours to achieve 80% confluence. The medium was then

removed from all the wells. To two of the wells in the 6 well plate either 10µl, 25µl or 50µl of plasmid in a total volume of 250µl of serum free medium was added. Six microlitres of lipofectamine 2000 (Invitrogen) made up to at total volume of 250µl with serum free medium was also added to each well. To each well a further 2ml of serum enriched medium is added. The cells were incubated for 24 hours at 37^0C and 5% CO_2. The cells were split in the usual manner and 50% were transferred to a new 6 well plate. To each well, serum enriched medium containing Zeocin at a concentration of 1:10 was added. The cells were incubated for a further 24 hours and split once more. This procedure was continued until the population of cells in each well grew in the presence of medium containing Zeocin.

11.15 Preparation of Infected and Non-Infected Cell Extracts

35mm plates were seeded with tissue culture cells at a required density and if indicated, infected with appropriate virus at various MOI. The cells were incubated for either 24 or 48 hours at 37^0C and 5% CO_2. To harvest the cells the medium was removed and 500ml of boiling mix was added. The cells were scraped off and stored in a micro-centrifuge tube at -20^0C.

11.16 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Cell protein extracts were separated using 10% SDS-PAGE (2.5% (w/v) crosslinker). Cell suspensions were heated to 100^0C for 2 minutes in a heating block prior to loading on a denaturing acrylamide gel. Glass gel forming plates were washed and cleaned in ethanol before assembly. The gels were cast vertically in a tightly scaled sandwich, consisting of 2 glass plates separated by 1.5 mm thick Perspex spacers. The resolving gel mix was prepared using running gel buffer which contained 375mM Tris-HCL, pH 8.9 and 0.1% (w/v) SDS. The addition of 0.06% (w/v) ammonium persulphate (APS) and 0.04% (v/v) N,N,N,N',tetramethlyenediamine (TEMED) polymerised the gel. 31.5 ml of resolving gel was poured gently between the glass plates, and a 2 ml layer of butan-2-ol was poured on top before setting. This excluded air and enabled

polymerisation of the gel. When the gel had set, butan-2-ol was poured off and thoroughly rinsed to remove any residue before the stacking gel was poured between the plates. The stacking gel was composed of 5% acrylamide crosslinked with the same ratio of N,N'-methylbisacrylamide used in the resolving gel. The stacking gel buffer was formed by 0.11 Tris-HCL, pH6.7 and 0.1% (w/v) SDS. Ammonium persuphate and TEMED were added to the gel prior to pouring. A Teflon comb was positioned to form the wells for the samples. The stacker was allowed to set, the comb was removed and the wells rinsed with running buffer. The reservoir was connected and filled with running buffer. Each sample was then loaded into the wells and separated by electrophoresis for 3-4 hours at 55mA. A pre-stained molecular weight marker was included onto each gel for identification of protein bands.

11.17 Western Blotting

Following SDS electrophoresis, the gel was analysed by Western blotting to identify protein expression. The gel was removed from between the plates and the stacking gel discarded. While the gel floated in transfer buffer, the Bio-Rad blotting apparatus was assembled using a Hybond Electrogenerated Chemiluminescence (ECL) nitrocellulose membrane (Amersham). With everything submerged in transfer buffer, a sandwich was assembled: mesh, sponge, card, gel, ECL membrane, card, sponge, mesh. The apparatus was submerged into the buffer filled transfer tank, with the gel nearest the negative electrode and the proteins transferred over 3 hours at 250mA.

Following transfer, the blots were placed in 3% blocking solution composed of dried milk in PBS and 0.05% Tween 20 for 2 hours, which reduced non-specific binding. Following a gentle wash in distilled H_2O, the blot was incubated in the primary polyclonal antibody (Ab-137 against ICP 34.5 protein), and incubated overnight on a shaker at 4^0C. After three 10 minute washes in PBS+10 µl Tween-20 (PBS-T), each blot was incubated in secondary antibody (Anti-rabbit IgG Horse Radish Peroxidase conjugate) 1:1000 in PBS for 2 hours. After vigorous

washing in PBS-T, followed by a 10-minute wash in PBS, the blots were then developed using ECL detection and exposed to Kodak X-OMAT film.

11.18 PCNA Localisation in Cell Lines

16mm cover-slips were sterilised by dipping them in 100% ethanol, blotting them to remove excess alcohol and then flaming dry. Two sterilised cover-slips were placed into a well of a 6 well plate. The wells were seeded with 2ml of cells suspension ($0.5x10^5$ cells/well) and incubated at 37^0C, 5% CO_2, and serum rich or serum free medium overnight. The medium was poured off and the cell monolayers washed twice with PBS. The cells were fixed by removing the medium from the dish, washing three times with PBS. To visualise the bound PCNA, the cells were also washed in hypotonic solution (10mM Tris-HCL, pH7.4, 2.5mM $MgCl_2$, 0.05% Nonidet P-40) for 10 min at 4^0C. The cells were fixed by transferring the cover-slip to 2ml methanol for 10 minutes at -20^0C. The methanol was poured off and the cells washed twice with 2ml of PBS. The cover-slips were blocked with PBS/1%BSA for 1hour at 4^0C and washed three times with PBS. 100µl of the primary antibody, monoclonal Ab PC10, diluted to 1/1000 in PBS/1%BSA, was added to each cover-slip and incubated at 4^0C overnight. The cover-slips were washed three times with PBS before adding the secondary antibody – goat anti-mouse IgG (conjugated to Rhodamine) diluted 1/100 in PBS/1%BSA, for 1 hour at 37^0C. Each cover-slip was washed twice with PBS before being placed cell side down onto a drop of mounting fluid on a microscope slide. The edges of the cover-slips were sealed using nail varnish.

12 Materials

12.1 Cells

Baby hamster kidney: 21 clone 13 (BHK) cells (MacPherson I and Stoker M, 1962) and mouse embryo fibroblast (3T6) (Todaro GL and Green H, 1963) cells were obtained from the European Collection of Cell Cultures (ECACC).

MOG-G-UVW (UVW) human brain astrocytoma, MOG-G-CCM human brain astrocytoma (MOG) (Frame M *et al.*, 1984) and U-373 MG (373) human

glioblastoma astrocytoma (Ponten J, 1968) were obtained from the department of Radiation Oncology, Beatson Institute, Glasgow. All three cell lines are available from ECACC.

12.2 Cell Culture Media

BHK21/C13 cells were grown in BHK-21 medium (Gibco) supplemented with 10% newborn-calf serum (Gibco); 10% (v/v) tryptose phosphate broth (Busby *et al.*, 1964); 10,000 IU/ml penicillin (Gibco); 10,000 UG/ml streptomycin (Gibco) and 250 UG/ml amphotericin B (Gibco).

3T6 cells were grown in Dulbecco's modified eagles medium (Gibco) supplemented with 10% foetal calf serum (Gibco); 10,000 IU/ml penicillin; 10,000 UG/ml streptomycin and 250 UG/ml amphotericin B.

MOG cells were grown in Minimum Essential Medium (MEM) (Gibco) supplemented with 10% new born serum; 10,000IU penicillin.

373 cells were grown in Minimum Essential Medium (MEM) supplemented by 10% new born serum; 10,000UG/ml streptomycin 250UG/ml; amphotericin B; 1% of MEM Sodium Pyruvate 100mM (Gibco) and 1% MEM Non essential amino acids solution x100 (Gibco).

EMC10 composed of Eagle's medium containing 1.5% carboxymethyl cellulose , 10% new-born calf serum and 10% tryptose phosphate broth, was used during viral titrations.

12.3 Viruses

Wild-type HSV-1 strain 17^+ (Brown SM *et al.*, 1973) and HSV1716 (MacLean A *et al.*, 1991) were used for laboratory studies. HSV1716 GMP grade, was prepared by Q1 Biotech, Glasgow and was used in the clinical trial.

12.4 Bacteria

E. coli used for cloning was the strain NM522. Bacteria were grown in L-broth (Lb) composed of, 170mM NaCl, 10g/l Difco bactotryptone and 5g/l yeast extract. Bacteria from glycerol stocks were plated out onto L-broth agar (L-broth containing 1.5% (w/v) agar).

12.5 Plasmids

pGEM-34.5, (McKie E *et al.*, 1994), was provided by Dr.E. McKie. pGEM34.5 is the pGEM-3Zf(-) plasmid (Promega) into which has been cloned the *Alu*1/*Rsa*1 HSV-1 fragment (bases 125074-126530) containing the RL1 gene, its promoter and flanking sequences.

The plasmid pGFP-34.5 was generated by introducing the GFP gene excised from the pGEFP-C3 plasmid (Clonetech) cloned in-frame between the RL1 promoter and the RL1 gene in pGEM34.5. This was kindly donated by Dr. P Dunn.

pcDNA4/*myc*-His was supplied by Invitrogen.

12.6 Enzymes

Restriction endonucleases were obtained from Promega and New England Biolabs. Buffers for the appropriate enzymes were supplied with the enzymes.

12.7 Solutions

12.7.1 Agorose Gel Reagents

TAE:	0.04M Tris, 0.14% acetic acid (v/v), 2mM EDTA (pH 8.0)
TBE:	89mM Tris. 89mM boric acid and 2mM EDTA
RE stop:	100mM EDTA, 10% (w/v) Ficoll 400, 0.25% (w/v) Bromophenol blue in 5x TBE

12.7.2 Tissue Culture Reagents

Tris-Saline:	140mM NaCl, 30mM KCL, 280mM Na_2HPO_4, 1mg/ml glucose, 0.0051% (w/v) phenol red, 25mM Tris-HCl (pH7.4), 100 units/ml penicillin, 0.1mg/ml streptomycin
Trypsin:	0.25% (w/v) Difco trypsin in Tris-Saline
HBSS:	Hanks Balanced Salt Solution

12.7.3 SDS PAGE and Western Blot Reagents

Boiling Mix:	151mM Tris-HCL (pH6.7), 6,28% (w/v) SDS, 0.15% (w/v) β-mercaptoethanol, 0.31% (v/v) glycerol, 0.1% (w/v) bromophenol blue.
Running Gel Buffer:	375mM Tris-HCl (pH8.9) and 0.1% (w/v) SDS
Stacking Gel Buffer:	0.1M Tris-HCl (pH6.7) and 0.1% (w/v) SDS
Tank Buffer:	6.32% (w/v) Tris, 4% (w/v) glycine, 1.33% (w/v) SDS
Towbin Buffer:	20% (v/v) MeOH, 25mM Tris-HCl (pH8.3), 192mM glycine

12.7.4 Cell Fixing Agents

PBS:	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4
Hypotonic Solution:	10mM Tris-HCL pH7.4, 2.5mMMgCl_2, 0.05% Nonidet P-40
Fix Solution:	4% (w/v) Paraformaldehyde in 0.1% PBS (pH7.3)
	100% Methanol

12.8 Plasmid Preparation

GFX *Micro* Plasmid Prep Kit – Amersham Biosciences (UK).

GFX PCR and Gel Band Purification Kit – Amersham Biosciences (UK).

12.9 Chemicals

All chemicals were of AnalaR grade and were obtained form BDH Chemicals Ltd, Poole, Dorset or Sigma Chemicals Ltd, Poole, Dorset.

Exceptions were:

APS and TEMED – supplied from Bio-Rad laboratories.

Histoclear – supplied by Fisher Chemicals

Platinum® P*fx* DNA polymerase, $MgSO_4$ – Invitrogen

Supersignal® West Pico Chemiluminescent Substrate – Pierce, Rockford, USA

Giemsa – Giemsa stain improved R66 solution 'Gurr'

12.10 Cytotoxicity Assays

Cell viability was measured using Cell Titer 96 ®AQ_{ueous} One Solution Cell Proliferation Assay (Promega).

Readings were taken using a Dynatech MR5000 96 well plate reader.

12.11 HSV Antibody Detection Kit

HSV IgM and IgG detection was performed using Elisa Kit, Virotech, Russelsheim, Germany.

12.12 Human Blood and Tissue DNA Extraction Kit

DNA was extracted from clinical trial patients' blood and tissue biopsies using Nucleon® genomic DNA extraction kit – Amersham Biosciences (UK).

12.13 Antibodies

Rabbit polyclonal anti – ICP34.5 (137) kindly provided by Dr. J. Harland and used in dilution of 1/100 to detect ICP34.5 and ICP34.5-GFP by Western blotting.

Mouse monoclonal anti-PCNA antibody (PC10), obtained from Santa Cruz Biotechnology Inc. and used at a dilution of 1/1000. The secondary antibody was goat anti-mouse IgG conjugated to Rhodamine, obtained from Vector Laboratories and used at a dilution of 1/1000.

12.14 Equipment and Plasticware

Gibco Europe, Life Technologies Ltd, Paisley:
Nunc 1 ml cryotubes
Nunc 6 and 12 well tissue culture plates
Nunc 25 cm^2, 75 cm^2, 175 cm^2 tissue culture flasks

Fisher Scientific UK, Loughborough:
Tissue culture dishes (35mm, 60 mm)

Leicestershire Corning Incorporated:
850 cm^2 tissue culture roller bottle

Greiner Bio-One Ltd, Stonehouse, Gloucestershire:

Micro-centrifuge tubes

Universal bottles

Bijoux bottles

96 well plates

Sterile pipettes

Becton and Dickson and Company:

BD Micro-Fine 1ml U-100 Insulin Syringe, 0.33mm (29G) x 12.7mm

12.15 Centrifuges

Volumes <1.5ml, up to 13,000rpm:	Micro-centrifuge
Volumes 1.5ml-45ml, up to 13,000rpm:	Beckman Centrifuge

12.16 Microscope

Images of cells were captured under a DMLB Fluorescent microscope using a DC 200 digital camera (Leica) and Leica IM 500 software.

12.17 Film

Autoradiograph X-OMAT film for Western blots was obtained from Kodak.

Autoradiographs were developed in an X-OGRAOH Compact X2.

12.18 Alcyon II Teletherapy Unit

Alcyon II Teletherapy Unit contains a 222 TBq (6000Ci) Co^{60} source situated at the Beatson Institute, Garscube, Glasgow.

Chapter 3

13 HSV1716 Injection into the Resection Cavity Rim Following Surgical Resection of High Grade Glioma in Patients with Recurrent and *de novo* Tumours

13.1 Introduction

Despite standard treatment, prognosis remains poor in patients with high-grade glioma and new therapies are required. The selectively replication competent herpes simplex virus, HSV1716 been shown to be safe and to replicate following injection into high-grade glioma (Rampling R *et al.*, 2000, Papanastassiou V *et al* 2002). Safety had not been previously been demonstrated following injection into brain rather than tumour. This section presents a phase 1 study of patients with such a paradigm.

It is envisaged that HSV1716 will be used in combination with standard therapies. One possible application would be in patients who have undergone surgical resection but who have residual tumour that cannot be excised. Following resection there is tumour regrowth and we reasoned that these actively dividing cells could present an ideal environment for replication of HSV1716. In this phase one study, virus was delivered into the rim of the resection cavity. The aims of the study were to assess the safety of HSV1716 when injected into predominantly brain adjacent to tumour, but it also allowed an assessment of a delivery strategy in which replicating virus might 'mop up' residual tumour unable to be removed by surgery. Patients were closely monitored for toxicity associated with HSV1716. Although not designed to assess efficacy, longitudinal follow-up has allowed assessment of overall survival. Retrospective comparison of survival of similar patients not treated with HSV1716, as reported in the literature was possible.

The study was performed in patients with newly diagnosed and recurrent disease. The enrolment period of nine months was from April 2001 until December 2001. In that period twelve patients were enrolled, ten men and two women. The age range was from 33 to 66 with the average age of 48.5 years.

Six patients (patients 1,2,3,4,6,9) had previously received treatment for a proven tumour of glial origin. Four of these tumours had previously been categorised as WHO grade IV glioblastoma. Patient 4's tumour was categorised as a WHO grade II Astrocytoma and patient 9's tumour had been categorised as a WHO grade II Oligodendroglioma. The demographics of the patients and the treatment they had received prior to entering into the study is detailed in table 13.1.

The other six patients (patients 5,7,8,10,11,12) presented with *de novo* tumours thought to be high-grade glioma on imaging alone. Following formal pathological analysis of the tumour resected from patient 7, it was noted that the high-grade tumour had arisen from a region of low-grade tumour lying adjacent. Patient 10 had been reviewed with serial imaging over 2 years following the discovery of a low-grade lesion and the high-grade glioma was considered to have arisen from the malignant transformation of low-grade tumour. Therefore tumours found in Patient 7 and Patient 10 are considered Secondary Glioblastoma.

Formal pathological analyses categorised ten of the tumours as WHO grade IV Glioblastoma. Patient 4's tumour was categorised as a WHO grade III Anaplastic Astrocytoma. Patient 9's tumour was categorised as a WHO grade III Anaplastic Oligodendroglioma.

Six of the tumours were located in the left hemisphere, five were located in the right hemisphere and one was noted to be a bi-frontal 'butterfly' tumour with new growth detected in the right frontal region.

Of the tumours in the left hemisphere two were in the temporal region, two were in the parietal region and two were in the parietal-occipital region. Of the tumours located in the right hemisphere two were in the frontal region, one was in the

fronto-temporal region, one was in the temporal region and one was in the parietal region.

Table 13.1; Newly diagnosed and recurrent high grade glioma patients

Pt	*Age*	*Sex*	*Original Pathology*	*Original Diagnosis*	*Previous Rx*	*Operation date*	*Pathology*	*Location*	*BS*	*KS*
1	61	M	GBM	1/8/00	Bx.Ch.DXT	18/4/01	GBM	L Pariet./Occip	8	60
2	43	M	GBM	15/11/99	S.DXT	26/4/01	GBM	R Front.	20	90
3	43	M	GBM	1/7/00	S.DXT	25/6/01	GBM	L Temp.	20	90
4	40	M	Astrocytoma	4/4/97	S.DXT	29/6/01	A.A.	R Front./Temp.	20	90
5	49	M	-	-	-	3/8/01	GBM	L Pariet./Occip	20	100
6	58	F	GBM	1/11/00	S.DXT	7/9/01	GBM	R Pariet.	19	90
7	41	M	-	-	-	10/9/01	*GBM	R Front.	20	90
8	53	M	-	-	-	10/9/01	GBM	L Temp.	20	90
9	40	M	Oliogodendroglioma	1/9/95	S(x2).DXT	6/11/01	A.O.	R Front.	20	90
10	33	F	-	-	-	30/11/01	**GBM	L Pariet.	11	80
11	66	M	-	-	-	3/12/01	GBM	L Pariet.	20	100
12	55	M	-	-	-	19/12/01	GBM	R Temp.	20	100

*patient had features on pathological analysis of a GBM arising in low grade glioma.
** patient had been diagnosed with a low grade tumour based on imaging and had been under surveillance for evidence of transformation

Key;
M – male
F – female
GBM – glioblastoma
A.A. – anplastic astrocytoma
A.O. – anaplastic oligodendroglioma
Bx – biopsy
Ch – chemotherapy
DXT – radiotherapy
S – Surgery

L – left
R - right
Pariet. – parietal lobe
Occip. – occipital lobe
Front. – frontal lobe
Temp. – temporal lobe
BS – Barthel score
KS – Karnofsky score

The following section gives a detailed account of each patient that was enrolled into the phase 1 trial. Information is presented that details the preoperative, intraoperative and postoperative period. Laboratory findings for the duration the patients were observed are similarly presented. Each patient was followed up formally from the period of enrolment to the time of death or February 2003 whichever came first.

13.2 Patient 1

Patient 1 was a 61 year old man who experienced sudden onset headache in March 2000. He was diagnosed as having suffered a sub-arachnoid haemorrhage. Over the subsequent two months he became confused with occasional periods of unsteady gait. A CT scan indicated a space occupying lesion in the left parieto-occipital region of the cerebral cortex. Pathological analysis of tumour samples obtained from stereotactic biopsy confirmed glioblastoma. Within the context of a clinical trial he received two cycles of Temozolamide chemotherapy prior to commencing a 6 week course of radical radiotherapy (60 Gray (Gy) in 30 fractions). Less than six months later he developed weakness down his right hand side. Neurological imaging confirmed tumour progression.

Preoperative condition and intra-operative procedure

Prior to his operation patient one was steroid dependent. He demonstrated poor memory and concentration span. Neurological assessment indicated he had reduced power of the right leg and an unsteady small step gait rendering him wheel chair bound. Indices of function indicated his clinical condition to be poor with a Karnofsky Score (KS) of 60 and a Barthel Score (BS) of 8. His preoperative clinical condition fulfilled the minimum eligibility criteria indicated in the inclusion criteria. Laboratory investigations and preoperative work up investigations were otherwise unremarkable. In April 2001, 9 months following formal diagnosis of glioblastoma, he underwent cytoreductive surgery and injection of 1 x 10^5pfu of HSV1716 into the resection cavity wall [HSV1716, Lot No. 9731057, 1 x 10^5 TCID 50/ml Vial No.23 1ml].

Post operative clinical course

Post operatively there was no obvious neurological deficit or suggestion of toxicity associated with the HSV1716 administration. Throughout the first week he remained well with no clinical evidence of toxicity. After the initial 7 postoperative days, he was transferred to the Beatson Oncology Centre for convalescence. During the second post-operative week he became progressively confused and refused adjuvant chemotherapy. His confusion was thought secondary to his steroid medication and this improved as the steroids were reduced. He was discharged to his local hospice for some respite care, three weeks following surgery and injection of HSV1716.

During this respite period, eleven weeks following surgery and HSV1716 injection, he suffered a grand-mal seizure and was admitted to hospital. He required endotracheal intubation and ventilation. On neurological imaging there was no indication of tumour recurrence. The seizures were controlled with anti-epileptic medication and he was discharged to the hospice. He remained on a regular dose of anti-epileptic medication and the steroids were gradually reduced. Over subsequent weeks he gradually improved clinically. This is reflected in the rise of KS and BS. Laboratory investigations were unremarkable. By the sixth post-operative month he was mobilizing independently and remained free from seizures.

During the post-operative period, in line with protocol, he was due to commence systemic chemotherapy. Against medical advice he refused chemotherapy. His clinical condition however continued to improve over the subsequent months. His mobility improved and he maintained a good quality of life. There was no evidence of disease recurrence on routine imaging at February 2003, 22 months since surgery and injection of HSV1716 and 29 months following diagnosis.

Markers of clinical performance

The following graphs present data on clinical performance up to the point when he was last reviewed in line with the protocol.

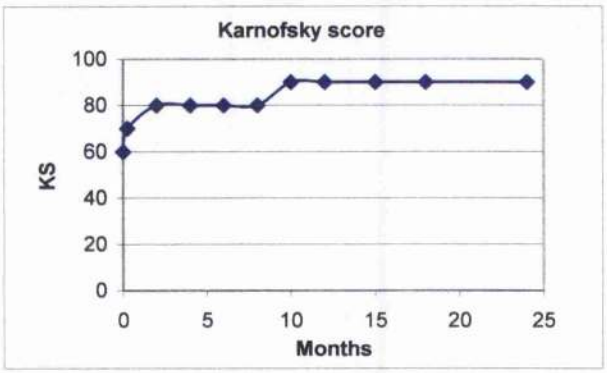


Figure 13.2(i); *Karnofsky Score patient 1*

Figure 13.2(ii); *Barthel Score Patient 1*

Laboratory Investigations

Paired vial titration

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of - **2.2×10^5 pfu/ml.**

HSV IgG and IgM by ELISA

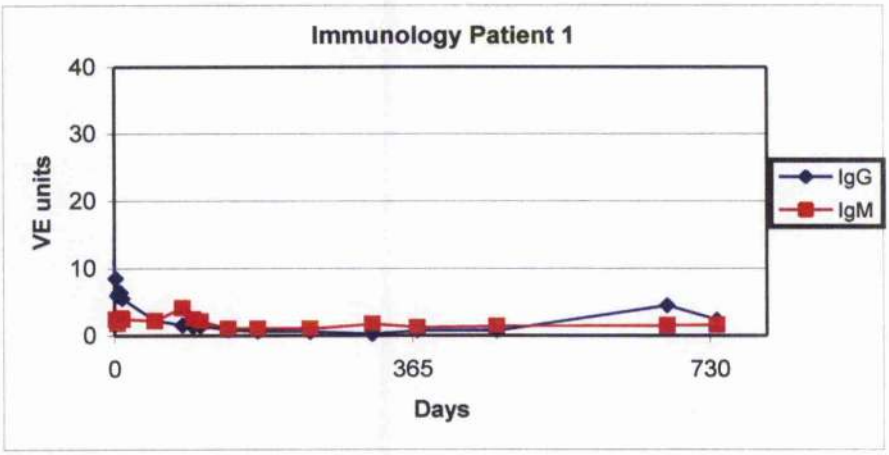


Figure 13.2(iii); *HSV IgG and IgM Patient 1*

According to the ELISA kit (Virotech) used to detect human HSV IgG and IgM Patient 1 was IgG and IgM positive for HSV antigen. A positive result is recorded if it is 11 VE units or greater. The IgG level before HSV1716 injection was recorded as 11.3. The results show that there was a fall in the IgG titre following the injection of HSV1716.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood – In line with the trial method indicated previously on each occasion that blood was obtained for assessment of HSV IgG and IgM it was also analysed for evidence of HSV DNA. On day 83 following HSV1716 injection HSV DNA was detected in blood by PCR analysis. This was the only sample received following admission to hospital following grand-mal seizures 78 days post surgery. There was no accompanying rise in HSV specific IgM or IgG. Due to the limitations of the PCR assay, it was not possible to distinguish between wild type HSV and HSV1716.

Virus shedding assay

Buccal swabs – no evidence of live HSV has been detected.

Serum – no evidence of live HSV has been detected.

Imaging data

Imaging data from patient 1 is shown in figure 13.2(iv) which illustrates T2 and T1 weighted post contrast axial MRI of two adjacent sections at four time periods with similar thallium-201, SPECT sections. The images taken pre-resection and pre-virus injection (a) shows a large enhancing necrotic left parietal glioblastoma recurrence, surrounded by vasogenic oedema with marked thallium uptake. Images taken forty-eight hours after resection and virus injection (b) show a resection cavity with a moderate enhancing margin containing a fluid level surrounded by vasogenic oedema. No thallium uptake is present on the SPECT images. Follow-up at 6 weeks (c) indicates that the resection cavity has reduced in size with reduction in mass effect on the adjacent left lateral ventricle. There is minimal marginal enhancement and marked reduction in vasogenic oedema. On the SPECT images there is no thallium uptake. The imaging at 22 months post resection and virus injection (d) shows that the resection cavity is minute, with *ex vacuo* enlargement of the left lateral ventricle and no marginal enhancement. Once again there is no abnormal thallium uptake on the SPECT images to suggest tumour regrowth.

Figure 13.2(iv) a; *Preresection and HSV1716 administration*

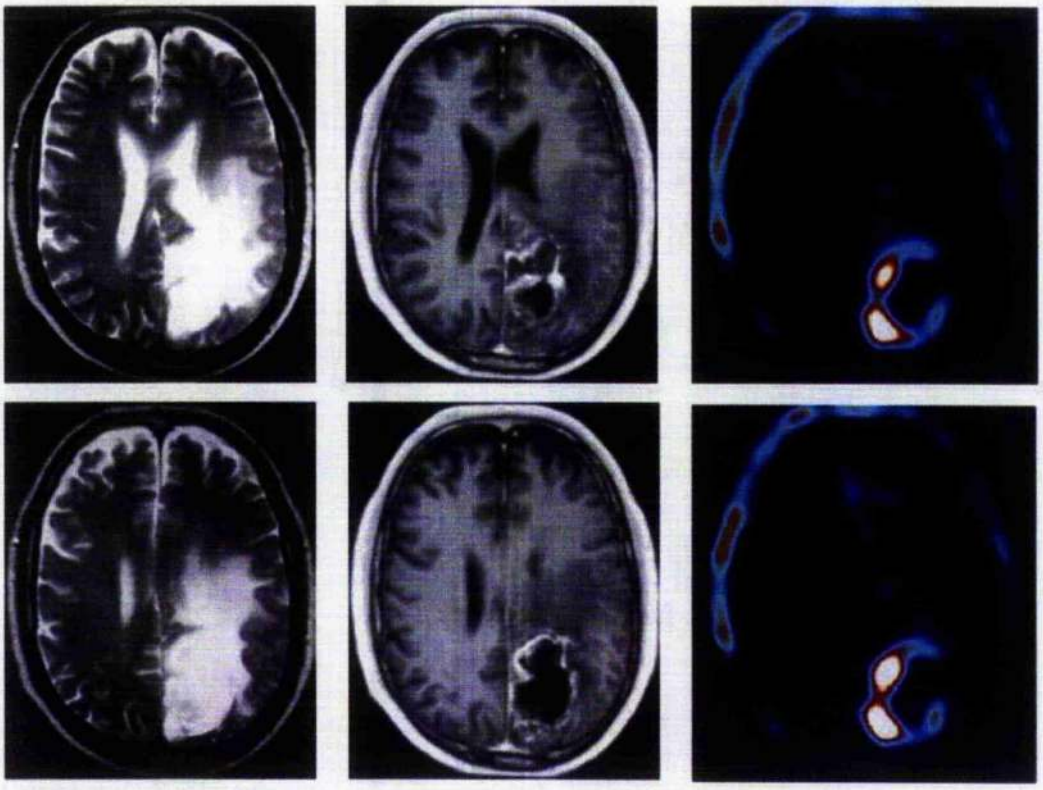


Figure 13.2(iv) b; *48 hours after tumour resection and HSV1716 injection*

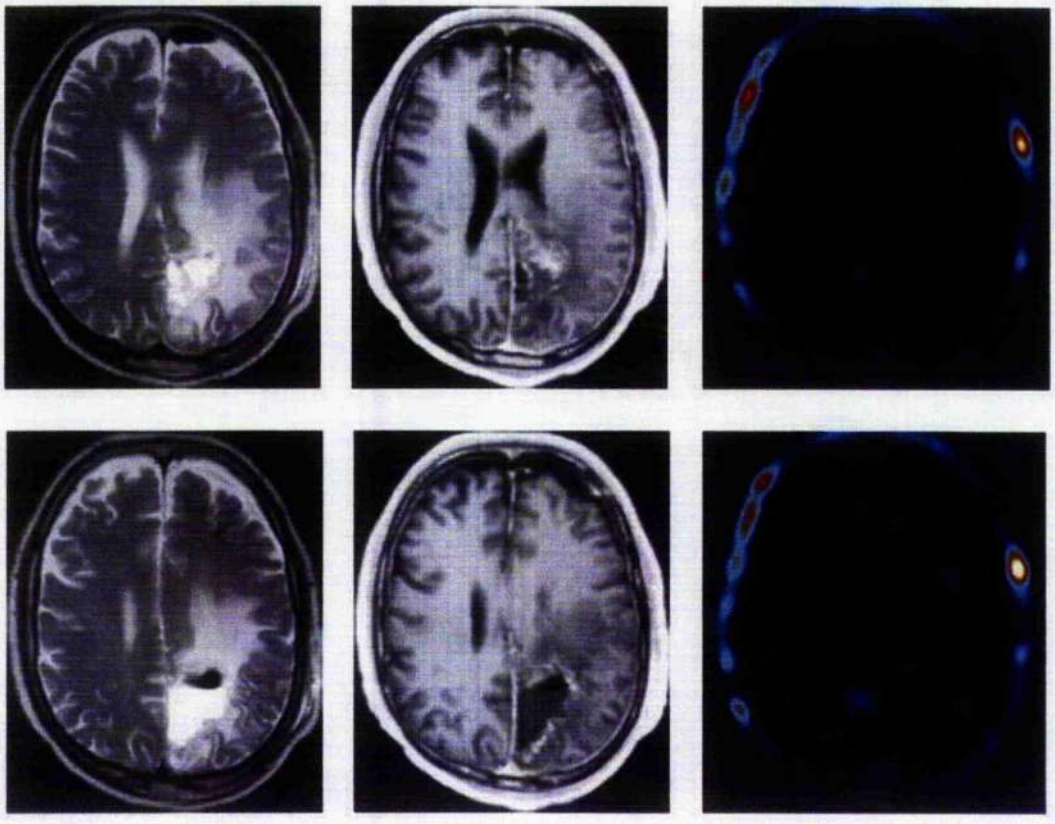


Figure 13.2(iv) c; *Six weeks following tumour resection and HSV1716 injection*

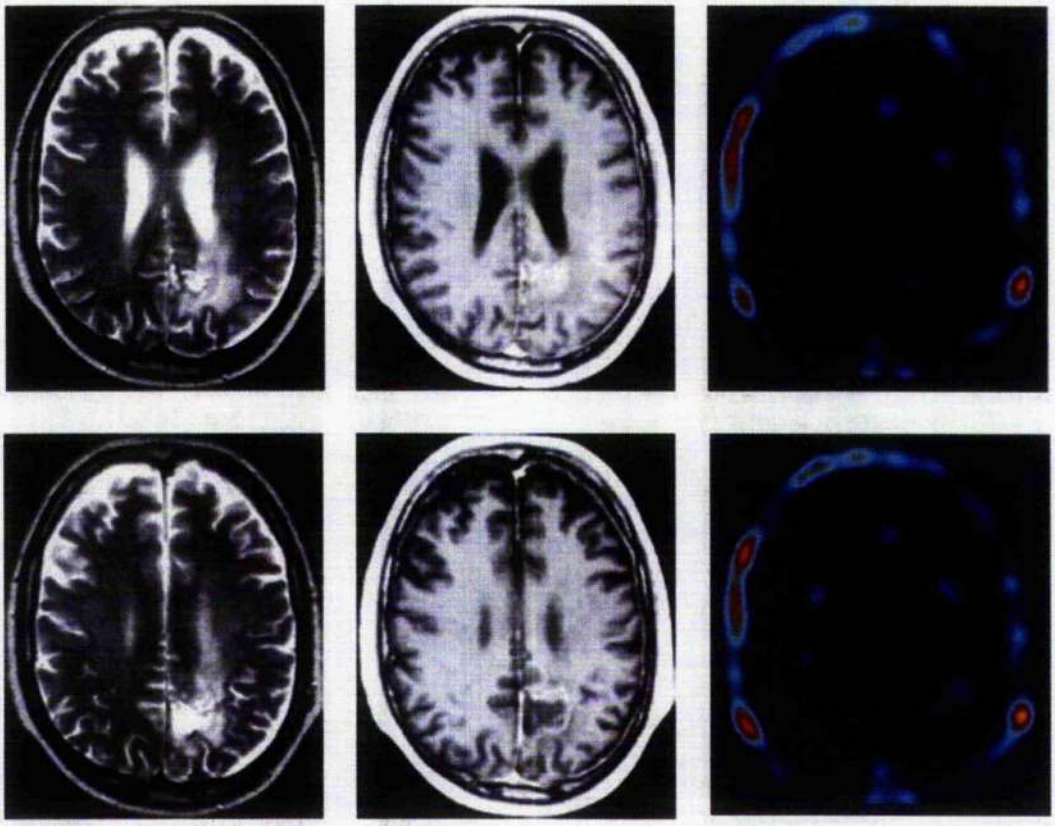
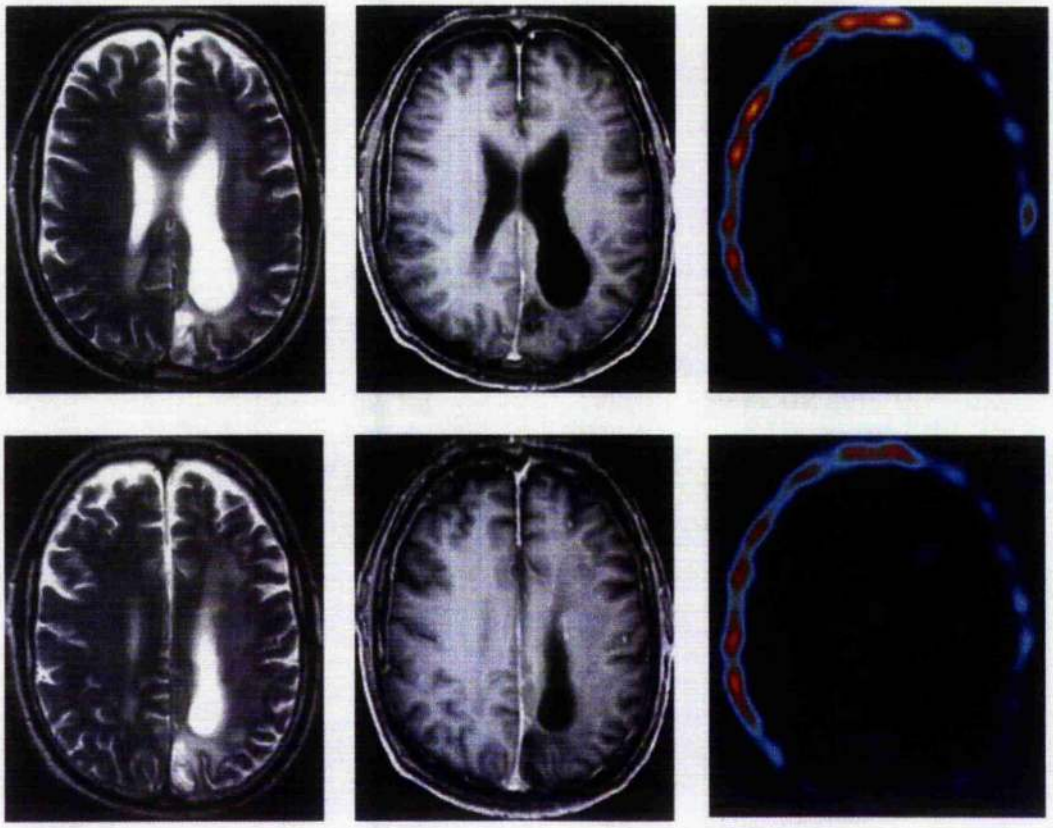


Figure 13.2(iv) d; *22 months following tumour resection and HSV1716 injection*



13.3 Patient 2

The second patient was a male aged 43 years. He was diagnosed with a bi-frontal 'butterfly' glioblastoma in November 1999. At that time he underwent cytoreductive surgery followed by a course of radiotherapy (45Gy in 20 fractions). He remained well until March 2001 when he began to experience tingling in his right arm and hand. These seizures were controlled with medication. Imaging indicated tumour re-growth in the right frontal region. He was enrolled in the study in April 2001.

Preoperative clinical condition and operative procedure

On entry into the study his symptoms were controlled with steroid medication. Clinical examination revealed mild right papilloedema. Indices of clinical function were KS-90 and BS-20. Laboratory investigations and preoperative workup were unremarkable. He underwent extensive frontal lobectomy and macroscopic excision of tumour. HSV1716 was injected into the resection cavity wall [HSV-1 1716 Lot No. 9731057 10^5 TCID 50/ml Vial No.3 1ml].

Post operative clinical course

Post operatively he made a slow recovery. Neurological examination revealed some left arm weakness. There were no signs or symptoms suggestive of toxicity associated with the virus. A few days following surgery, his wound was swollen and inflamed. In addition he was pyrexial and slightly confused. The wound was thought to be infected and was treated with appropriate antibiotics. The wound healed and he made a full recovery.

One week following discharge he developed a cough associated with rigors. He was noted to be pyrexial, tachycardic and tachpnoeic. Chest x-ray findings were consistent with an infective process. There was no evidence of an elevated white cell count or neutrophilia. He was commenced on appropriate antibiotics and given controlled oxygen therapy. He responded to treatment and was soon discharged home.

In line with the protocol he was commenced on CCNU chemotherapy. After the second cycle of chemotherapy he experienced headache, vomiting and decreased consciousness. A CT scan indicated an increase in the residual tumour in the right frontal region. Tumour progression was confirmed 5 months following the surgery and HSV1716 injections. Further surgical intervention was not indicated and he rapidly deteriorated and died 6 months following the initial procedure.

Markers of clinical performance

The following graphs present data on clinical performance for the duration the patient remained in the study assessed using the Karnofsky score and Barthel score.

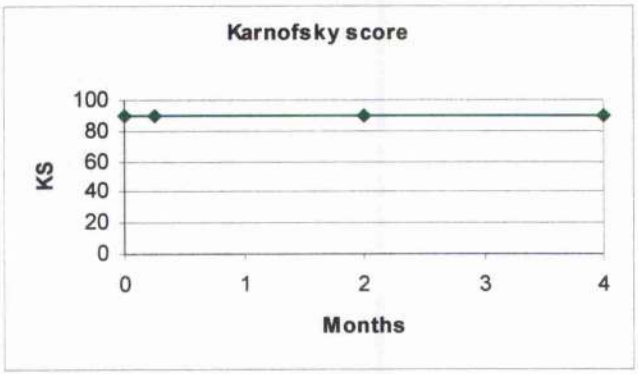


Figure13.3(i); *Karnofsky Score Patient 2*

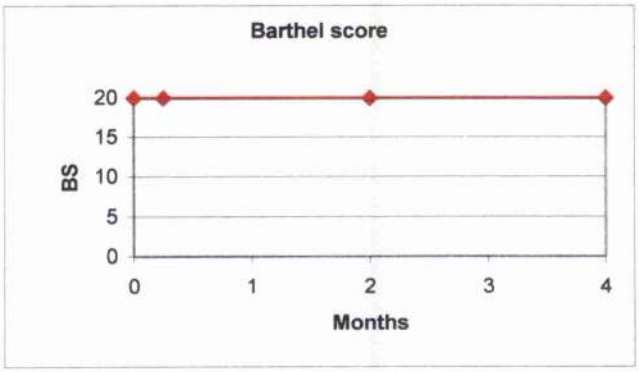


Figure13.3(ii); *Barthel Score Patient 2*

Laboratory Investigations

Titration of paired vial

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of - **3 x 10^5 pfu/ml.**

HSV IgG and IgM by ELISA

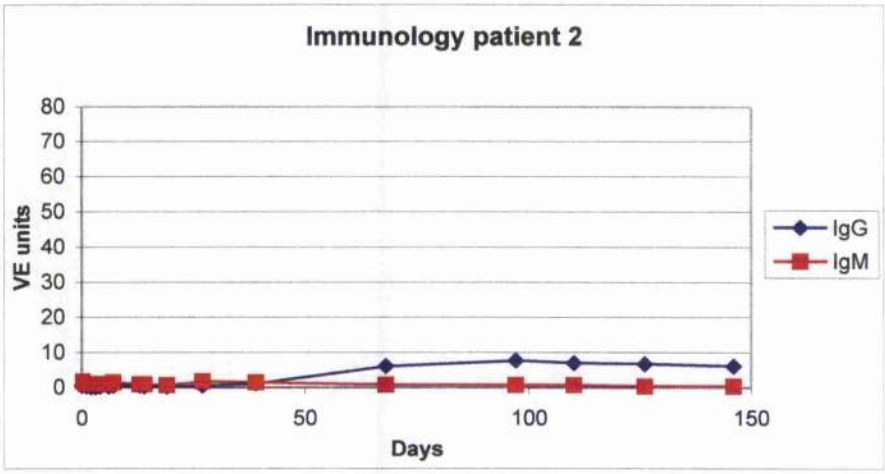


Figure13.3(iii); *IgG and IgM Patient 2*

Patient 2 was sero-negative to HSV antigen prior to the injection of HSV1716. He failed to mount an immune response. This was possibly a consequence of being iatrogenically immunosupressed with oral steroids through-out his follow up.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood – there was no evidence of HSV DNA detected in the blood.

CSF – there was no evidence of HSV DNA in the CSF sample taken at the time that he had the craniotomy wound infection.

Virus shedding assay

Buccal swabs - no samples had evidence of live HSV.

Serum – no samples had evidence of live HSV.

CSF – there was no evidence of live HSV in the CSF sample taken at the time of the craniotomy wound infection immediately post surgery.

13.4 Patient 3

The third patient was a 43 year old man. In June 2000 he began to experience headaches. Neuro-imaging indicated a lesion in the left temporal region. He underwent tumour debulking surgery followed by a radical course of radiotherapy (60Gy in 30 fractions) in July 2000. His symptoms of headache returned in April 2001. MRI indicated tumour progression. He was entered into the study in June 2001.

Preoperative clinical condition and operative procedure

Despite tumour re-growth he was symptom free on a maintenance dose of oral steroids. Indices of clinical performance were KS-100 and BS-20. There was no evidence of neurological deficit on clinical examination. Laboratory investigations and preoperative work up were unremarkable. He underwent further cytoreductive surgery in June 2001. At surgery the tumour was noted to be heavily calcified and adherent to dura and cavernous sinus resulting in some macroscopic tumour being unresected. HSV1716 was injected into the resection cavity rim [HSV1716 Lot.No. 907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 2].

Post operative clinical course

In the immediate post-operative period he remained well with no new neurological deficit. There were no signs or symptoms suggestive of toxicity associated with the virus. Patient 3 lived out with Glasgow and as a result the first four weeks of his follow-up was conducted by telephone. During this time he indicated that he was well. At his 6-week hospital review he was noted to be lethargic and have reduced mobility. His wound was markedly swollen and 'boggy' on palpation. Neurological examination revealed that he had reduced power in his right upper and lower limbs and fundoscopy revealed mild papilloedema. Contrary to medical advice he had rapidly reduced his oral dexamethasone. MRI indicated a CSF meningocoele but no evidence of tumour regrowth. His wound was aspirated and a lumbo-peritoneal shunt was inserted. He

required three lumbar punctures to remove excessive CSF. Dexamethasone medication was re-instated. Over the course of the next few weeks his lumbar spine wound dehisced. The lumbo-peritoneal shunt was replaced by a Ventriculo-peritoneal shunt. This procedure was uneventful. Over the course of the next few months he continued to reduce his oral steroid dose contrary to medical instruction. Consequently his clinical performance would deteriorate and to control his symptoms a higher dose of dexamethasone would have to be reinstated. He was commenced on CCNU chemotherapy following his surgery in line with the protocol.

In December 2001, six months following HSV1716 injection he developed expressive dysphasia. Clinical examination revealed new neurological signs, papilloedema, right homonymous hemianopia and diplopia on right and left lateral gaze. MRI indicated tumour progression. The tumour recurrence was not amenable to further surgery. He died 9 months following entry into the study.

Markers of clinical performance

The following graphs present data on clinical performance, assessed by Karnofsky score and Barthel score, for the duration the patient was in the study.

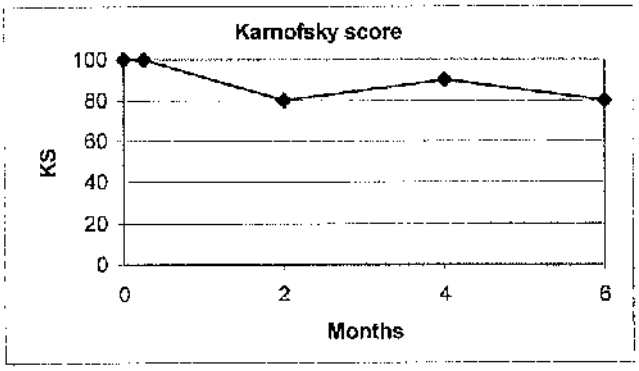


Figure 13.4(i); Karnofsky Score Patient 3

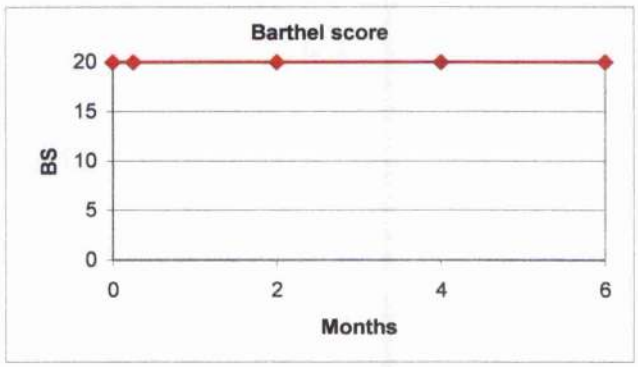


Figure 13.4(ii); *Barthel Score Patient 3*

Laboratory investigations

Paired vial titration

The sample of HSV1716 paired to the aliquot of HSV1716 injected had a titre of - **1.1×10^5 pfu/ml**.

HSV IgG and IgM by ELISA

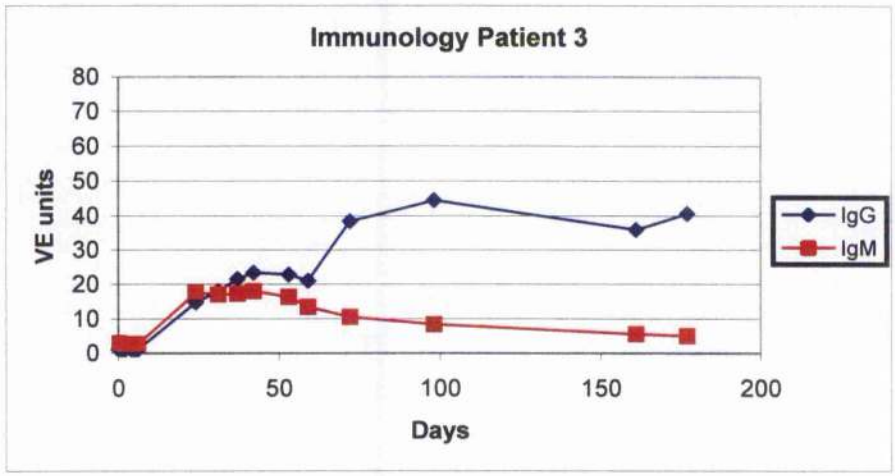


Figure 13.4(iii); *IgG and IgM Patient 3*

Patient 3 was seronegative prior to the injection of HSV1716. On day 24 analysis of blood serum indicated that he had seroconverted.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood – HSV DNA was detected in the serum on day 24 following HSV1716 injection. Patient 3 was seronegative prior to the injection of HSV1716 and sero-converted at the same time as PCR detected HSV DNA in his blood.

Virus shedding assay

Buccal swabs – no evidence of live HSV was detected.

Serum – no evidence of live HSV was detected.

13.5 Patient 4

This 40 year old man's symptoms date back to 1997 when he began to experience auras. These were diagnosed as temporal lobe epilepsy. In 1998, imaging indicated a lesion in the right temporal region. He underwent macroscopic resection of the lesion and a radical course of radiotherapy (60Gy in 30 fractions). Pathological diagnosis confirmed an astrocytoma. In May 2001 he experienced a recurrence of auras and developed expressive dysphasia. Neuro-imaging indicated tumour recurrence and he entered into the study in June 2001.

Preoperative clinical condition and operative procedure

On entry into the study, patient 4 was clinically stable. Markers of clinical performance were KS-90 and BS-20. Neurological examination revealed a left upper quadrantic hemianopia. Laboratory investigations and pre-operative work-up were unremarkable. He underwent surgical resection following pathological confirmation of a high-grade glioma by smear preparation. Although the surgery was extensive, insular vessels limited deep resection. HSV1716 was injected into the resection cavity wall [HSV1716 Lot. No. 907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 3].

Post-operative clinical course

Post operatively he recovered well with no new neurological deficit. There was no toxicity related to the HSV1716 injection. Pathological analysis of the tumour identified it as an Anaplastic Astrocytoma. Post-operative laboratory investigations indicated a rise in the levels of the liver transaminases, alanine transaminase (ALT) and aspartate transaminase (AST). The levels returned to within the reference range over time. The rise was probably related to the anaesthetic procedure.

In line with protocol he received PCV combination chemotherapy up to the maximum of 6 cycles without any complications. He remained free from recurrence for 10 months post procedure when tumour recurrence was noted on routine MRI. The suspicion of tumour recurrence was supported by increased thallium uptake on the Thallium SPECT images. In line with current clinical practice he was offered second line chemotherapy, Temozolamide. He received 3 cycles of Temozolamide. Due to symptoms associated with continued tumour progression, further Temozolomide was not indicated. Patient 4 died 15 months following the injection of HSV1716.

Markers of clinical performance

The following graphs present data on clinical performance, as assessed by Karnofsky score and Barthel score, for the duration the patient remained in the study.

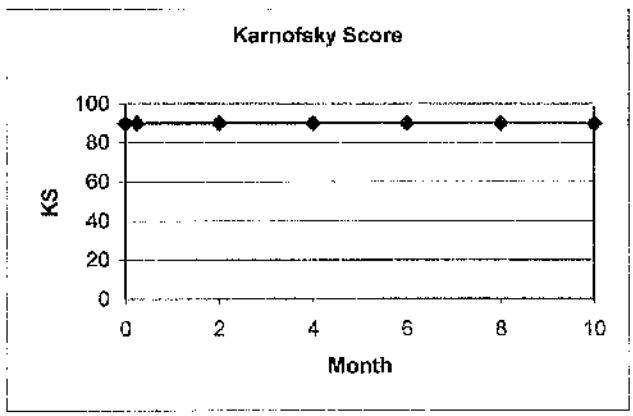


Figure 13.5(i); *Karnofsky Score Patient 4*

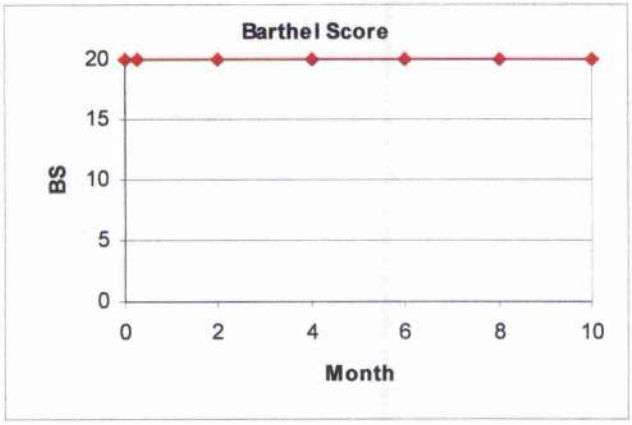


Figure 13.5(ii); *Barthel Score Patient 4*

Laboratory Investigations

Paired vial titration

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of - **1.0×10^5 pfu/ml**.

HSV IgG and IgM by ELISA

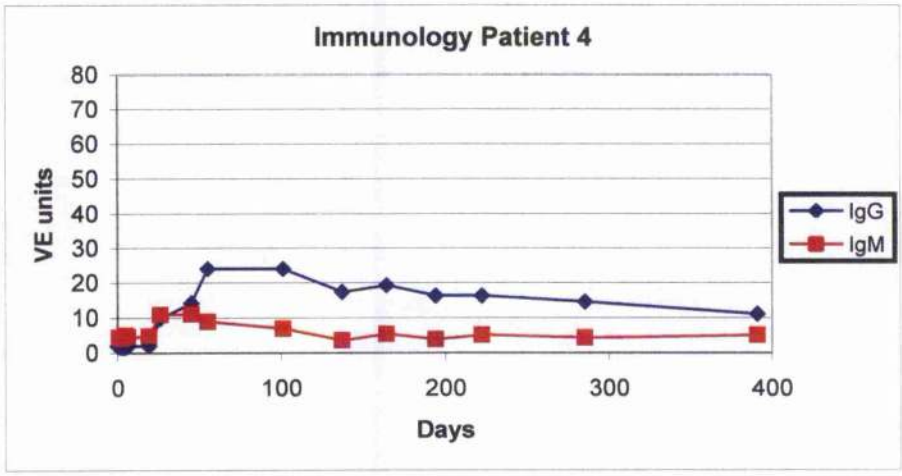


Figure 13.5(iii); *IgG and IgM Patient4*

Patient 4 was seronegative for HSV antibodies prior to the injection of HSV1716. He mounted an immune response and by day 49 post injection had seroconverted.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood – no evidence of HSV DNA was detected by PCR despite evidence of sero-conversion on day 49 following injection of HSV1716.

Virus shedding assay

Buccal swabs – There was no evidence of live HSV.

Serum – There was no evidence of live HSV.

13.6 Patient 5

This 49-year old gentleman first developed symptoms in April 2001 following a fall whilst at work which resulted in the amputation of a finger. A month later he developed severe headaches that were not relieved by analgesia. Initially the headaches were considered related to the fall. By June 2001 he began to develop weakness in his right leg. Following an unexpected collapse he was admitted to hospital. A CT scan indicated a lesion in the left paricto-occipital region. He underwent a diagnostic biopsy prior to being enrolled in the study. This confirmed a Glioblastoma, WHO grade IV. He entered into the study in August 2001.

Preoperative clinical condition and operative procedure

On entry to the study patient 5 was clinically stable. Markers of clinical performance were KS-90 and BS-20. The right-sided weakness had resolved as a result of oral dexamethasone and he had no new neurological deficit. Laboratory investigations and pre operative work-up were unremarkable. Following macroscopic tumour resection HSV1716 was injected into the resection cavity rim [HSV1716 Lot.No. 907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 7].

Post operative clinical course

Post operatively he recovered well. There was no clinical evidence of toxicity associated with the injection of HSV1716. Post-operatively he was noted to have a right homonymous hemianopia thought to be a consequence of the surgical intervention. He received a radical course of radiotherapy (60Gy in 30 fractions)

in line with standard treatment of de novo Glioblastoma. Three months after virus injection and shortly after completing the course of radiotherapy, patient 5 suffered a complex partial seizure which was medically managed using, Carbamazepine. Within one week of the seizures, he was re-admitted with pyrexia of unknown origin and a generalised rash. Clinical examination and laboratory investigations failed to confirm a diagnosis. There was no evidence of HSV DNA by PCR analysis (this test being sensitive at levels equivalent to 10^2 pfu per ml) in blood or cerebrospinal fluid samples. Despite there being no evidence to implicate HSV1716 in this illness, Acyclovir was administered as a precautionary measure as he continued to deteriorate clinically despite broad-spectrum intravenous antibiotics and supportive treatment. Following the Acyclovir, he remained unwell with a spiking temperature. Two weeks following hospital admission, a positive ELISA test for Candida antigen, confirmed invasive candidiasis. He was treated with intravenous Fluconazole and clinically improved.

Routine imaging four months post surgery and HSV1716 injection suggested tumour re-growth although clinically he remained well. Neurological examination was unremarkable. As he had just completed a course of radiotherapy and was continuing to recover form invasive candidiasis, no new treatment was instituted at that time. Over the subsequent few months he developed headaches, which were controlled with steroid. Magnetic Resonance Imaging 6 months following the procedure showed evidence of marked tumour growth. He was commenced on CCNU chemotherapy. He continued to deteriorate following the first cycle of chemotherapy and was unfortunately unfit to receive a second dose. He died 8 months following tumour resection and HSV1716 injection.

Markers of clinical performance

The following graphs present data on clinical performance as assessed by the Karnofsky score and Barthel score, for the duration the patient remained in the study.

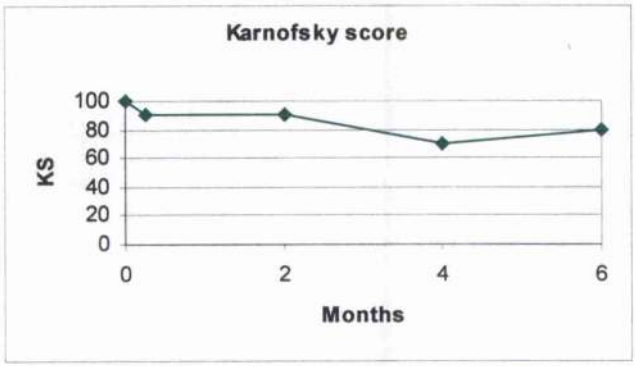


Figure 13.6(i); *Karnofsky Score Patient 5*

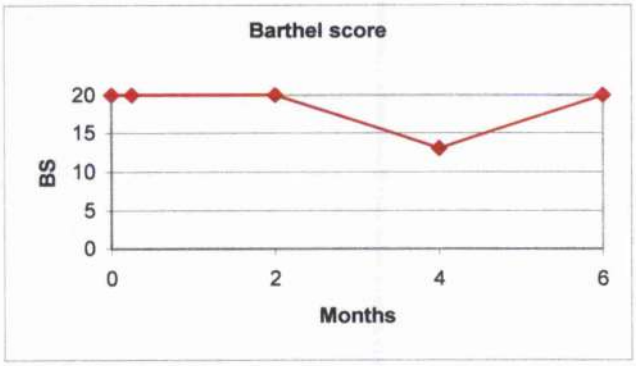


Figure 13.6(ii); *Barthel Score Patient 5*

As indicted by the KS and BS his clinical condition deteriorated during his admission to hospital with 'pyrexia of unknown origin' although they did recover.

Laboratory Investigations

Paired vial titration

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of - **7.5×10^5 pfu/ml**.

HSV IgG and IgM analysis by ELISA

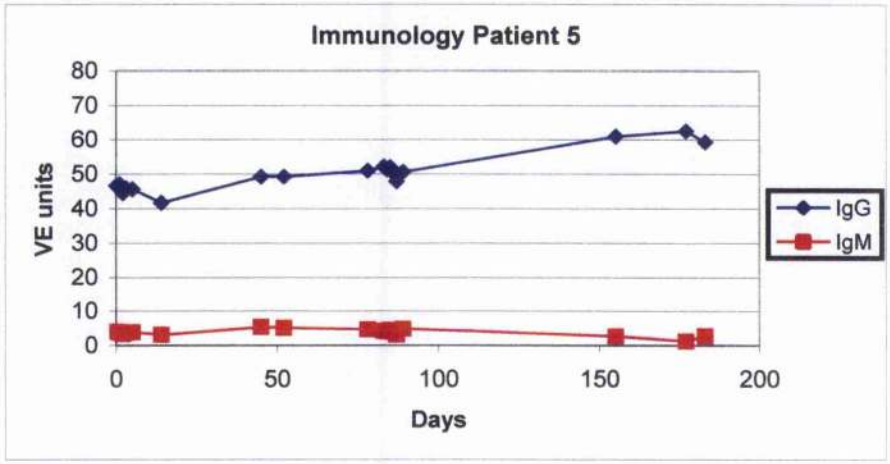


Figure 13.6(iii); *IgG and IgM Patient 5*

Patient 5 was seropositive prior to the injection of HSV1716. During the period he was hospitalised with 'pyrexia of unknown origin', which began on day 78 post surgery and HSV1716 injection, numerous blood samples were analysed for evidence of a change in antibody titre in response to HSV in the blood. No change in the antibody titre was detected.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood – serum tested positive for evidence of HSV DNA on day 155 following HSV1716 injection. There was no evidence of an immunological response on ELISA analysis in the serum.

CSF – none of the samples analysed demonstrated evidence of HSV DNA.

Virus shedding assay

Buccal swabs – there was no evidence of live HSV in any of the samples.

Serum – there was no evidence of live HSV in any of the samples.

Lip sore – there was no evidence of live HSV in the sample analysed.

HSV replication assay in tumour samples

Tumour samples taken at the time of surgical resection were dissociated and cultured. After 17 days in culture and one passage a multicycle growth experiment was carried out.

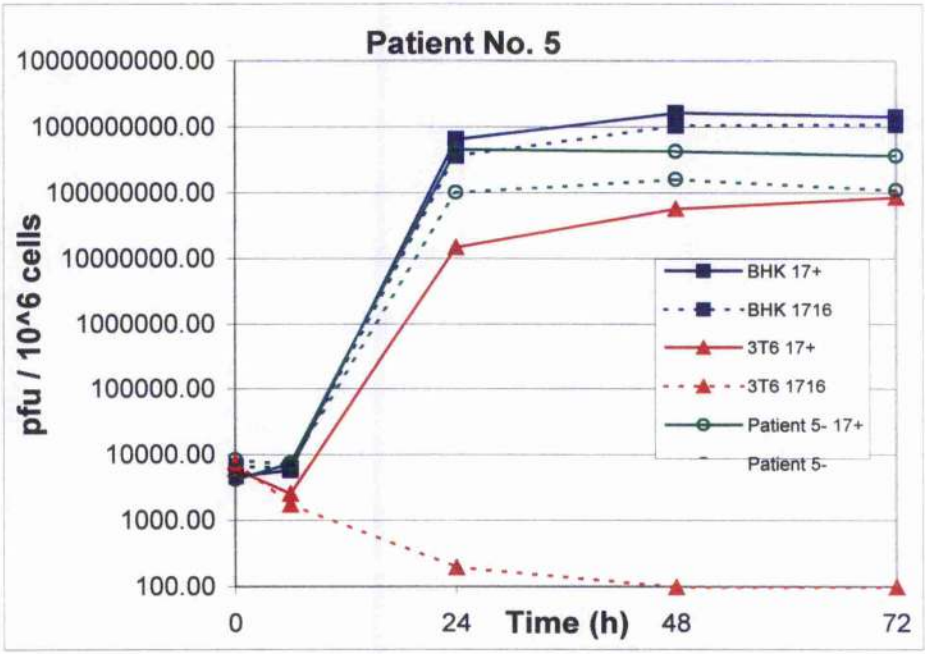


Figure 13.6(iv); *Multicycle growth curve using tumour sample from Patient 5*

It is shown that the tumour sample from patient 5 was fully permissive for the growth of HSV1716. The growth of HSV1716 in the tumour cells was as efficient as the growth of wild type HSV. The yield of wt HSV and HSV1716 was approximately one log less than that of the same viruses in the fully permissive BHK cell line. Although the cell suspensions were counted prior to plating this is probably a reflection of fewer tumour cells compared to BHK cells. Therefore the reduction in virus yield probably reflects the number of cells available for viral replication rather than a reduction in permissivity of cells for viral replication. 3T6 cells are included in this experiment to act as a control. $HSV17^{+}$ is known to

replicating in 3T6 cells, where as it is recognised that 3T6 is not permissive to HSV1716 replication.

13.7 Patient 6

The sixth patient was a 58 years old female. In February 2000 she developed left partial motor seizures. Imaging at that time identified a right parietal lesion, thallium scan negative and considered to be of low grade. She was kept under surveillance by routine imaging. By November 2000 tumour growth was confirmed on imaging. She underwent craniotomy and excision of the lesion. Pathological analysis confirmed the tumour to be a glioblastoma. Surgery was followed by a radical course of radiotherapy (60Gy in 30 fractions). She remained well and independent until August 2001 when she began to experience weakness down her left hand side. In addition she had word finding difficulties and loss of memory. Imaging confirmed the clinical suspicion of tumour recurrence. Of note in her past medical history was a diagnosis of breast cancer in 1994 for which she had surgery and radiotherapy.

Preoperative clinical condition and intra-operative procedure

Her pre-operative clinical assessment indicated a mild left sided weakness. Indices of function indicated that her clinical condition was satisfactory, KS of 90 and a BS of 19. Laboratory investigations and preoperative work up were unremarkable. High-grade glioma was confirmed intra-operatively by smear preparation. Following maximal cyto-reductive surgery she received HSV1716 into the cavity rim [HSV1716 lot No.907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 8].

Postoperative clinical course

Post operatively, neurological examination confirmed extension of the left sided hemiparesis, in addition to a left upper motor neuron seventh nerve palsy and left homonymous hemianopia. There were no signs or symptoms of toxicity associated with the HSV1716 administration. The neurological changes were

considered secondary to the cytoreductive surgery. Patient 6 was unable to be discharged home due poor clinical performance status and required respite care. As she had recurrent disease she received a cycle of CCNU chemotherapy, in line with protocol. Two months following the operation the left sided weakness became more pronounced and imaging indicated further tumour progression. Her symptoms progressed rapidly and she did not receive any further chemotherapy. She required hospice care in the later stages and died three months following the surgery and HSV1716 administration.

Markers of clinical performance

The following graphs present data on clinical performance as assessed by the Karnofsky score and Barthel score, for the duration the patient remained in the study.

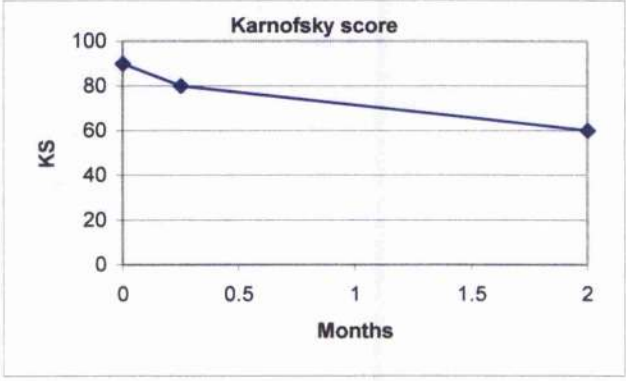


Figure 13.7(i); *Karnofsky Score Patient 6*

Figure 13.7(ii); *Barthel Score Patient 6*

As can be seen by the above graphs soon, after surgery her clinical condition deteriorated and this is highlighted in the reduction in the scores of function.

Laboratory Investigations

Paired vial titration

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of – **8.3 x 10^4 pfu/ml**.

HSV IgG and IgM by ELISA

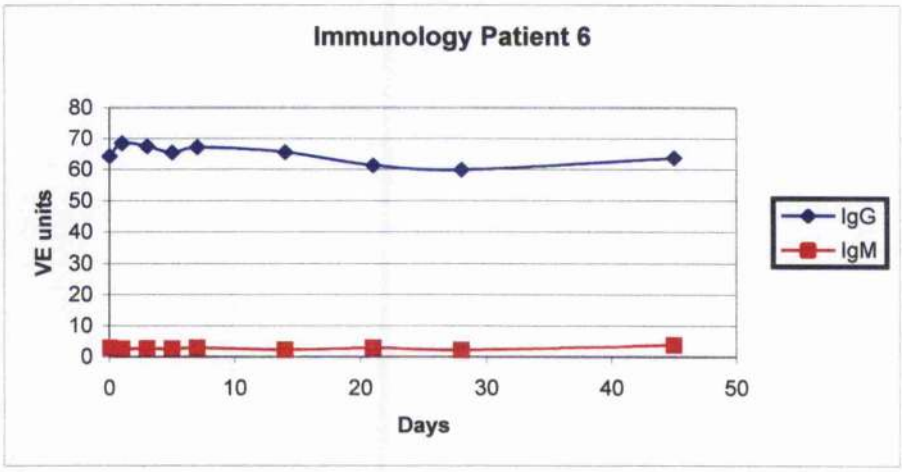


Figure 13.7(iii); *IgG and IgM Patient 6*

Patient 6 was sero-positive for HSV IgG and IgM. Subsequent results were unremarkable.

PCR evidence of HSVDNA

Blood – there was no evidence of HSVDNA in any of the samples taken.

Virus Shedding Assay

Buccal mucosa – there was no evidence of live HSV in any of the swabs analysed.

Serum – there was no evidence of live HSV in any of the samples taken.

HSV replication assay in tumour samples

Tumour samples taken at the time of surgical resection were dissociated and cultured. After 87 days in culture and one passage, a multicycle growth experiment was carried out.

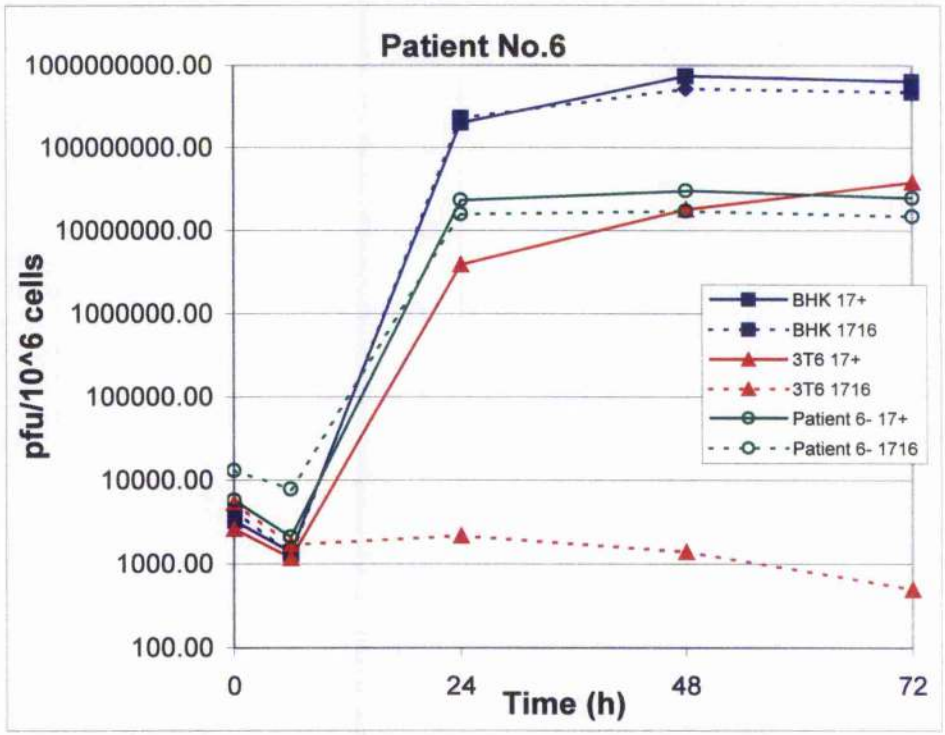


Figure 13.7(iv); *Multicycle growth curve using tumour sample from Patient 6*

It is shown that the tumour sample from patient 6 was fully permissive for the growth of HSV1716. The growth of HSV1716 in the tumour cells was as efficient as the growth of wild type HSV. The growth of wild type HSV and HSV1716 was approximately one log less than that of the same viruses in the fully permissive BHK cell line. Although the cell suspensions were counted prior to plating this is probably a reflection of fewer tumour cells compared to BHK cells. Therefore the reduction in virus yield probably reflects the number of cells available for viral replication rather than a reduction in permissivity of cells for viral replication. The 3T6 cell line was used as a control for $HSV17^{+}$ and HSV1716 growth.

13.8 Patient 7

This 40 year old man presented in July 2001 following the onset of vomiting in addition to headaches and left arm weakness. In September he developed a left upper motor neurone facial palsy. Neurological imaging demonstrated a lesion in the right frontal region. He was enrolled into the study in September 2001.

Preclinical condition and intra-operative procedure

On entry into the study, he was clinically stable with his symptoms maintained on dexamethasone. Indices of clinical performance were KS-90 and BS-20. Neurological examination revealed a left upper motor seventh cranial nerve partial palsy. Laboratory investigations and preoperative work up were unremarkable. At surgery the initial biopsy sent for confirmation of a malignant glioma was graded as a low-grade astrocytic tumour. The low-grade component of the tumour was removed and revealed tumour of a higher-grade beneath. The lesion was macroscopically excised and HSV1716 was injected into the resection cavity rim [HSV1716 Lot. No. 907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 9]. Formal pathological analysis of the tumour reclassified it as a WHO grade IV, glioblastoma. Postoperatively there was partial resolution of the left upper motor seventh nerve palsy and increased power of the left arm.

Postoperative clinical course

He received a radical course of radiotherapy (60Gy in 30 fractions) in line with the protocol. Within a few days of completing his radiotherapy he began to develop new symptoms attributable to tumour progression. Initially he experienced seizures that were controlled with medication. Three months post procedure he developed a left homonymous hemianopia, reduction in power of his left arm and leg in addition to extension of the left upper motor neurone VII cranial nerve palsy. Imaging confirmed the clinical suspicion of tumour re-growth. The tumour was re-excised and the patient subsequently received CCNU chemotherapy. He has remained stable since then 18 months from diagnosis to February 2003.

Markers of clinical performance

The following graphs present data on clinical performance as assessed by the Karnofsky score and Barthel score, for the duration the patient remained in the study.

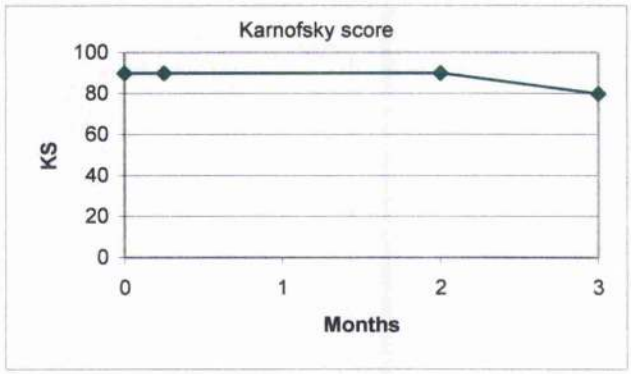


Figure 13.8(i); *Karnofsky Score Patient 7*

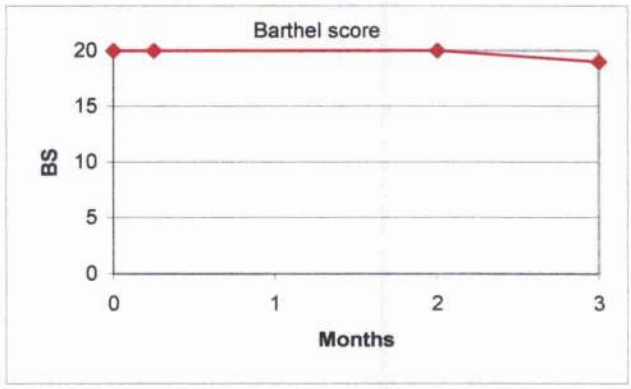


Figure 13.8(ii); *Barthel Score Patient 7*

Laboratory investigations

Titration of paired vial

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of - **1.2×10^5 pfu/ml.**

HSV IgG and IgM by ELISA

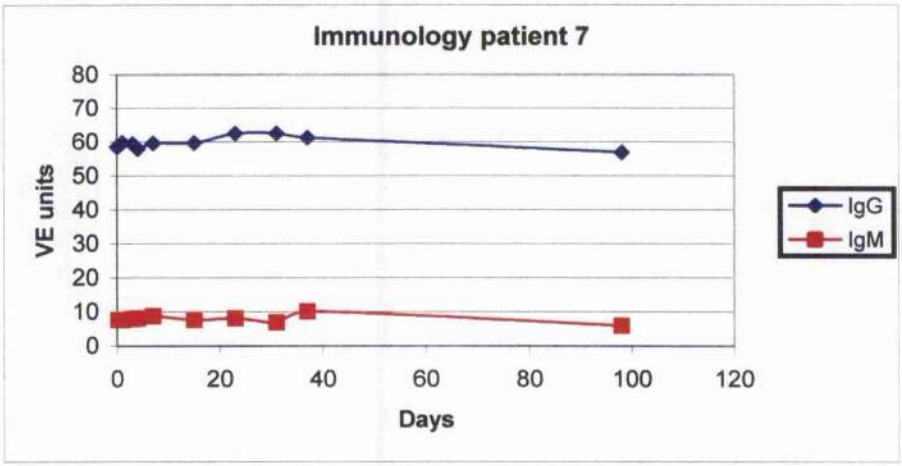


Figure 13.8(iii); *IgG and IgM Patient 7*

Patient 7 was sero-positive prior to the injection of HSV1716. Immunoglobulin levels remained stable for the duration of the study.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood –there was no evidence of HSV DNA in the blood using PCR analysis.

Biopsy – at the time of the second tumour resection, biopsy material from areas surrounding the tumour and from areas distal to the initial HSV1716 injection sites were obtained and assayed for HSV DNA using PCR. There was no evidence of HSV DNA in those samples.

Virus shedding assay

Buccal swabs – no evidence of live HSV was detected.

Serum – no evidence of live HSV was detected.

Biopsy – at the time of the second tumour resection, biopsy material from areas surrounding the tumour and from areas distal to the initial HSV1716 injection sites were obtained and assayed for evidence of live HSV using the virus shedding assay. There was no evidence of live HSV1716 in those samples.

HSV replication assay in tumour samples

Tumour samples taken at the time of surgical resection were dissociated and cultured. After 29 days in culture and no passages a multicycle growth experiment was carried out.

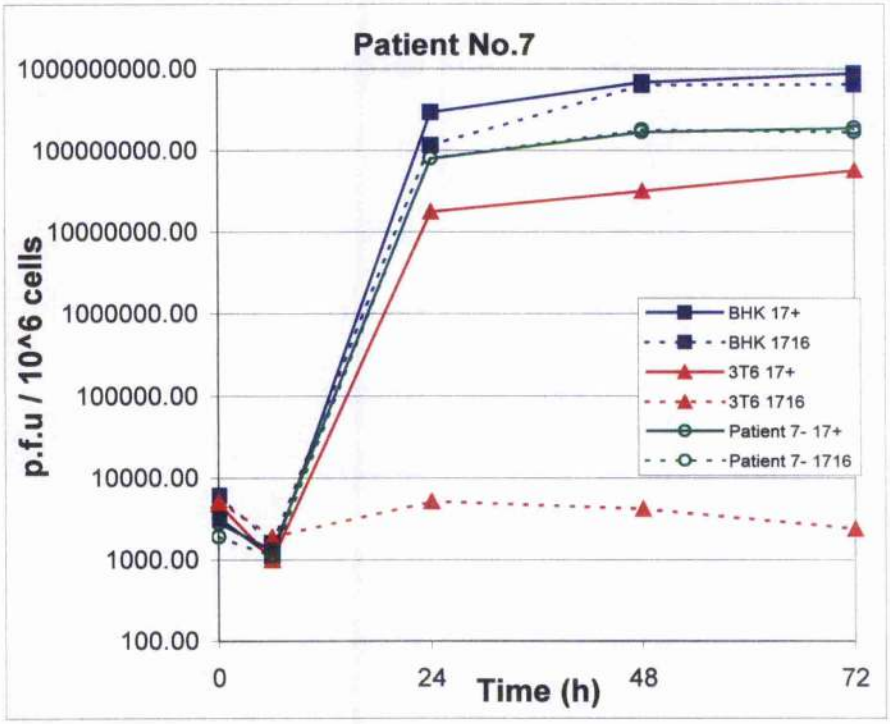


Figure 13.8(iv); *Multicycle growth curve using tumour sample from Patient 7*

It is shown that the tumour sample from patient 7 was fully permissive for the growth of HSV1716. The growth of HSV1716 in the tumour cells was as efficient as the growth of wild type HSV. The 3T6 cell line was used as a control for $HSV17^{+}$ and HSV1716 growth.

13.9 Patient 8

Patient 8 was a 53 year old man who presented initially with severe headaches. Imaging demonstrated a lesion in the left temporal region. He was referred for neurosurgical intervention and was enrolled into the study in September 2001.

Preoperative condition and operative procedure

Prior to the operation he was clinically stable and fulfilled the entry criteria. His markers of clinical performance were satisfactory with a KS of 90 and BS of 20. Neurological examination revealed papilloedema in the left fundus. Laboratory investigations and pre operative work-up were unremarkable. Pathological analysis of an intra-operative smear preparation confirmed a high-grade glial tumour. He underwent left temporal craniotomy and partial lobectomy to remove the tumour. HSV1716 was injected into the resection cavity wall [HSV1716 Lot. No. 907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 12]. Post operatively he recovered with no new signs of neurological deficit. There were no signs of toxicity associated with the HSV1716 injection.

Postoperative clinical course

In line with the protocol he received a course of radical radiotherapy (60Gy in 30 fractions). He had an uneventful period until eight months following surgery and HSV1716 injection when he began to experience some mild expressive dysphasia. Clinical examination revealed a right seventh upper motor neurone partial palsy. CT scan indicated a large mass in the left temporo-parietal region extending into the left fronto-parietal region. He underwent further tumour resection and commenced PCV chemotherapy. He died 3 months following the second resection and 11 months from the initial surgery and HSV1716 injection.

Markers of Clinical Performance

The following graphs present data on clinical performance as assessed by the Karnofsky score and Barthel score, for the duration the patient remained in the study.

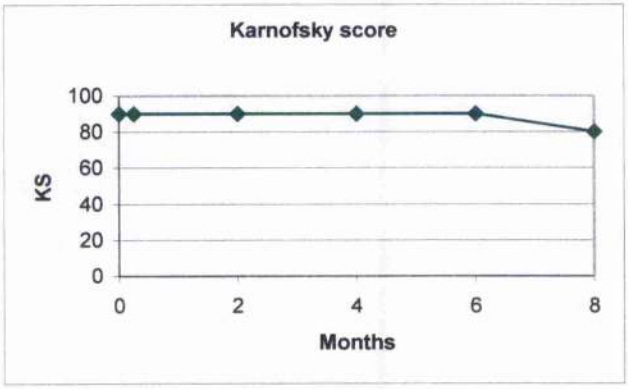


Figure 13.9(i); *Karnofsky Score Patient 8*

Figure 13.9(ii); *Barthel Score Patient 8*

Laboratory Investigations

Titration of paired vial

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of – **9.4×10^4 pfu/ml.**

HSV IgG and IgM by ELISA

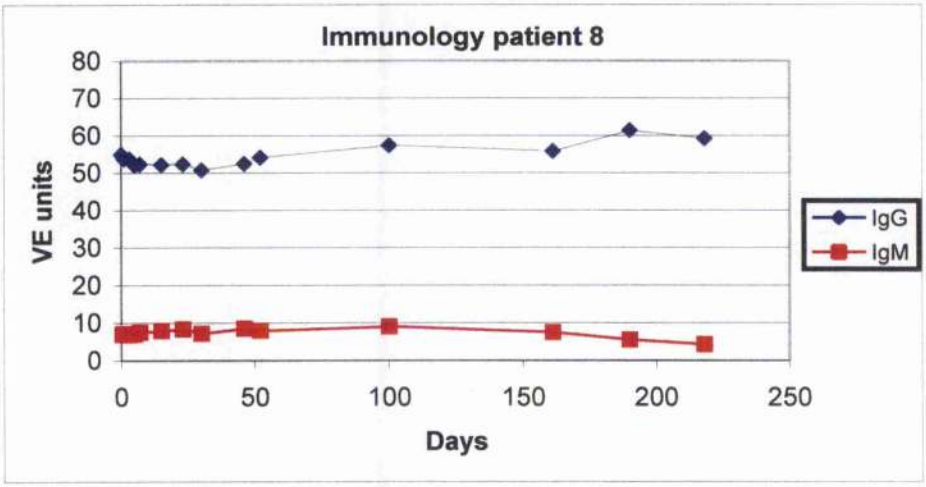


Figure 13.9(iii); *IgG and IgM Patient 8*

Patient 8 was sero-positive prior to the injection of HSV1716. His immunoglobulin levels remained relatively stable for the duration of the study.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood – There was no evidence of HSV DNA in blood by PCR analysis.

Cerebral biopsy - Prior to the second, tumour resection biopsy material from the resection cavity and from sites distal to the initial HSV1716 injection were assayed for HSV DNA using PCR. There was no evidence of HSV DNA in the samples.

Virus shedding assay

Buccal swabs – There was no evidence of live HSV in any of the samples.

Serum – There was no evidence of live HSV in any of the samples.

Cerebral biopsy - Prior to the second tumour resection, biopsy material from the resection cavity and from sites distal to the initial HSV1716 injection was analysed for evidence of live virus. There was no evidence of HSV in those samples.

HSV replication assay in tumour samples

Tumour samples taken at the time of surgical resection were dissociated and cultured. After 38 days in culture and no passages, a multicycle growth experiment was carried out.

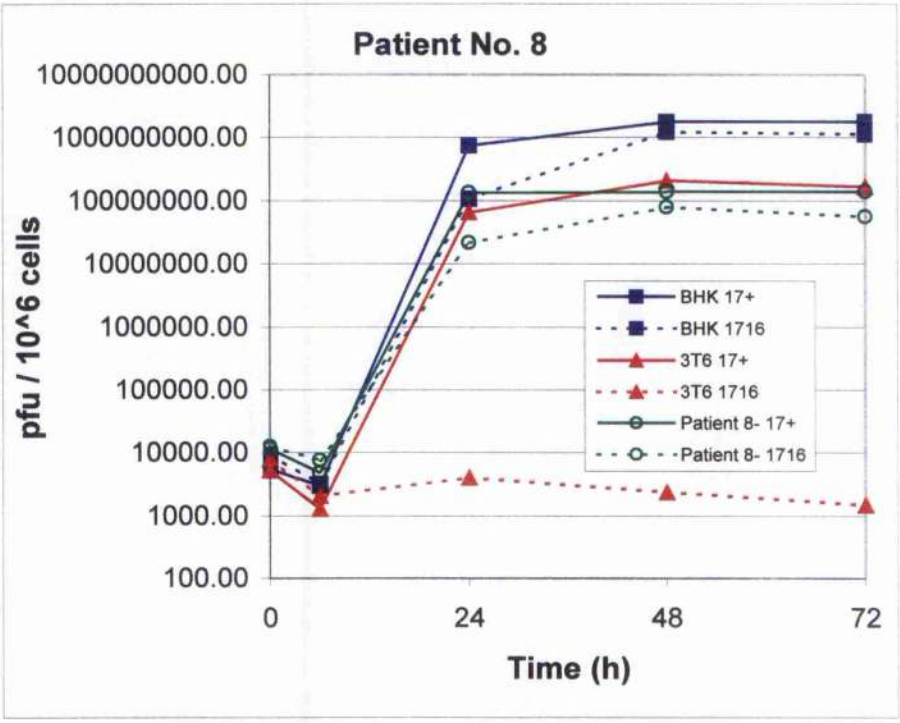


Figure 13.9(iv); *Multicycle growth curve using tumour sample from Patient 8*

It is shown that the tumour sample from patient 8 was fully permissive for the growth of HSV1716. The growth of HSV1716 in the tumour cells was as efficient as the growth of wt HSV. The growth of wild type HSV and HSV1716 was approximately one log less than that of the same viruses in the fully permissive BHK cell line. Once again this is probably a reflection of fewer cells in the tumour samples petri dish compared to those containing BHK cells. The 3T6 cell line was used as a control for $HSV17^{+}$ and HSV1716 growth.

13.10 Patient 9

Patient 9 was a 40 year old man who initially presented in 1995 having suffered a grand-mal seizure. Neurological imaging indicted a lesion in the right frontal region. The tumour was resected and pathological analysis identified the tumour

as a WHO grade II, oligodendroglioma. He received no additional treatment at that time but was kept under review with serial neuro-imaging. In February 2000 he presented with worsening headaches. Imaging confirmed tumour progression. He underwent further cytoreductive surgery, which was followed by radical radiotherapy (60Gy in 30 fractions). In September 2001 he represented with increasing seizure activity and imaging indicated an increase in the tumour size. He was enrolled into the study in November 2001.

Preoperative condition

On entry to the study, patient 9 was clinically stable on a regular dose of oral dexamethasone. Markers of clinical performance were KS-90 and BS-20. Neurological examination revealed no deficits. Laboratory investigations and pre-operative work-up were unremarkable. Intra-operative pathological analysis of a smear preparation diagnosed an anaplastic glial tumour. Cytoreductive surgery was carried out and 1ml of 1 x 10^5 pfu/ml was injected into the resection cavity rim in line with the protocol [HSV1716 Lot.No. 907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 14].

Postoperative clinical condition

Post operatively neurological examination revealed a partial left upper motor neurone seventh nerve palsy and minimal left hemiparesis. These signs resolved in the immediate post-operative period. Initial biochemistry results post operatively indicated elevation of the liver transaminases ALT and AST. This resolved in the first post operative week and were considered a result of the anaesthetic. There was no evidence of toxicity associated with the injection of HSV1716. Formal pathological analysis categorised the lesion as an Anaplastic Oligodendroglioma. One month following the surgical intervention he was admitted with headache and feeling non-specifically unwell. On clinical examination he had a raised temperature in addition to a tense and inflamed craniotomy wound. Culture of blood and CSF samples failed to reveal any bacterial infection. In view of the wound infection he was commenced on broad-spectrum antibiotics. He made a good recovery and was discharged home.

In line with the protocol he was commenced on PCV chemotherapy. Six months following entry into the study and after three cycles of PCV chemotherapy clinical deterioration was noted. Neurological examination indicated that there was extension of the previously noted seventh upper motor neurone palsy and reduced power of his left arm and leg. Despite clinical deterioration the imaging indicated that his disease was stable. A clinical decision was made to change his chemotherapy to second line, Temozolamide. He completed three cycles of chemotherapy before clinical deterioration precluded him from receiving further cycles. He died 14 months following HSV1716 intervention.

Markers of clinical performance

The following graphs present data on clinical performance as assessed by the Karnofsky score and Barthel score, for the duration the patient remained in the study.

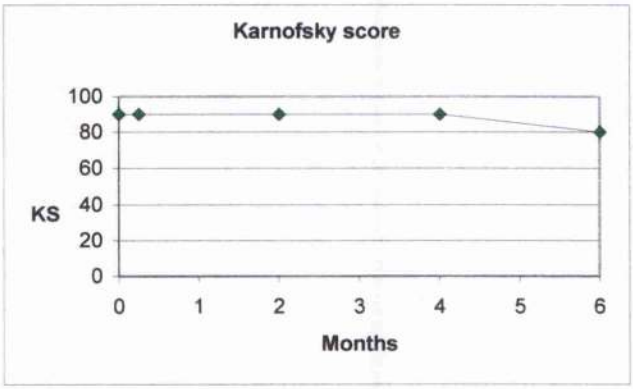


Figure 13.10(i); *Karnofsky Score Patient 9*

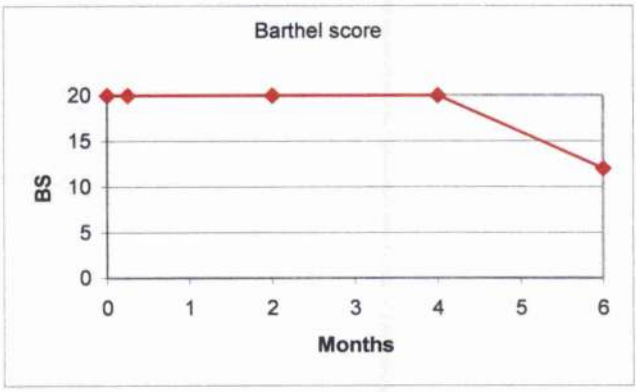


Figure 13.10(ii); *Barthel Score Patient 9*

Laboratory investigations

Paired vial titration

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of – **1.1×10^5 pfu/ml.**

HSV IgG and IgM by ELISA

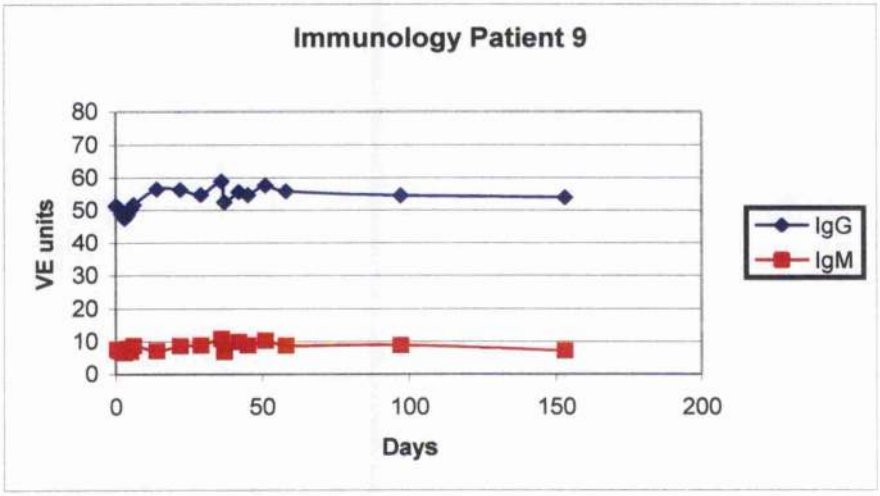


Figure 13.10(iii); *IgG and IgM Patient 9*

Patient 9 was sero-positive prior to the injection of HSV1716. There was no immunological response detected related to the injection.

Polymerise Chain Reaction (PCR) for evidence of HSVDNA

Blood –There was no evidence of HSVDNA in the blood using PCR analysis.

Virus shedding assay

Buccal swabs – no evidence of live HSV was detected.

Serum – no evidence of live HSV was detected.

13.11 Patient 10

The tenth patient to be enrolled into the study was a 33 year old female. Her symptoms dated back ten years to when she first became aware of intermittent weakness of her right arm. In 1999 neurological imaging identified a lesion in the left parietal region, which was considered low grade. No intervention was required at that stage. She began to experience focal seizures limited to her right arm, which were controlled by anti-convulsants. Her symptoms progressed two weeks prior to entry into the study when she developed severe headaches. Imaging confirmed tumour progression. Immediately prior to her surgery there was rapid deterioration in her conscious level and clinical condition despite supportive medical care. She underwent emergency surgery to drain a cyst and her symptoms improved. Following this intervention she was clinically stable and underwent definitive surgery and received an injection of HSV1716 injection into the cavity rim in November 2001.

Pre-clinical condition and intra-operative procedure

Following the emergency surgery to drain the cyst that had developed patient 10 was noted to be slightly drowsy. Neurological examination revealed nystagmus on right lateral gaze in addition to a right hemiparesis. Markers of clinical performance were influenced by her recent operation and were recorded as KS-70 and BS-11 at 24 hours pre-operatively. Laboratory and pre-operative work up investigations were unremarkable and did not preclude surgery. At surgery it was noted that the tumour had undergone anaplastic transformation within the core with low-grade tumour surrounding this. Pathological analysis of a smear

preparation at surgery confirmed a high-grade glioma. HSV1716 was injected into the resection cavity rim [HSV1716 Lot.No. 907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 6].

Post-operative clinical condition

Post operatively neurological examination revealed some decrease in right leg power. Formal pathological analysis categorised the tumour as a glioblastoma. In line with the protocol she underwent a radical course of radiotherapy (60Gy in 30 fractions). Her long-term follow up has been unremarkable. She remained well 15 months from surgery and HSV1716 injection with no evidence of tumour re-growth at February 2003.

Markers of clinical performance

The following graphs present data on clinical performance as assessed by the Karnofsky score and Barthel score, for the duration the patient remained in the study.

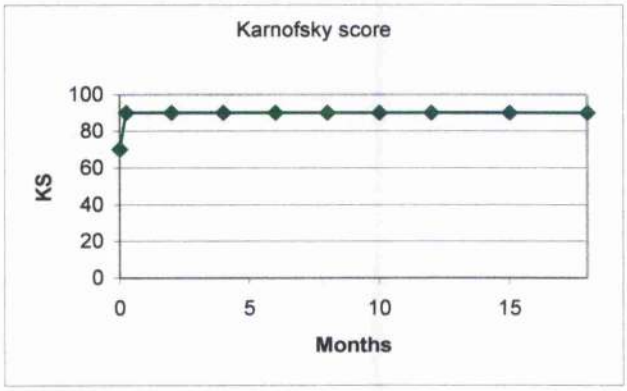


Figure 13.11(i); *Karnofsky Score Patient 10*

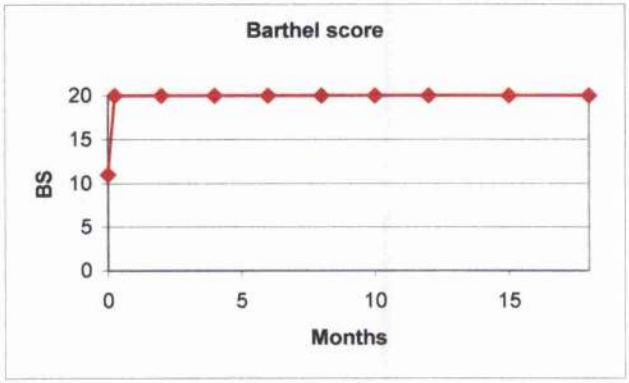


Figure 13.11(ii); *Barthel Score Patient10*

Immediately following surgical drainage of the cyst and prior to tumour resection the markers of clinical performance were low. Subsequently she has made a good recovery and has remained clinically stable as indicated by the markers of clinical performance.

Laboratory investigations

Paired viral titration

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of – **1.1×10^5 pfu/ml.**

HSV IgG and IgM by ELISA

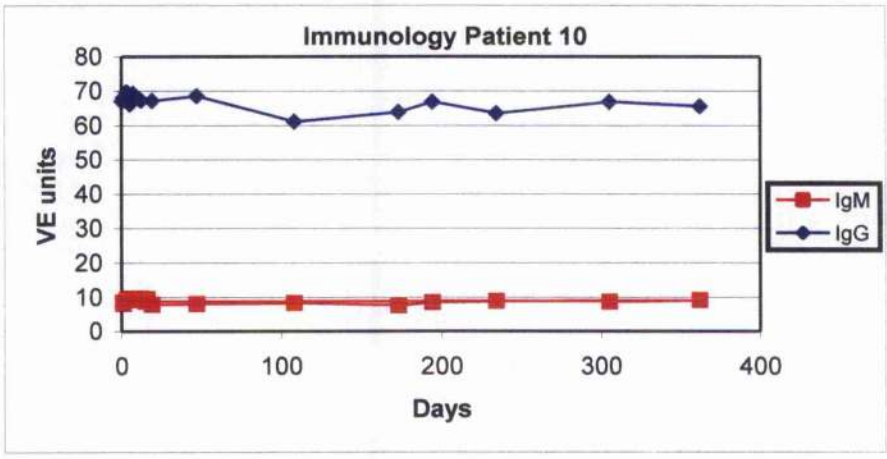


Figure 13.11(iii); *IgG and IgM Patient 10*

Patient 10 was sero-positive prior to the injection of HSV1716. There was no significant change in the immunoglobulin levels for the duration of the study.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood – serum tested positive for evidence of HSV DNA on day 108 following HSV1716 injection. There has been no evidence of an immunological response on ELISA analysis of serum.

Virus shedding assay

Buccal swabs – there was no evidence of live HSV in any of the samples.

Serum – there was no evidence of live HSV in any of the samples.

13.12 Patient 11

This 66 year old man presented to hospital following a collapse and loss of consciousness. Further enquiry revealed a one month history of headache and word finding difficulties. Computed Tomography demonstrated a lesion in the left parietal region with surrounding oedema.

Pre-clinical condition and intra-operative procedure

On entry to the study patient 11 was clinically stable with no signs or symptoms of disease. His markers of clinical performance were KS of 90 and BS of 20. Laboratory and pre-operative investigations were unremarkable. He underwent left parietal craniotomy and excision of the tumour following pathological identification of a high-grade glioma by smear preparation. HSV1716 was injected into the resection cavity rim. [HSV1716 Lot.No.907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 17]. Formal pathological analysis indicated that the lesion was a Glioblastoma.

Post-operative clinical course

Post operatively he was well with no new neurological disability or evidence of toxicity associated with the injection of HSV1716. Within three weeks of

discharge he developed a rash over his legs which was diagnosed as eczema in addition to steroid induced acne. The dermatological conditions were controlled with medical management.

In line with the protocol he received a radical course of radiotherapy (60Gy in 30 fractions). He remained well until 10 months post surgery when he developed weakness of his right leg. Neuro-imaging demonstrated significant change suggestive of tumour recurrence. Following confirmation of tumour progression (clinically and radiologically) he was offered Temozolomide chemotherapy. Following two cycles of chemotherapy tumour growth was not controlled and he died 11 ½ months following the surgery and HSV1716 injection.

Markers of clinical performance

The following graphs present data on clinical performance as assessed by the Karnofsky score and Barthel score, for the duration the patient remained in the study.

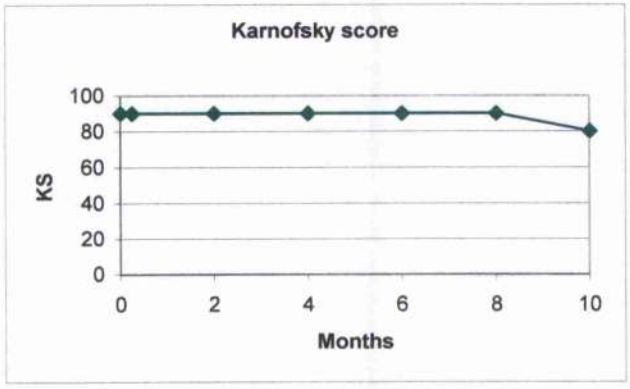


Figure 13.12(i); *Karnofsky Score Patient 11*

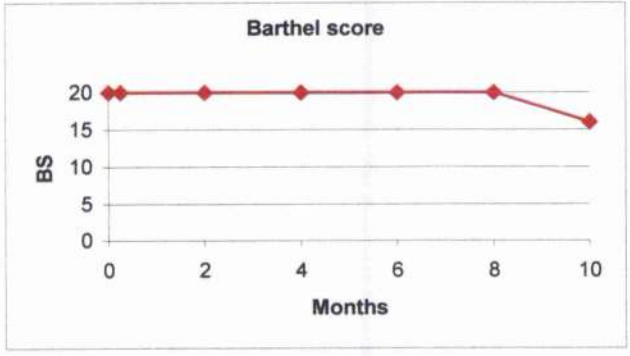


Figure 13.12(ii); *Barthel Score Patient 11*

Laboratory Investigations

Titration of paired vial

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of – **8.9×10^4 pfu/ml.**

HSV IgG and IgM by ELISA

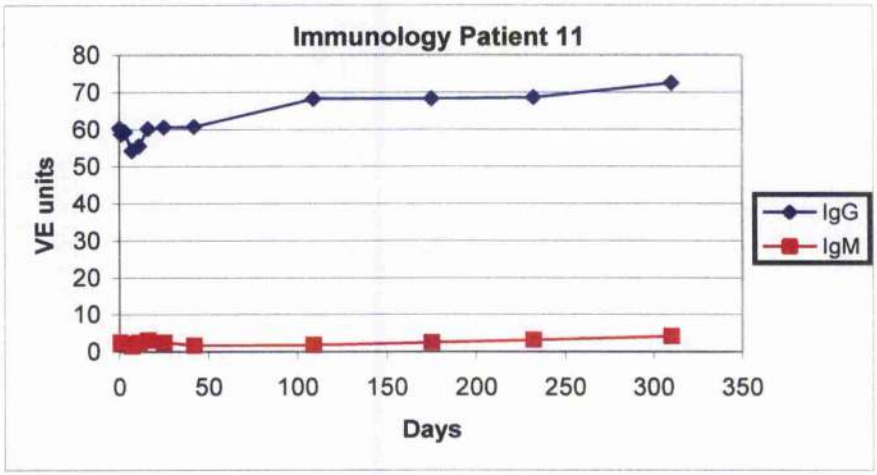


Figure 13.12(iii); *IgG and IgM Patient 11*

Patient 11 was sero-positive prior to the injection of HSV1716. There was no significant change in the immunoglobulin levels for the duration of the study.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood –There was no evidence of HSV DNA in the blood using PCR analysis.

Cerebral biopsy – Post mortem tissue was assessed by PCR for evidence of HSV DNA. No HSV DNA was detected from ten samples.

Virus shedding assay

Buccal swabs – no evidence of live HSV was detected.

Serum – no evidence of live HSV was detected.

13.13 Patient 12

Patient 12 was a 55 year old man whose symptoms developed following an occipital head injury after a fall. In the weeks following the fall he developed headache associated with vomiting. Initial imaging suggested an occipital cerebral contusion. Further analysis of the imaging discovered a lesion in the right temporal region. He was entered into the study in December 2001. He had a past medical history of type II diabetes mellitus with secondary vascular disease that had resulted in a right below the knee amputation. Of note in his family history was that his brother had died of a brain tumour.

Pre-clinical condition and intra-operative procedure

Prior to the operation he was clinically stable. His markers of clinical performance were recorded as KS-90 and BS-20. Laboratory investigations revealed an elevated blood glucose level that required medical management. Preoperative assessment was unremarkable. He underwent a right temporal craniotomy and resection of tumour following confirmation of a high-grade malignant glioma on a smear preparation. HSV1716 was injected into the resection cavity wall [HSV1716 Lot. No. 907.01 Dilution Lot 2 10^5 pfu/ml, 1ml dose 14.7.99 Vial 18].

Post-operative clinical condition

Post operatively he recovered well with no new neurological deficit. There was no associated toxicity with the injection of HSV1716. His diabetic control proved problematic and eventually to control his blood glucose the oral hypoglycaemic medication was substituted by subcutaneous insulin.

At the three week assessment, it was noted that he had a rash over both elbows. The rash was vesicular in nature and appeared to be in limited to the fifth and sixth cervical dermatomes. This was confirmed as bilateral C5/C6 varicella zoster virus (VZV) rash. As there was no associated pain, treatment with Acyclovir was not indicated. Swabs and vesicular fluid samples were taken in order to

investigate if live HSV1716 (by virus shedding assay) or HSV DNA (by PCR analysis) could be detected. None of the tests gave a positive result.

Furthermore he developed ulceration and cellulitis around his left ankle. In view of the vascular risk to his remaining limb he was transferred to a medical ward for further assessment and broad-spectrum intravenous antibiotics. The wound healed following optimisation of diabetic control. This complication was considered to be secondary to his poor diabetic control and not related to HSV1716.

In line with the protocol he underwent a radical course of radiotherapy (60Gy in 30 fractions). During routine follow-up he remained well. Imaging at his 8 month review demonstrated tumour progression. Despite the lack of clinical symptoms or signs it was decided that he had tumour progression and went on to receive Temozolamide chemotherapy.

Unexpectedly he died from a presumed myocardial infarction shortly after his second cycle of Temozolamide. He survived 11 months following surgery and injection of HSV1716.

Markers of clinical performance

The following graphs present data on clinical performance as assessed by the Karnofsky score and Barthel score, for the duration the patient remained in the study.

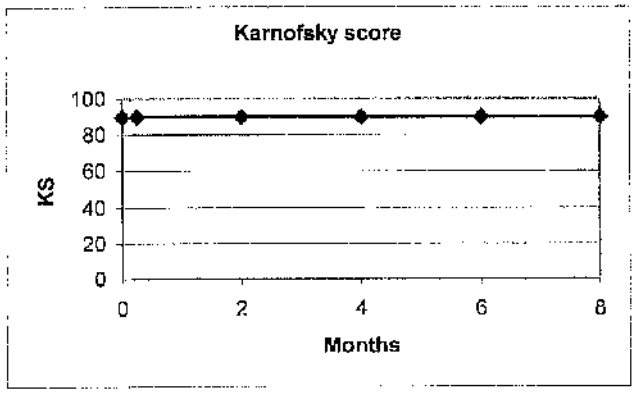


Figure 13.13(i); *Karnofsky Score Patient 12*

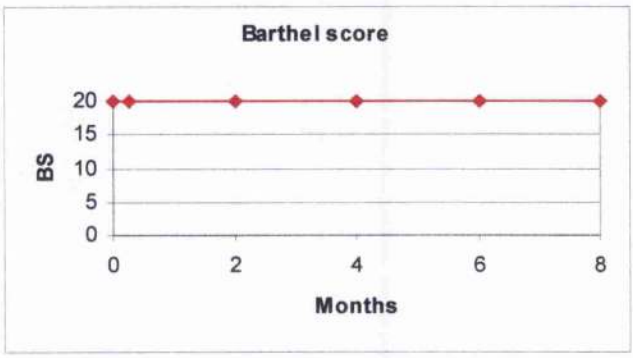


Figure 13.13(ii); *Barthel Score Patient 12*

Laboratory investigations

Titration of paired sample

The sample of HSV1716 that was paired to the injected aliquot of HSV1716 had a titre of – **8×10^4 pfu/ml.**

HSV IgG and IgG by ELISA

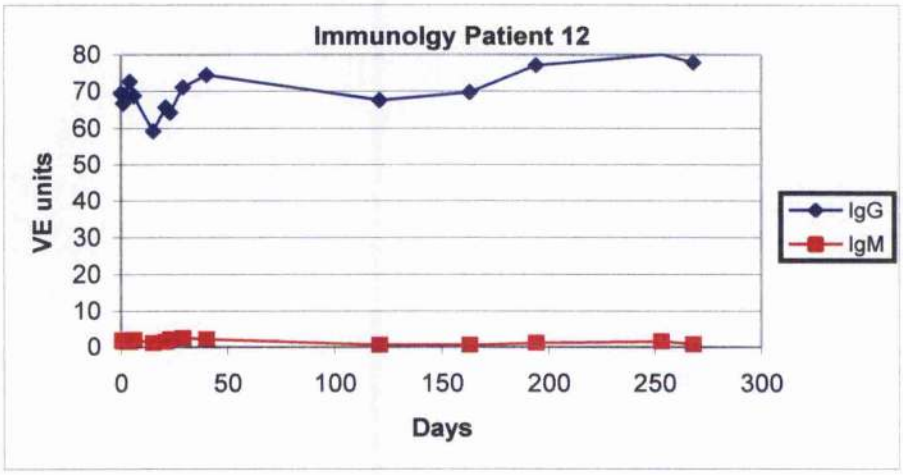


Figure 13.13(iii); *IgG and IgM Patient 12*

Patient 12 was sero-positive prior to the injection of HSV1716. There was no significant change in the immunoglobulins levels for the duration of the study.

Polymerise Chain Reaction (PCR) for evidence of HSV DNA

Blood – There was no evidence of HSV DNA in blood by PCR analysis.

Vesicular fluid – there was no evidence of HSV DNA in the vesicular fluid samples.

Virus shedding assay

Buccal swabs – there was no evidence of live HSV in any of the samples.

Serum – there was no evidence of live HSV in any of the samples.

Vesicular fluid – there was no evidence of live HSV in any of the samples.

13.14 Summary of Results

Between April 2001 and December 2001 twelve patients with high grade glioma were enrolled into this phase one clinical trial. Six patients had previously received treatment for a proven tumour of glial origin. The remaining six patients presented with *de novo* tumours thought to be high grade on imaging alone. Following resection of the tumour HSV1716 was delivered into the rim of the resection cavity. The primary aim of the study was to assess the safety of HSV1716 when injected into predominantly brain adjacent to tumour. Although not designed to assess efficacy longitudinal follow-up allowed assessment of overall survival.

Formal patient review was completed by February 2003. There was no evidence of local or systemic toxicity associated with the procedure. At study completion ten patients had demonstrated evidence of tumour progression; one (patient 12) died from a non-tumour related cause and one (patient 7) was still alive18 months from initial diagnosis and treatment. Patient 1 and 10 were clinically well and free of tumour progression at 22 and 15 months respectively following HSV1716 administration.

After each appointment at the clinic, patient serum samples were analysed for routine biochemistry and haematology. In addition, blood samples were analysed

in our laboratory for infectious HSV; HSV DNA by PCR and for HSV-1 IgG and IgM by ELISA. Patients 2, 3 and 4 were HSV seronegative at the time of enrolment. Of these, patients 3 and 4 mounted an immune response and seroconverted between the third and fourth weeks post virus injection. Patient 3 showed evidence of disease progression at 10 months following injection of HSV1716 and died at 11 months. Patient 4 also died at 11 months after virus injection. The remaining patient (2) did not mount an immune response and died six months after injection of virus

Serum samples from four patients have been positive for HSV DNA. Of these, Patients 1, 5 and 10 were seropositive, and had a prior clinical history of HSV infection (cold sores). They were PCR positive some time after injection of HSV1716. Patient 3 was seronegative prior to injection of HSV1716 and seroconverted at day 24, the same time at which HSV DNA was detected in his serum.

Chapter 4

14 Investigation of the Interaction Between Ionising Radiation and HSV1716 *in vitro*.

14.1 Introduction

The rational for this research was based on the publication of data indicating a potentially synergistic relationship of tumour cell kill between a HSV ICP34.5 null mutant, R3616 and ionising radiation *in vivo* (Advani SJ *et al.*, 1998). No data on the *in vitro* interaction of the two modalities was presented and possible reasons of the modes of cell kill being synergistic were unclear. The aim of this research was to develop a tissue culture system to investigate, the possibility of enhanced cell kill when ionising radiation is employed in conjunction with the ICP34.5 null mutant HSV1716 and if possible to elucidate the mechanisms responsible for the enhanced cell kill.

It became apparent at an early stage that the initial cell viability assay of choice, the clonogenic assay, was not suitable to investigate the combined effects of a replication competent virus such as HSV1716 and ionising radiation. The clonogenic assay is used effectively to investigate the effects of radiation alone on a population of cells. Radiation causes a proportion of a population of cells to be sterilised. Those that retain their replicative potential and can produce a population of cells numbering more than 50 are termed clonogens. However when replication competent virus is added to a population of cells the effect is not finite, as is the case for radiation. Each infected cell has the potential to produce hundreds of infectious progeny with the potential to kill other cells. Therefore viral infection in tissue culture cells tends to be an all or nothing phenomenon. When HSV1716 was added to cells in clonogenic assays there was complete lysis of all clonogens so that any additional effect exerted by radiation could not be detected.

The relationship between HSV1716 and ionising radiation was investigated using the Promega MTS cytotoxicity assay. This assay is a colorimetric method for determining the number of viable cells. At the outset there was no available published data detailing the use of the MTS assay with an oncolytic virus. Initial experiments were performed to determine the reduction in cell viability following ionising radiation or HSV1716 infection in isolation. Following the initial characterisation, HSV1716 and radiation were then combined to investigate if any possible relationship between the two modalities could be identified *in vitro*.

Baby Hamster Kidney cell line (BHK) and Mouse fibroblast cell line (3T6) were used throughout this research project. Both cell lines are well characterised as they have been used over many years in research involving HSV1716. BHK has been shown to be fully permissive to HSV infection and supports lytic replication of HSV1716 and wild type HSV17^{+}. In comparison 3T6 cells, although capable of infection with HSV1716 cannot support its replication cycle and therefore the cells are not lysed. 3T6 cells support wild type HSV17^{+} lytic replication (Brown SM *et al.*, 1994b).

As the clinical studies involved the use of HSV1716 in human malignant brain tumours it was considered necessary to investigate the characteristics of ionising radiation and HSV1716 replication in an appropriate human cell line. The human high grade glioma MOG and 373 cell lines were selected. Both cell lines grew well under optimum conditions and preliminary investigations indicated their suitability for investigation in combination experiments with ionising radiation and HSV1716. Details of these experiments are presented in this chapter.

14.2 Establishment of MTS Assay Parameters

Cell viability was assessed using the Promega MTS cytotoxicity assay. The Cell Titre 96® AQ_{ueous} Assay uses the novel tetrazolium compound (3-4,5-dimethlythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and the electron coupling reagent, phenazine methosulphate (PMS). MTS is chemically reduced by cells into formazan, which

is soluble in tissue culture medium. Measurement of the absorbance of the formazan can be carried out using 96 well microplates at 492nm. The assay measures dehydrogensase enzyme activity found in metabolically active cells. Since the production of formazan is proportional to the number of living cells, the intensity of the produced colour is a good indication of the viability of the cells.

Promega suggest the addition of 20µl of MTS substrate to 100µl of medium per well of a 96 well plate, the absorbance being recorded after a period of between 1 and 4 hours. However initial experiments using 20µl of MTS per 100µl of medium failed to provide a discernable difference between cell monolayers that were approximately 80% to 100% confluent on microscopic analysis. 20µl of MTS substrate produced too intense a colorimetric reading when large numbers of viable cells were present.

The cytotoxicity experiments proposed were to be analysed by MTS assay daily for up to one week. It was reasoned that the control monolayers and monolayers that were exposed to minimal radiation or HSV1716 would probably be 80-100% confluent by one week and that 20µl of MTS substrate would not distinguish subtle differences at higher monolayer densities. It was therefore thought that a smaller volume of substrate might allow such differences to be detected.

10µl of MTS solution per 100µl of medium was assessed to ensure that this volume provided enough substrate for viable cells to convert over the four hours incubation period. Readings taken two and four hours after MTS substrate addition were compared.

BHK or 373 cells were seeded at $3x10^3$ cells and incubated at 37^0C and 5% CO_2 for 24 hours.

BHK cells;

	Colorimetric reading obtained with various quantities of MTS solution per 100µl medium	
Time between MTS addition and plate reading (Hours)	10µl	20µl
2	0.539	0.642
4	0.728	0.863

Table 14.2(i); *MTS reading with BHK cells*

373 cells;

	Colorimetric reading obtained with various quantities of MTS solution per 100µl medium	
Time between MTS addition and plate reading (Hours)	10µl	20µl
2	0.597	0.705
4	0.907	1.04

Table 14.2(ii); *MTS reading with 373 cells*

The data demonstrate that after two hours there was still substrate available for conversion given the increase in the colorimetric readings after four hours of incubation.

The longer time period is required to ensure that the viable cells can convert all the available MTS substrate and produce a colorimetric reading that reflects the true number of viable cells in the monolayer. Using 10µl of MTS substrate per 100µl of medium enabled the detection of differences in cell viability even when the monolayers were more than 80% confluent.

Subsequent cytotoxicity assay experiments were carried out using 10µl of MTS substrate and the colorimetric readings were acquired after four hours.

14.3 MTS Reading With Respect to Number of Viable Cells

Interpretation of the MTS colorimetric reading requires that there is a linear relationship between the number of live cells and the MTS colorimetric reading. It was therefore necessary to confirm the relationship was linear and intersected the zero.

The cell lines used in this experiment were 3T6 and 373. In this experiment 3T6 cells were investigated as they do not form clumps following trypsinisation of monolayers and therefore could be counted accurately using a haemocytometer. 373 cells were also investigated as this tumour cell line was proposed for use in future analyses.

3T6 and 373 cells were seeded in six well columns of a 96 well plate in a range of cell numbers - 1000, 2000, 5000, 10000 and 20000 in 100µl of medium.

The results (figure 14.3a) demonstrate a linear relationship between the number of 3T6 cells and the MTS reading. Over this range of cell densities it can be seen that an MTS reading of 1.2 has double the number of viable cells present than an MTS reading of 0.6.

This relationship is the same for the 373 cell line (figure 14.3b).

Subsequent experiments were all conducted at cell densities within this linear range.

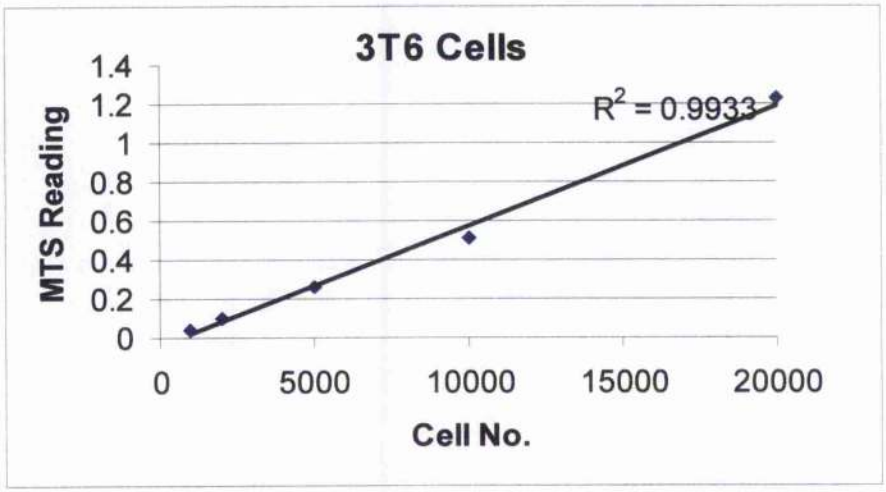


Figure 14.3(a); *relationship between 3T6 cells and an MTS reading*

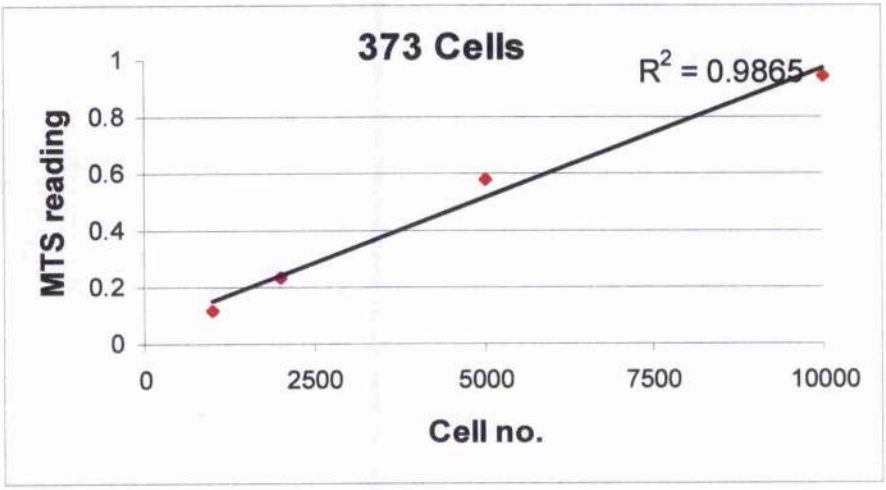


Figure 14.3(b); *relationship between 373 cells and an MTS reading*

14.4 Assessment of Permissivity of MOG Cells for Replication of HSV1716

To conduct *in vitro* studies on the possibility of synergy between cell kill from the lytic replication of HSV1716 and cell kill from external beam radiation it is necessary to have a suitable cell line. Whilst the cells must be permissive to HSV1716 infection, the virus must not kill all of them before the radiation has had an effect. The effects of ionising radiation (IR) on cell viability are not manifested immediately, so the cell kill afforded by HSV1716 alone should preferably be less than 100% after approximately one week, if a synergistic relationship is to be detected. In practice this will probably require a low multiplicity of infection allowing the possibility of several cycles of virus replication.

To select suitable cell lines, capable of infection but not killed too rapidly, a number of human glioma cell lines were tested for their ability to support HSV1716 infection i.e. UVW, MOG and 373. For this selection process, cells were assayed in multicycle growth experiments for their ability to support HSV1716 replication over 72 hours following a low multiplicity of infection (MOI = 0.1pfu/cell).

Of the cells tested, MOG cells appeared the most suitable for the use in cytotoxicity assays to investigate synergy between HSV1716 and ionising radiation. They were permissive for HSV1716 replication (figure 14.4) but the 72 hour yield was approximately one log lower than that for the parental wild type strain, $HSV17^{+}$. The finding that both HSV1716 and $HSV17^{+}$ apparently replicated less efficiently in MOG cells than in BHK cells could be a true reflection of the ability of the cells to support a HSV infection. Alternatively it may be due to a cell counting error or the failure of the cells to settle in the well as efficiently, resulting in the number of MOG cells infected being lower than the number of BHK cells infected. However the difference between the 72 hour yields of $HSV17^{+}$ and HSV1716 in the MOG cultures must reflect less efficient replication of HSV1716 in these cells. This may indicate that the size of viral burst was smaller or that HSV1716 did not infect and lyse as many MOG cells as $HSV17^{+}$. Whatever the cause of the reduced efficiency of HSV1716 replication it

is apparent that additional cell kill could be realised if combination with radiation has an additive effect.

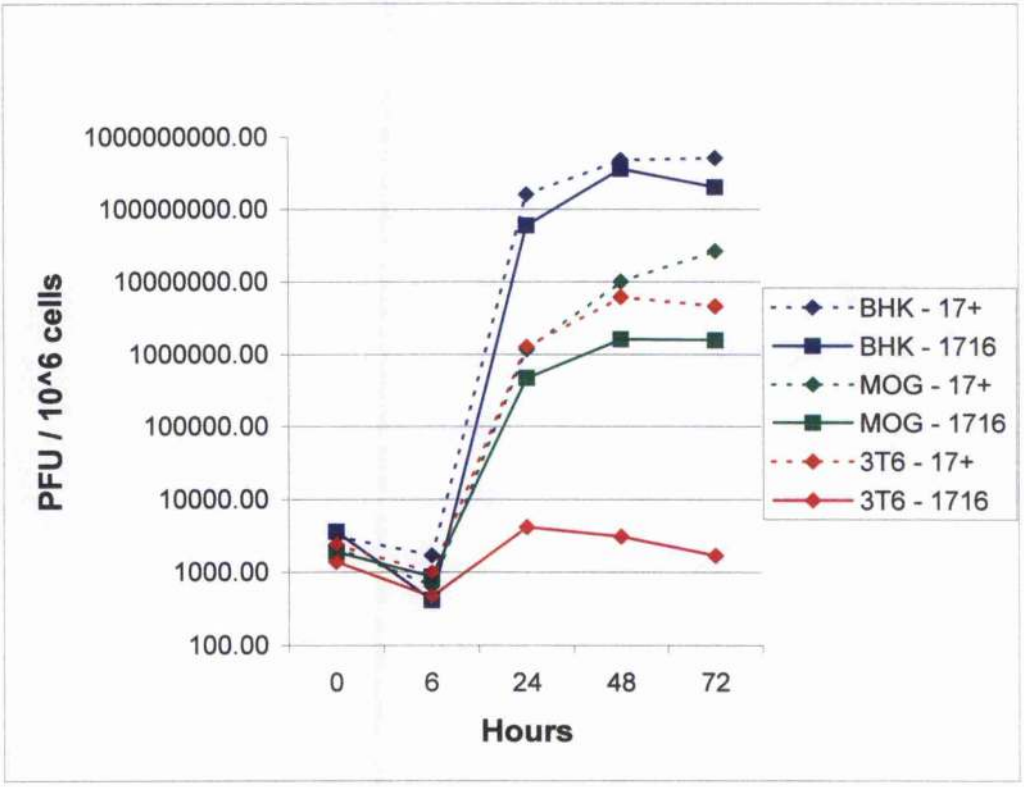


Figure 14.4; *Multicycle growth experiment of HSV1716 and HSV17^{+} BHK, 3T6 and MOG cells.*

14.5 Cell Density for Propagation of Viral Infection

At the outset it was necessary to create conditions *in vitro* that were similar to those of a tumour *in vivo*. It was necessary that the cells had the ability to replicate for the duration of the experiment to ensure that the impact of HSV1716 and ionising radiation on the monolayer were similar to when these modalities are used in tumours *in vivo*. It was reasoned that the effect of radiation on a cell monolayer would have an impact detectable by the MTS assay by approximately one week at low radiation doses and earlier if higher doses were used. The density of the cells seeded initially had to allow exponential growth to continue for about one week to ensure the level of cell kill exerted by radiation could be detected colourimetrically by the MTS assay. If the cell monolayer reached confluence earlier than one week, natural cell death might occur prior to the cell kill caused by the radiation, making detection of cell death from radiation more difficult.

Initially each experimental well of the 96 well plate was seeded with $1x10^3$ MOG cells in 100µl of medium. The following day, the cells were very sparse and over the 72 hour course of the experiment the control plates did not achieve confluence. However, no cell kill was detected in the experimental samples infected at a MOI of 0.1pfu/cell HSV1716 indicating that such sparse monolayers were unable to support lytic viral infection.

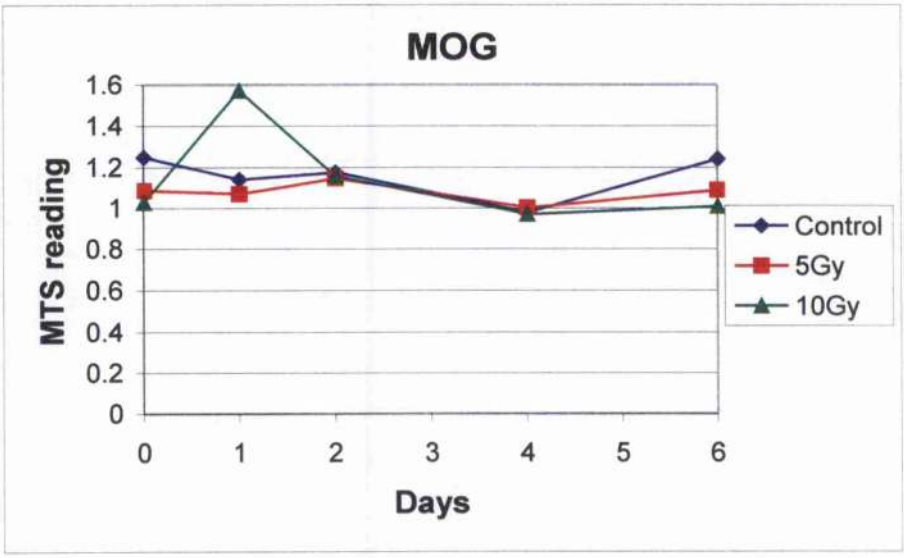
Following initial viral infection of a cell monolayer, lytic HSV replication results in progeny particles being released into the extracellular medium. As a result, cells distant to the site of the released virions may become infected and further lytic cycles initiated. However, observation of cell monolayers following infection with HSV1716 shows that cells adjacent to infected cells are most at risk of subsequent infection. As the predominant process propagating HSV1716 infection is through cell-to-cell spread it was decided that a greater density of cells in a monolayer would be required at the outset of an MTS cytotoxicity assay to give a viable infection.

14.6 Cytotoxicity Assay with 1 x 10^4 Cells and Ionizing Radiation

As $1x10^3$ MOG cells per well of a 96 well plate formed too sparse a monolayer to support a low MOI infection of HSV1716, a higher number of cells ($1x10^4$) was seeded at the outset. The cell lines 3T6 and BHK, which are well characterised by our laboratory, were analysed at the same time as the malignant glioma, MOG cell line. The effect on cell viability of 5Gy or 10Gy of external beam radiation was compared in the three cell types (figure 14.6).

It appeared that $1x10^4$ cells seeded at the outset were too great. For the MOG and 3T6 cells a confluent monolayer was achieved following incubation for 24 hours. Therefore there was no period of exponential growth achieved. The cells at this density appear to be resistant to the effects of high doses of ionising radiation as there is no impact on cell number detected by the MTS cytotoxicity assay.

Figure 14.6 *– MTS cytotoxicity assay of (a) MOG, (b) 3T6 and (c) BHK cells following exposure to ionising radiation doses of 5Gy and 10Gy. Initial density of cells was $1x10^4$ per 100µl in each well of a 96 well plate. Twenty-four hours after seeding the cells, time point 0, the cells were irradiated and the first MTS recording made.*



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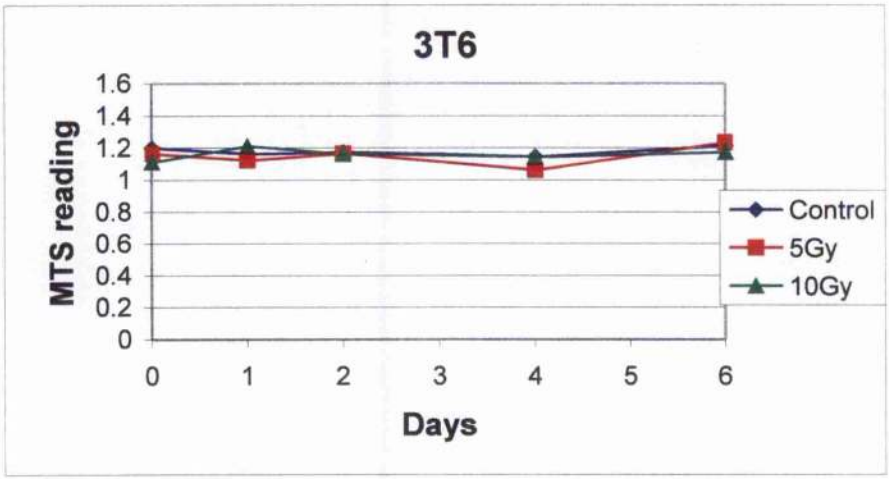

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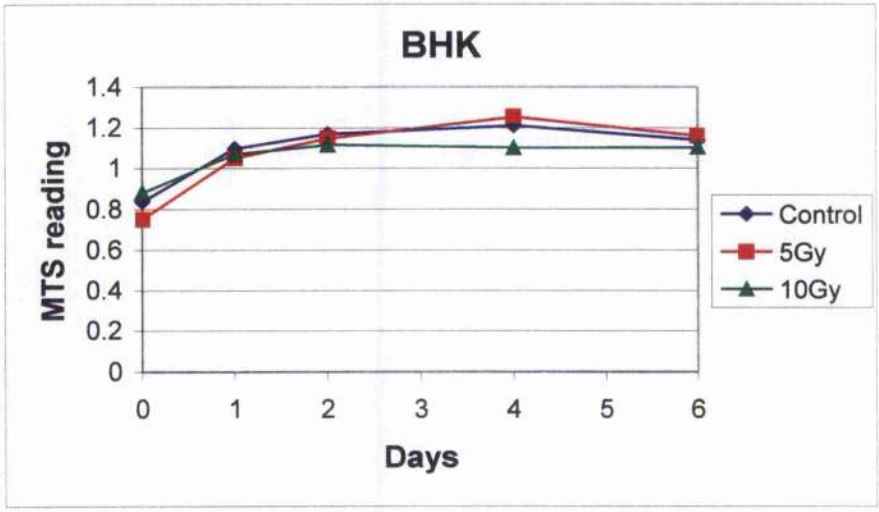


(c)

14.7 Cytotoxicity Assay with 5 x 10^3 Cells and Ionizing Radiation

As an initial MOG and 3T6 cell density of $1x10^4$ cells per well of a 96 well plate resulted in a confluent monolayer by 24 hours a lower number of cells, $5x10^3$ cells was seeded to allow a longer period of exponential cell replication (figure 14.7).

In the MOG cell line (figure 14.7a) there was an initial period of exponential growth with confluence being reached in control and irradiated wells by day 2. After day 2 there was a steady reduction in the number of viable cells over the subsequent six days in the control and irradiated wells. These results suggest that once confluence was reached, a healthy state could not be maintained and cells either died due to over confluence or exhaustion of nutrients from the medium. This reduction in cell number after reaching confluence was not identified in

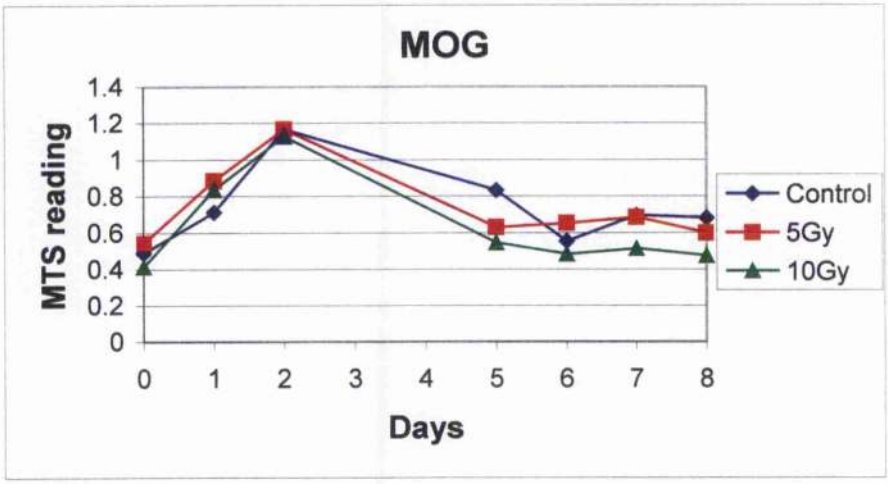
experiment 14.6. It is postulated that as the MOG cells were actively growing for 2 days, they consumed essential nutrients from the medium and that when confluent there was a lack of nutrients to support quiescence. In experiment 14.6 the high number of cells seeded at the outset meant that no active replication was achieved thus ensuring available nutrients to support a period of quiescence.

The 3T6 cells (figure 14.7b) grew exponentially reaching confluence at day 2 when efficient contact inhibition halted further cellular division. The ability to convert the MTS substrate for the six days of the experiment demonstrates that 3T6 monolayer despite being confluent remained viable. The 3T6 cells appear to be relatively radio-resistant with no evidence of a reduction in cell viability detected by MTS assay following ionising radiation doses of 5Gy or 10Gy.

The BHK cells (figure 14.7c) appear to have been initially seeded with fewer than the intended $5x10^3$ cells as the initial MTS reading when compared to the day 0 reading with MOG or 3T6 was significantly reduced. In this experiment, where the initial cell number was presumably less than $5x10^3$, the exponential growth was maintained for 7 days at which point the cells reached confluence. BHK cells seeded at this low density were sensitive to ionising radiation. 5Gy resulted in a delay in exponential growth with an apparent surge in growth at day 5 to day 7, reaching confluence by day 8. Ten Gray had a marked effect on the cell replication and growth with the earliest signs of growth detected by day 7.

$5x10^3$ cells appeared to be too many cells to achieve exponential growth over a suitably long time frame such as 7 days. An initial cell density of less than $5x10^3$ appears necessary to provided extended exponential growth over a period of 7 days for all three cell lines.

Figure 14.7; *MTS cytotoxicity assay of (a) MOG, (b) 3T6 and (c) BHK cells following ionising radiation at a dose of 5Gy or 10Gy following initial cell density of $5x10^3$ cells per 100µl in a 96 well plate. Twenty-four hours after seeding the cells, time point 0, the cells were irradiated and the first MTS recording made.*



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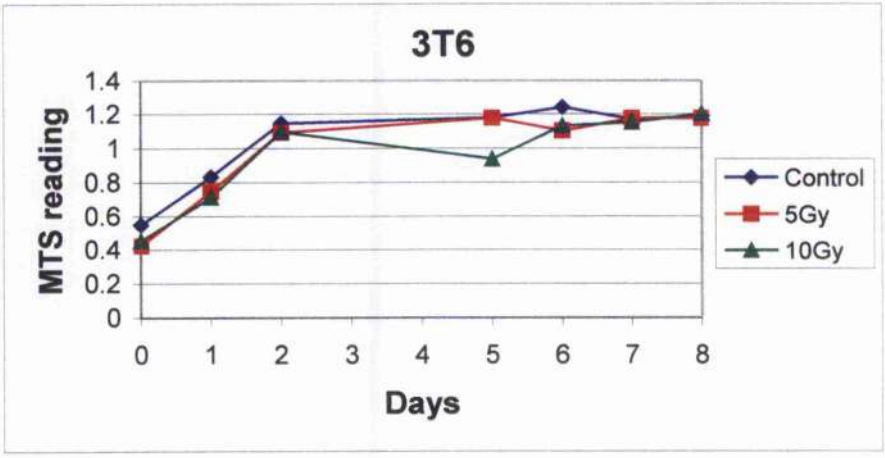

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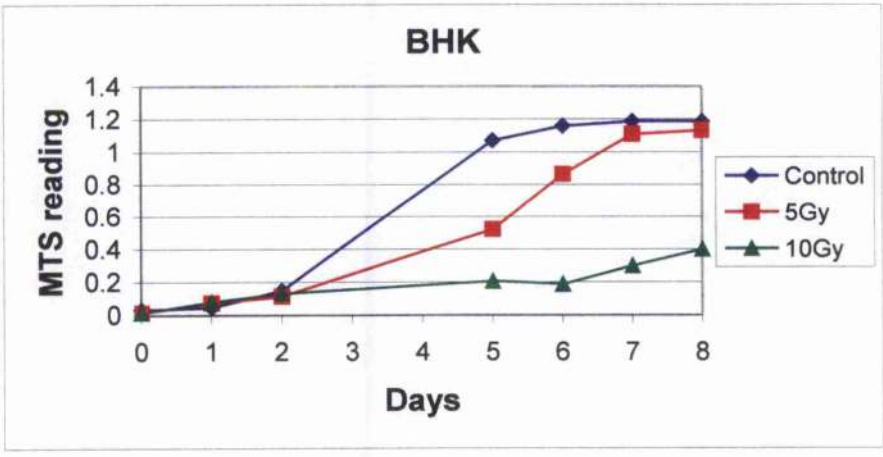

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14.8 Cytotoxicity Assay with 2.5 x 10^3 Cells and Ionizing Radiation

From the data in experiment 14.7 it appears that $5x10^3$ cells per 100µl of a 96 well plate is too high a quantity to initially seed if exponential growth is required for up to one week. Following the serendipitous seeding of the BHK cells at a density less than $5x10^3$, the results obtained enabled the effects of the radiation to be detected by the MTS assay. Therefore the initial number of cells seeded at the outset was reduced further to $2.5x10^3$ per well.

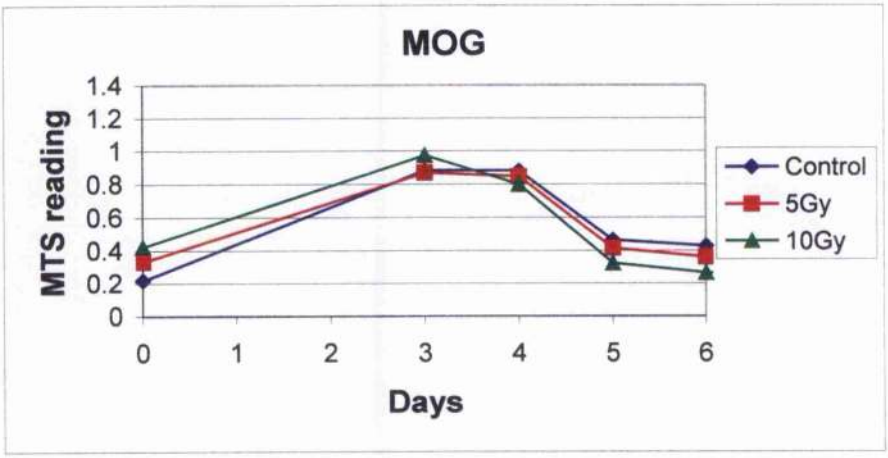
It can be seen that the MOG cells (figure 14.8a) reached confluence by day 3 then began to die. Ionising radiation appears to have had little impact on the rate of growth or cell kill when compared to the control.

3T6 cells (figure 14.8b) grew exponentially until day 3 when they too reached confluence. As with the BHK cells, ionising radiation appears to have had no impact on the rate of growth or the number of viable cells by day 6.

BHK cells (figure 14.8c) grew well reaching confluence by day 3 but in contrast to the MOG cells, remained as a healthy monolayer over the following 3 days. Radiation appears to have had little impact on the rate of cell growth, or impact on the number of viable cells by day 6. This is in contrast to the results obtained in experiment 14.7. The MTS reading at time point 0 was higher in experiment 14.8 compared to experiment 14.7 indicating that there were more cells present prior to irradiation. In experiment 14.7 there were probably fewer than $2.5x10^5$ cells present at the outset, consequently the doses of radiation had a more significant effect in delaying exponential growth until day 5.

$2.5x10^3$ cells per well in a 96 well plate gave a confluent monolayer 3 days after seeding. To achieve a longer period of exponential growth a still lower seeding density is required.

Figure 14.8 *– MTS cytotoxicity assay with (a) MOG, (b) 3T6 and (c) BHK cells at an initial density of $2.5x10^3$ cells in 100µl per well exposed to 5Gy and 10Gy ionising radiation. Twenty-four hours after seeding the cells, time point 0, the cells were irradiated and the first MTS recording made.*



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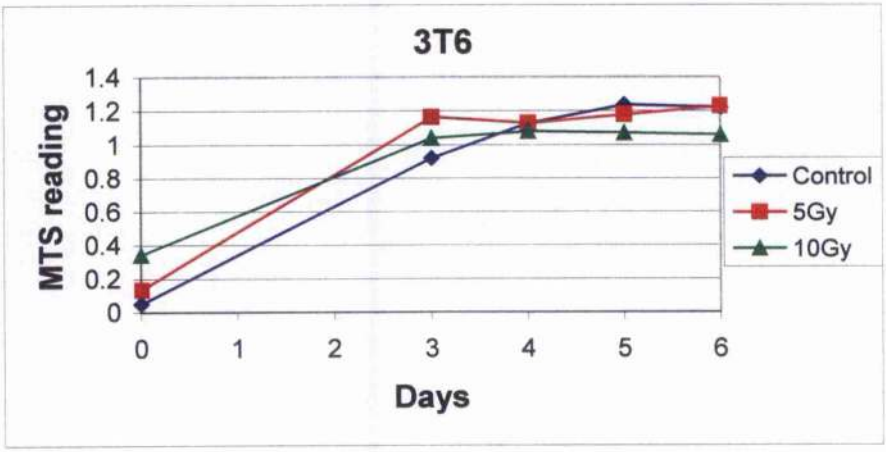

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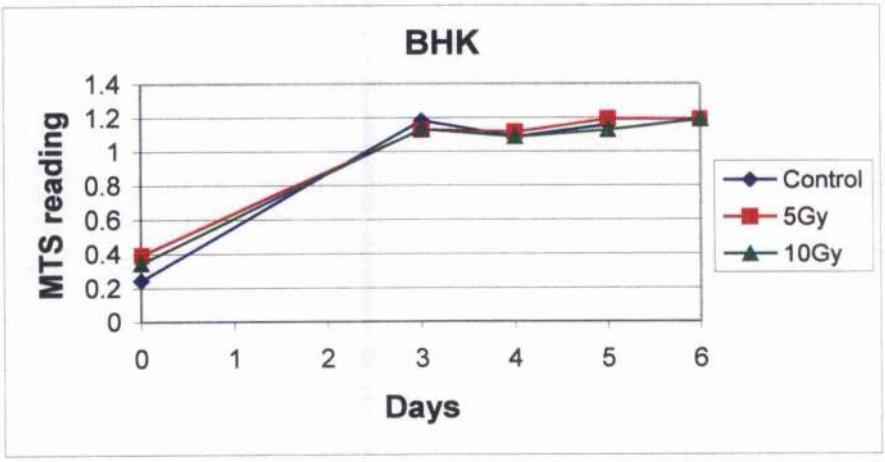

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14.9 Cytotoxicity Assay with 2 x 10^3 Cells and Ionizing Radiation

On the basis of the previous series of experiments it was apparent that the initial cell density was crucial. In this experiment $2x10^3$ cells in 100µl per well were seeded.

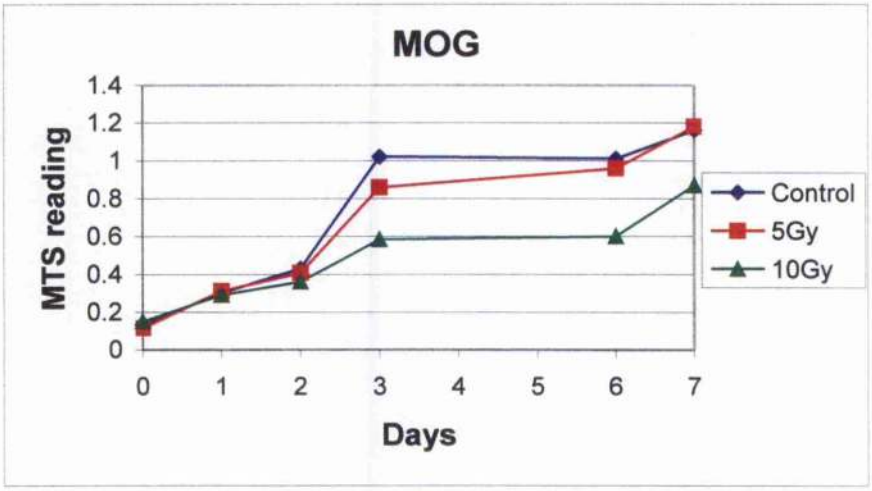
It can be seen that the MOG cells (figure 14.9a) grew to form a confluent monolayer in the control arm by between 3-5 days. There does not appear to have been significant cell kill or delayed growth with 5Gy. However there appears to have been a significant effect on the viability of MOG cells following 10Gy of radiation with a marked reduction in cell growth seen for the duration of the seven days.

The 3T6 cells (figure 14.9b) appear to have grown at a faster rate than the MOG or BHK cells reaching a confluent monolayer by 3 days. At this cell density, as with the other two cell lines there does appear to have been some effect on the rate of exponential growth following a radiation dose of 10Gy.

The rate of growth with the BHK cells (figure 14.9c) seeded at this density resulted in confluence being achieved again at around 4-5 days. Radiation seems to have had an effect with reduced growth rates seen following 5Gy or 10Gy.

Therefore a seeding quantity of between $2\text{-}3x10^3$ cells per 100µl per well seemed optimal to allow exponential growth for the duration of 3-5 days and enable the cytotoxic effects of radiation to be detected. However given that the HSV replication is dependent on the cell density it may be difficult to identify a suitable initial cell density that will demonstrate the cytotoxic effects of HSV1716 and ionising radiation when combined.

Figure 14.9 *– MTS cytotoxicity assay with MOG, 3T6 and BHK cells at an initial density of $2x10^3$ cells in 100µl per well exposed to 5Gy or 10Gy ionising radiation. Twenty-four hours after seeding the cells, time point 0, the cells were irradiated and the first MTS recording made.*



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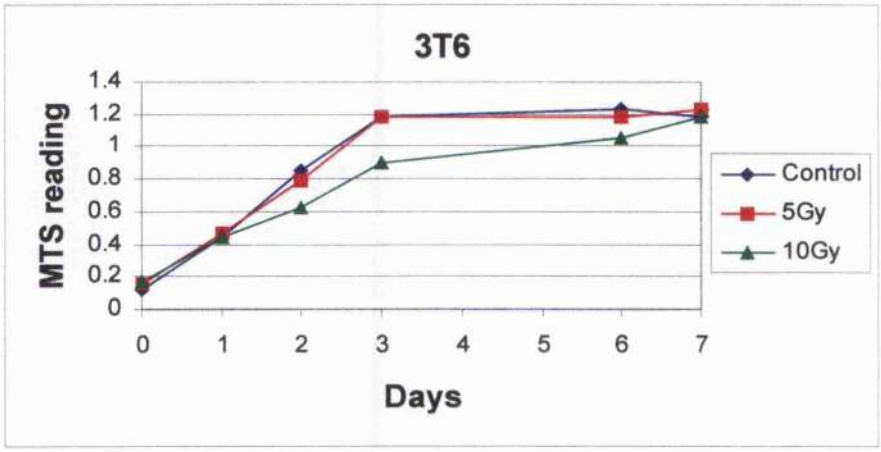

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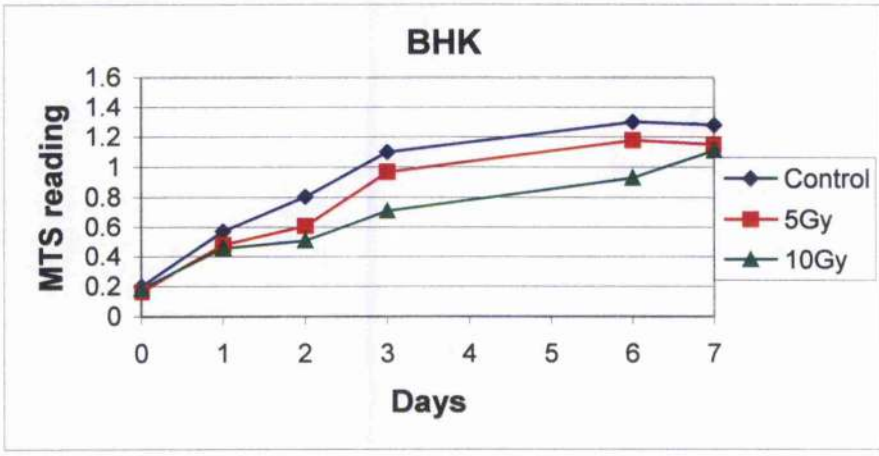

(c)

14.10 Comparison of Ionising Radiation Dose Rate on a Cellular Monolayer as Assessed by the MTS Cytotoxicity Assay

The effect of external beam radiation on the cell lines used in the previous experiments had demonstrated only a modest reduction in cell viability, leading to the presumption that the cell lines under investigation were relatively radio-resistant. It has been proposed that the level of cytotoxicity attributable to ionising radiation is related to the rate at which the dose is delivered. With the same dose delivered at a lower dose rate there is a reduction in the cell killing because sub-lethal damage is repaired during the protracted delivery of the radiation. This phenomenon varies between different cell lines and is most marked between 0.01Gy and 1Gy per minute (Hall EJ and Brenner DJ, 1994). The published data that indicated an additive or synergistic relationship between ionising radiation and replication competent HSV mutants delivered the ionising radiation at much faster rates, ranging from 0.75Gy/min to 2.77Gy/min (Spear M *et al.*, 2000).

The Alcyon II teletherapy unit available for use during these experiments delivered ionising radiation at a dose rate of 0.3Gy/minute.

In order to ascertain if dose rate was a significant factor in these experiments it was necessary to compare the use of the normal dose rate, 0.3Gy/minute with a higher dose rate. By decreasing the distance between the source and the experimental cells by half, the dose rate is increased by a factor of four. The distance between the source and the cells in culture could not be reduced further due to the casing surrounding the Alcyon II teletherapy unit, therefore the dose rate could not be increased higher than 1.2Gy per minute. For this experiment cells were seeded at $1x10^3$ cells per well of a 96 well plate.

It can be seen in figure 14.10a, that 5Gy had a greater effect on the replicating 3T6 monolayer than had been seen using 5Gy in previous experiments. 10Gy had a significant effect on the growth of the 3T6 cells while 20Gy had such a profound effect on the growth of the cells that it resulted in sterilisation. When the dose rate was increased by a factor of four to 1.2 Gy/min (figure 14.10b) the

effect of ionising radiation appears to have been the same with no obvious decrease in the number of viable cells compared to the results when ionising radiation was delivered at our normal rate of 0.3Gy/min.

The results seen in the BHK cell line are similar to those seen with the 3T6 cells. 5Gy resulted in a slight impairment in cell growth, however a more marked reduction in cell growth was detected following doses of 10Gy and 20Gy (figure 14.10c). The higher dose rate did not have a greater impact on the growth of cells when compared to ionising radiation delivered at a slower rate (figure 14.10d).

The apparent decrease in the viability of cells irradiated at the standard dose rate of 0.3Gy/min in this experiment compared to the radiation resistance seen in previous experiments is perhaps due to the low number of cells seeded. The cells were seeded at a low density ($1x10^3$ cells/well) to ensure that they could grow exponentially over a time scale that would allow the effect of a higher dose rate of ionising radiation to be compared with the standard dose rate. The smaller number of cells present at the start of the experiment, perhaps by increasing the number of cell doublings required to generate a confluent monolayer, had the effect of accentuating the effect of the ionising radiation.

Increasing the dose rate by a factor of four to the maximum for the Alcyon II teletherapy unit did not result in any additional reduction in cell viability and no reduction on the growth rates of either 3T6 or BHK cells.

Figure 14.10a *– MTS cytotoxicity assay in 3T6 cells exposed to ionising radiation of 5Gy, 10Gy and 20Gy at a dose rate of 0.3Gy/min. Twenty-four hours after seeding the cells, time point 0, the cells were irradiated and the first MTS recording made.*

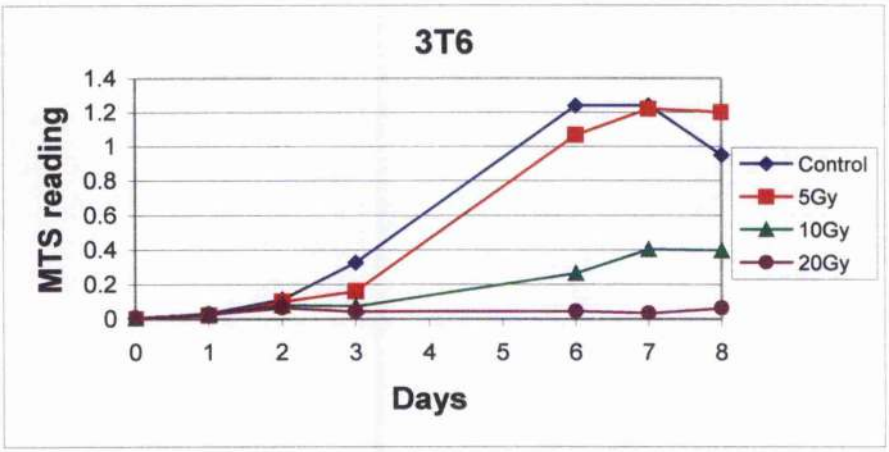

Figure 14.10b *– MTS cytotoxicity assay 3T6 cells exposed to ionising radiation of 5Gy, 10Gy and 20Gy at a dose rate of 1.2Gy/min.*

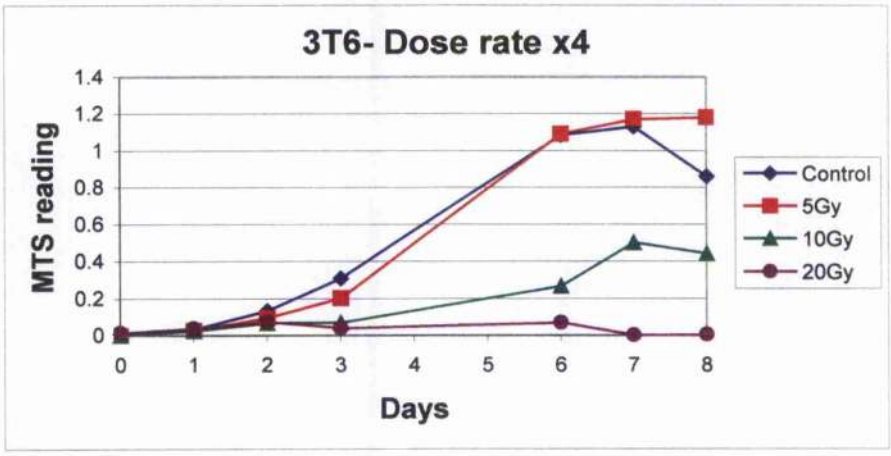

Figure 14.10c *– MTS cytotoxicity assay in BHK cells exposed to ionising radiation of 5Gy, 10Gy and 20Gy at a dose rate of 0.3Gy/min.*

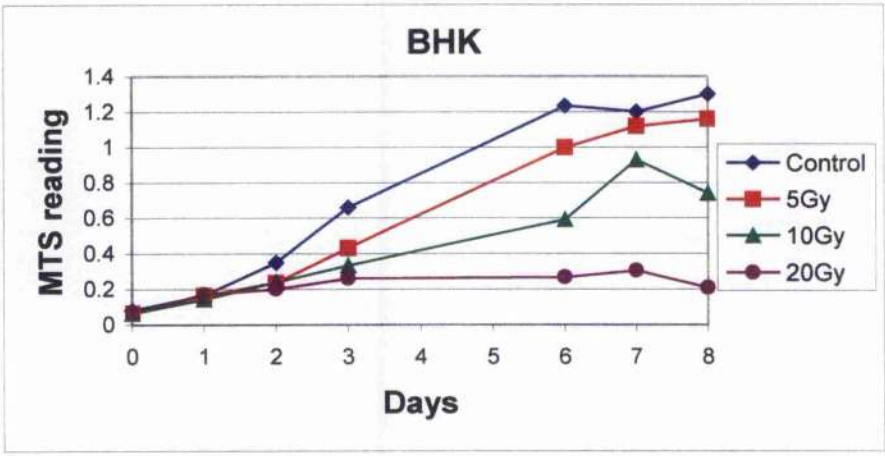

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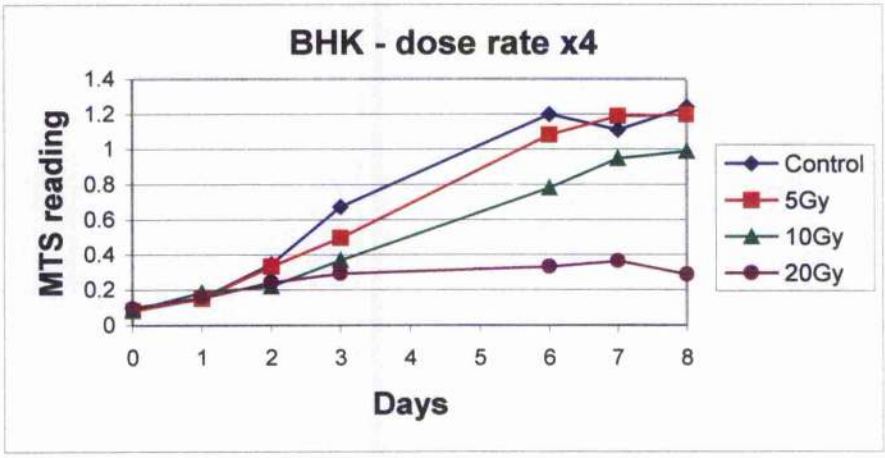
Figure 14.10d *– MTS cytotoxicity assay in BHK cells exposed to ionising radiation of 5Gy, 10Gy and 20Gy at a dose rate of 1.2Gy/min.*



14.11 Cytotoxicity Assay and HSV

It was necessary to demonstrate the effect of HSV1716 alone in the *in vitro* tissue culture MTS assay prior to investigating the relationship between HSV1716 and ionising radiation in combination.

The MTS cytotoxicity assay was used to assess cell kill generated by lytic replication of HSV1716. The subsequent experiments sought to identify a multiplicity of infection (MOI) of HSV1716 that would exert an *in vitro* reduction in cell viability detectable using the MTS assay, without resulting in eradication of all cells in the monolayer. In view of the time that it took ionising radiation to exert a level of cell kill on a cell monolayer detectable by the MTS assay, it was necessary for the experiment to continue for approximately 7 days. Therefore to investigate the effect of combining HSV1716 infection and radiation on cells in culture it was necessary to find conditions under which HSV1716 did not lyse all of the cells by 7 days.

As indicated earlier it was necessary to identify an initial cell density that would allow exponential cell division for at least 3-5 days.

As shown earlier however for IISV1716 to propagate a lytic infection, the cells in a monolayer must be of sufficient density. Following initial infection of a cell monolayer, lytic replication results in progeny being released into the extracellular medium. As a result cells distant to the site of the released virions may become infected and a further lytic cycles initiated. Observations of cell monolayers following infection with HSV1716 highlighted that cells adjacent to the virally infected cells are at most risk of subsequent infection. Therefore the predominant process that propagates the lytic infection is through cell-to-cell spread between neighbouring cells.

When cells are seeded too sparsely the cells may be unable to support HSV1716 lytic replication as the ability of the virus to infect adjacent cells through cell-to-cell spread is diminished. However, if the cells of the monolayer were initially too

dense, cell division would be diminished due to contact inhibition. A non-dividing, quiescent, monolayer would be less permissive to HSV1716 infection and less susceptible to radiation kill.

It was anticipated that the MOI of HSV1716 would have to be low, in order to ensure that there was not rapid cell kill of the exponentially growing monolayer. Initial experiments were aimed at identifying an inoculating dose of HSV1716 that would independently cause less than 100% kill of the cell monolayer by 6-8 days. In addition it was necessary that the inoculating dose would be supported by an initial cell density of between 2-3 x 10^3 cells per 100µl in a 96 well plate previously shown to be optimal for the detection of radiation cell kill.

14.12 Cytotoxicity Assay and HSV1716 and HSV17$^+$

Replicating monolayers of 3T6, BHK and MOG were investigated for cell kill following infection with HSV1716 and HSV17$^+$. 3T6 cells are known to be fully permissive for HSV17$^+$ but not for HSV1716. BHK cells are permissive for HSV17$^+$ and HSV1716 as was the MOG cell line (shown in section 14.4).

2.5x10^3 cells were seeded and infected the following day with either HSV1716 or wild type HSV17$^+$ at MOI of 0.1,1 or 10. The time of infection was taken as day 0, and MTS readings were taken over the course of 6 days.

In 3T6 cells HSV1716 did not cause any significant cell kill (figure 14.12a). HSV1716 at MOI 10 did slow the rate of initial exponential growth but ultimately a confluent monolayer was achieved only one day later than the control. This probably is indicative of minimal HSV1716 replication in the exponentially growing 3T6 cells. It has been shown that HSV1716 replication can be supported in replicating 3T6 cells but not in confluent monolayers where contact inhibition stops cell division (Brown SM *et al.*, 1994b).

The density of 3T6 cells replicating prior to virus infection is crucial. Additional experiments (not shown) indicated that HSV1716 at an MOI of 10 resulted in

complete cell kill, preventing the monolayer from replicating. In those experiments it was noted that the initial MTS reading was lower indicating fewer cells at the outset, possibly explaining the different results.

With HSV17^{+}, the reduction in the MTS reading indicates that an MOI of 1 generated cell kill between the third and sixth days post inoculation. No effect following an MOI of 0.1 was seen, however an MOI of 10 generated cell kill of 100%.

BHK cells were more permissive to lower concentrations of HSV17^{+} and HSV1716 as is indicated by the low MTS readings at day 6 for all the multiplicities of infection investigated when compared to the control (figure 14.12c and 14.12d). With the fully permissive BHK cell line, HSV1716 at a MOI of 0.1 allowed some cell replication to occur in contrast to the higher multiplicity of infections investigated. Unfortunately in this experiment the seeding density of MOG cells appears to have been higher than intended causing the monolayers to reach confluence by day 3. However, it is clear that HSV1716 at a MOI of 10 killed all of the cells early in infection and that a lower MOI would be required if added cell kill from radiation was to be investigated.

In the MOG cell line an HSV1716 MOI of 10 prevented the cell monolayer from entering a period of exponential growth (figure 14.12e). The lower multiplicities of infection (1 and 0.1 pfu/cell) of HSV1716 did not cause sufficiently greater kill to be detectable by the MTS assay when compared to the control. On reaching confluence at day 3 the monolayers once again began to die, as reflected in the decreasing MTS readings. When HSV17^{+} was used to infect MOG cells an MOI of 0.1 generated some cell kill by day 3, however after this point the control cell cultures began to die and therefore no conclusions can be drawn (figure 14.12f).

From the data from all 3 cell types it seems reasonable, that an MOI of 0.1 when inoculating a cell number that can support its replication, will not cause complete cell kill very early and will allow any additional kill by radiation to be detected by the MTS assay.

Figure 14.12a; *MTS cytotoxicity assay in 3T6 cells at various concentrations of HSV1716.*

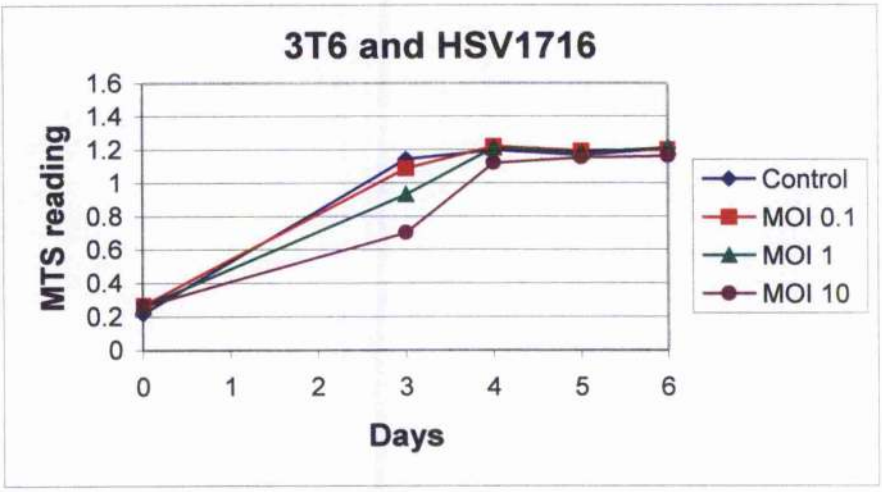


Figure 14.12b; *MTS cytotoxicity assay in 3T6 cells at various concentrations of HSV17^{+}.*

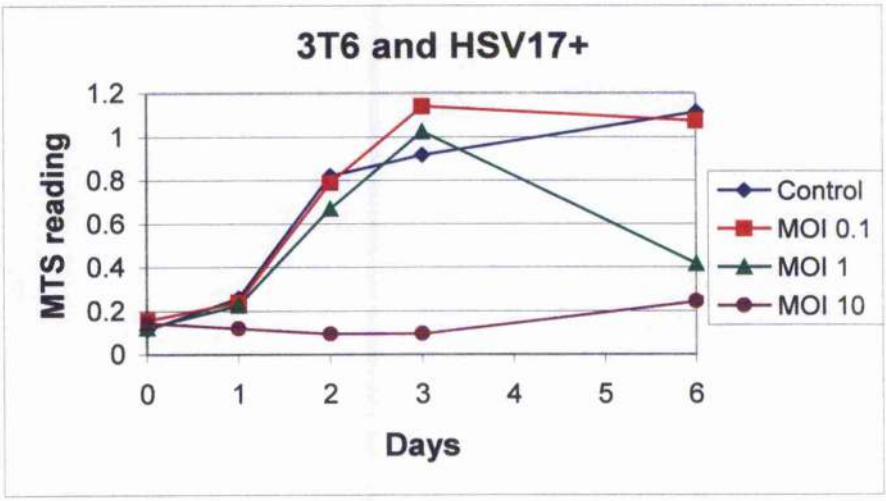


Figure 14.12c; *MTS cytotoxicity assay in BHK cells at various concentrations of HSV1716.*

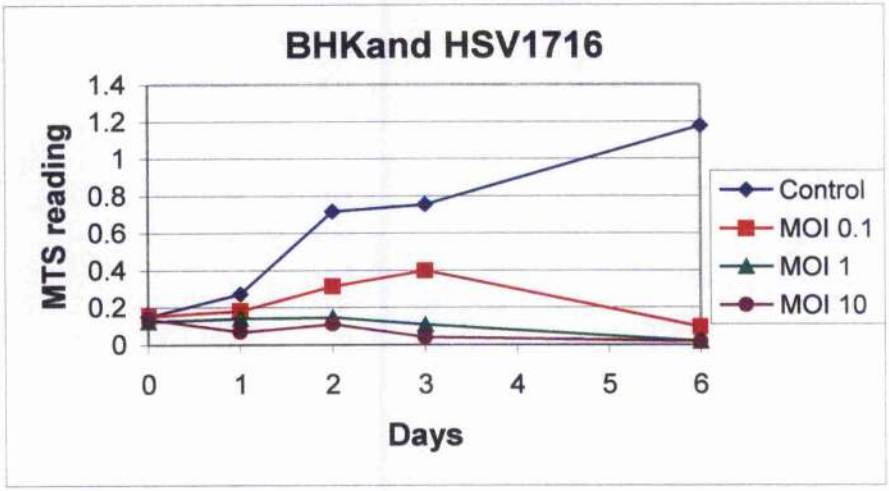


Figure 14.12d; *MTS cytotoxicity assay in BHK cells at various concentrations of HSV17^{+}.*

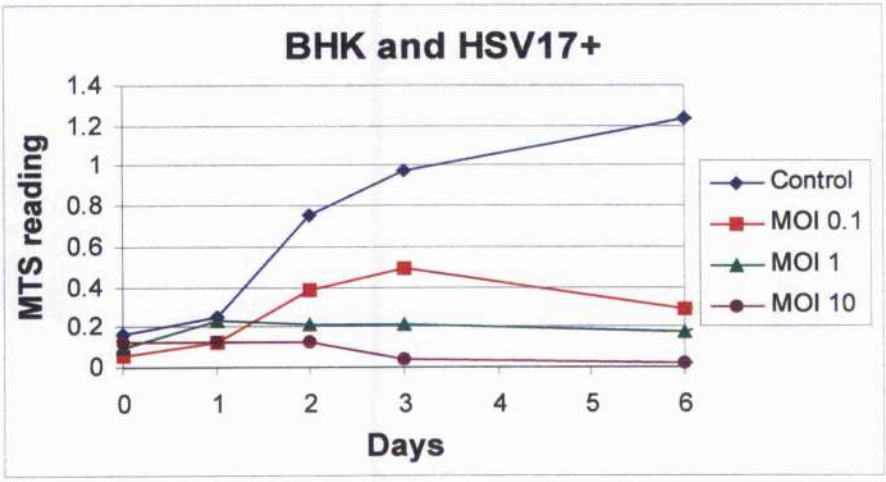


Figure 14.12e; *MTS cytotoxicity assay in MOG cells at various concentrations of HSV1716.*

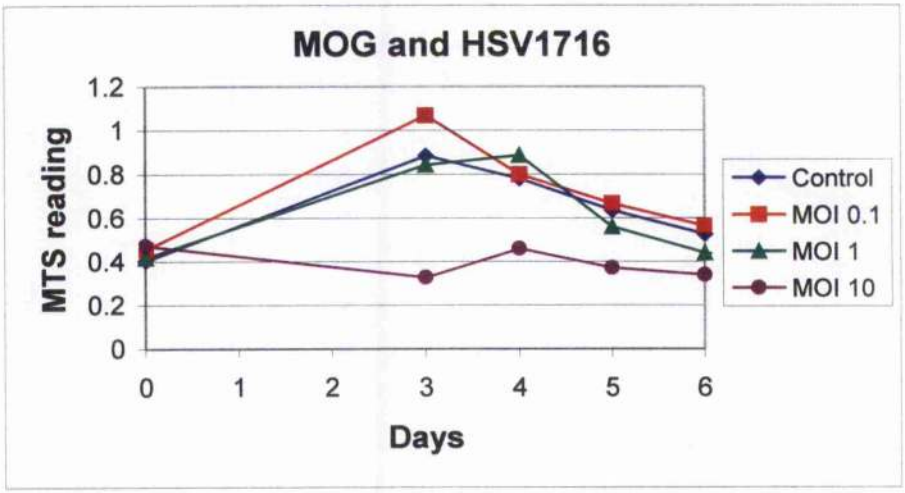
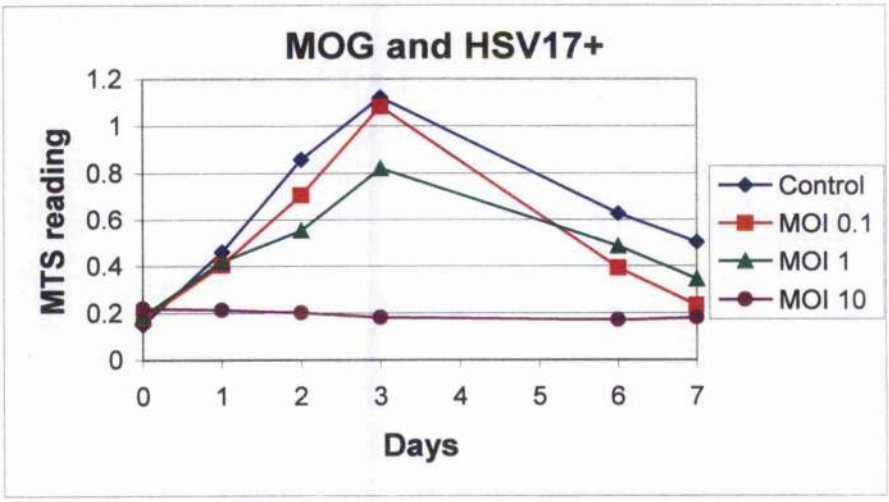


Figure 14.12f; *MTS cytotoxicity assay in MOG cells at various concentrations of HSV17^{+}.*



14.13 Assessment of Infectious Viral Titre Following Irradiation of HSV1716 and HSV17^{+}

The response in various cell cultures to ionising radiation and HSV1716 administered separately had been characterised and demonstrated in the preceding sections.

The published data demonstrating enhanced cell kill when an ICP34.5 null mutant was combined with ionising radiation had been done exclusively *in vivo*. In those studies, xenografts were irradiated at either 6 or 24 hours after virus inoculation. Based on those data, initial experiments were devised with the inoculation of replicating cells with HSV1716 six hours prior to irradiation.

As the proposed schedule to combine ionising radiation and HSV1716 *in vitro* was to infect the replicating monolayer 6 hours prior to delivery of radiation it raised the question as to whether the ionising radiation could possibly damage the viral particles either in an intracellular state or whilst still in the medium prior to adsorbing onto the surface of the cell in culture.

To investigate whether irradiating HSV1716 or HSV17^{+} in medium affected the ability of the virus to infect and lyse cells in culture; samples of the virus in solution were irradiated and compared to non-irradiated controls in a virus titration assay as detailed in section 11.4.

The results, indicated below, demonstrate that irradiating HSV1716 or HSV17^{+}at a dose of 5Gy did not decrease the 72 hour yield, indicating that the replicative potential of the virus was not impaired by prior radiation treatment.

HSV1716	8.6×10^5 pfu/ml
Irradiated HSV1716	9.8×10^5 pfu/ml

HSV17+	5.0×10^5 pfu/ml
Irradiated HSV17+	7.8×10^5 pfu/ml

14.14 Cytotoxicity Assay in 3T6 and BHK Cells Administering Ionising Radiation Six Hours After HSV1716 or HSV17^{+} Infection

In view of the fact that the glioma cell line MOG and BHK cells are fully permissive for HSV1716, it is probable that no enhanced kill by radiation would be detected in cultures of these cells as the viral lytic infection alone is too efficient. Therefore initial experiments also used the non-permissive 3T6 cell line. The decision to use 3T6 cells was based on the knowledge that HSV1716 can enter 3T6 cells but fails to undergo lytic replication (Brown SM *et al.*, 1994b). The idea was to investigate if treatment of these cells with ionising radiation would allow HSV1716 to replicate causing cell kill detectable by an MTS assay. The fully permissive BHK cells were used as a control.

The cells were seeded at a density of $2x10^3$ cells per well. Six hours after virus infection, experimental plates were irradiated with either, 5Gy, 7Gy or 10Gy delivered in one fraction.

In 3T6 cells the various doses of radiation alone caused a modest degree of delayed cell growth (figure 14.14.1a).

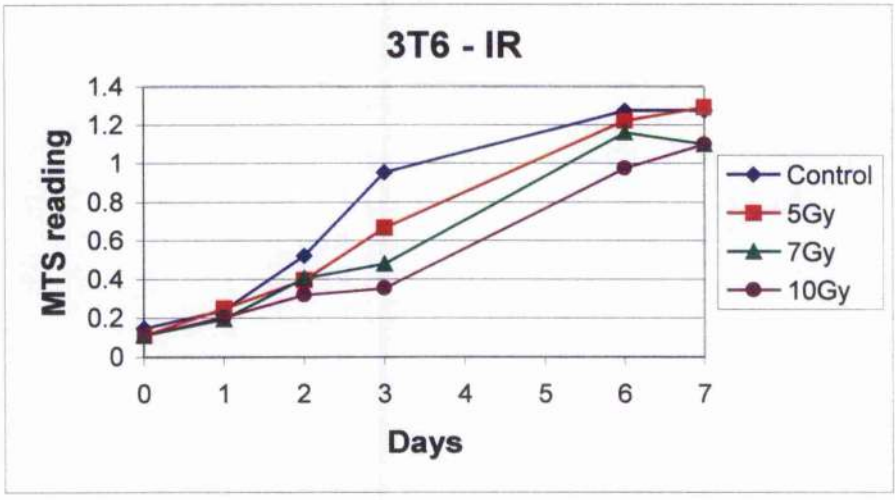
An HSV17^{+} MOI of 0.1 in 3T6 cells generated a lytic infection and caused cell kill, which independently virtually killed the whole monolayer by day 7 (figure 14.14.1b). The delayed cell kill seen over the same seven day period in the irradiated 3T6 cells inoculated with the same MOI of HSV17^{-} can be explained by the observation that there is a delay in initial growth of the monolayers irradiated with 5Gy, 7Gy and 10Gy. It is proposed that the delayed cell growth in the irradiated cells means that they were unable to support the viral replication as efficiently as their non-irradiated counterparts.

The 3T6 cells exposed to an MOI of 0.1 of HSV1716 alone grew in a similar manner to the control cells. The combination of HSV1716 and radiation did not generate any additional reduction in cell viability compared to the 3T6 cells that were only irradiated with 5Gy, 7Gy or 10Gy (figure 14.14.1c).

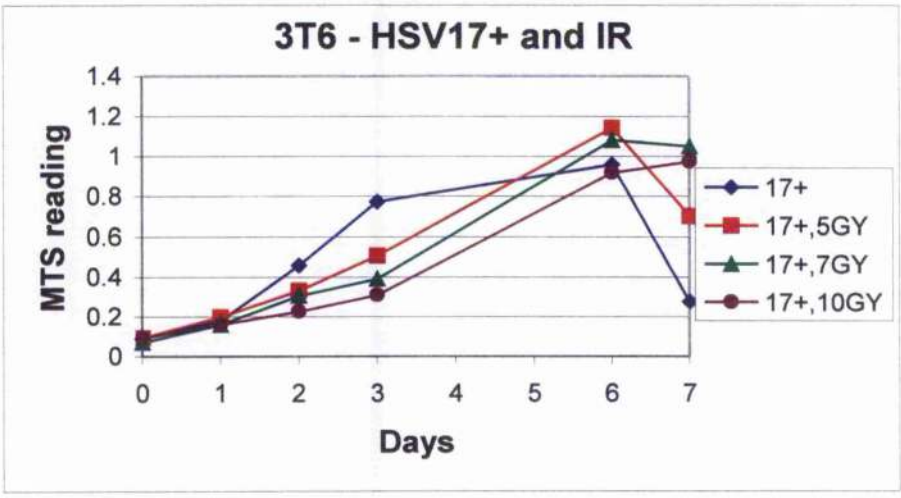
It appears that ionising radiation does not increase the permissivity of the 3T6 cells to infection with HSV1716, nor increase the replication efficiency of HSV1716 in 3T6 cells.

BHK cells were also affected by the radiation with marked reduction in cell growth in the irradiated plates (figure 14.14.2a). With both $HSV17^{+}$ and HSV1716 the BHK cells were completely killed and were unable to enter a phase of exponential growth (figure 14.14.2b and c). Once again it appears, that due to the difficulty in assessing cell numbers in suspensions of BHK cells, the initial cell number seeded into each well probably was lower than the intended $2x10^3$ cells, as indicated by the very low initial MTS reading. The BHK cells in the control and irradiated plates were able to increase in number. Where virus was added to the monolayers there was no indication that the BHK cells replicated. If the number of cells is assumed to have been less than the intended $2x10^3$ then the MOI of HSV1716 or $HSV17^{+}$ added was higher than the intended MOI 0.1. The BHK cells were therefore overwhelmed with virus and lysed preventing exponential growth from being achieved.

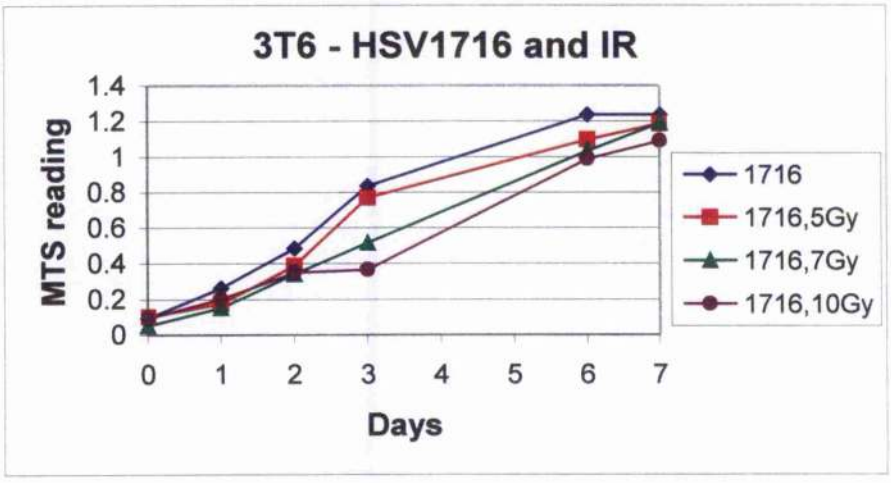
Figure 14.14.1; *MTS analysis of 3T6 cells following, radiation (IR) alone (a), HSV17^{+} and radiation (b) and HSV1716 and radiation (c).*



(a)

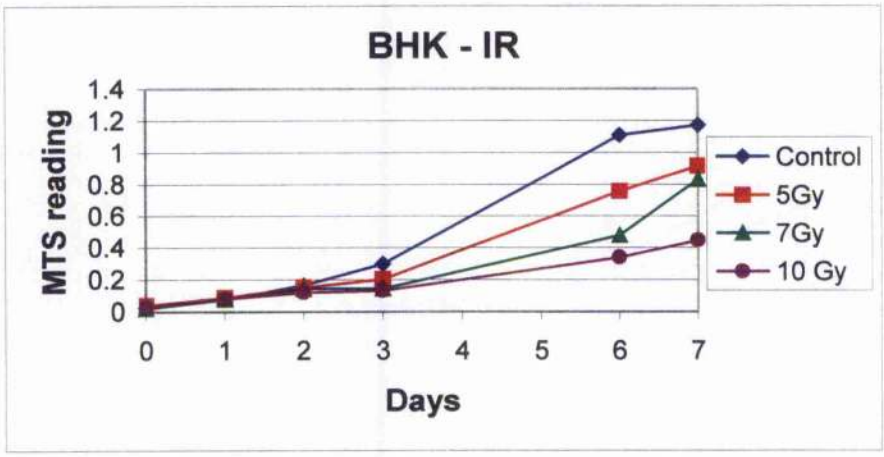


(b)

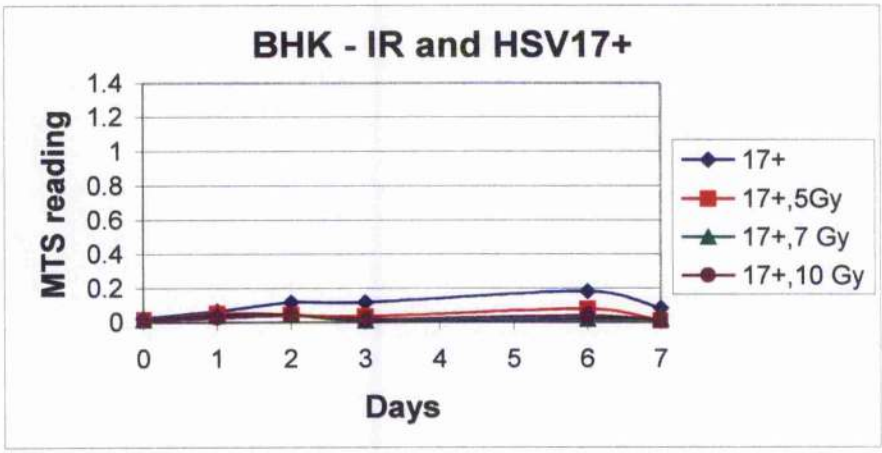


(c)

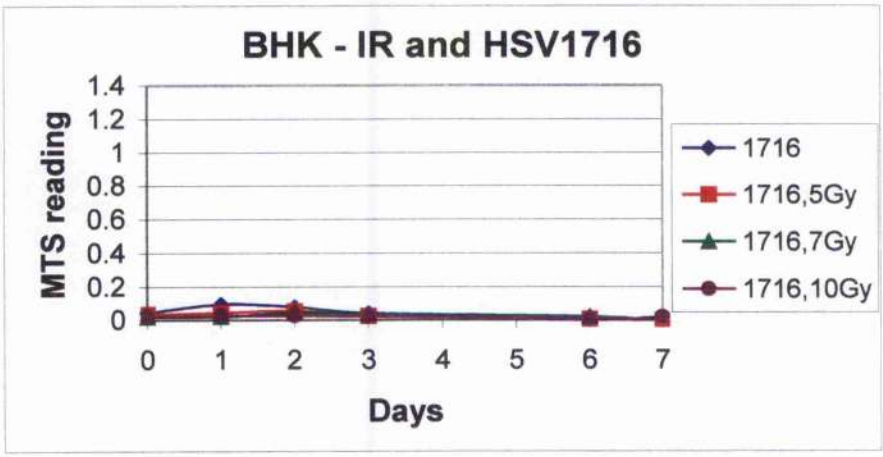
Figure 14.14.2; *MTS analysis of BHK cells following radiation alone(a), HSV17^{+} and radiation(b) and HSV1716 and radiation (c).*



(a)



(b)



(c)

14.15 Multicycle Growth Experiment Using 373 Cell Line

Given that cells fully permissive to HSV1716 infection are not suitable for studying the possibility of enhanced cell kill because the virus alone kills all of the cells, and that the non-permissive cell line 3T6 showed no more cell kill with HSV1716 infection over radiation alone, it was necessary to try and find a suitable semi-permissive cell line in which to study enhanced cell kill.

Work within the laboratory suggested that the human malignant glioma cell line 373 was slightly less permissive to HSV1716 infection than the MOG cell line. To demonstrate this, a multicycle growth experiment was performed. The results are demonstrated in the figure below (figure 14.15).

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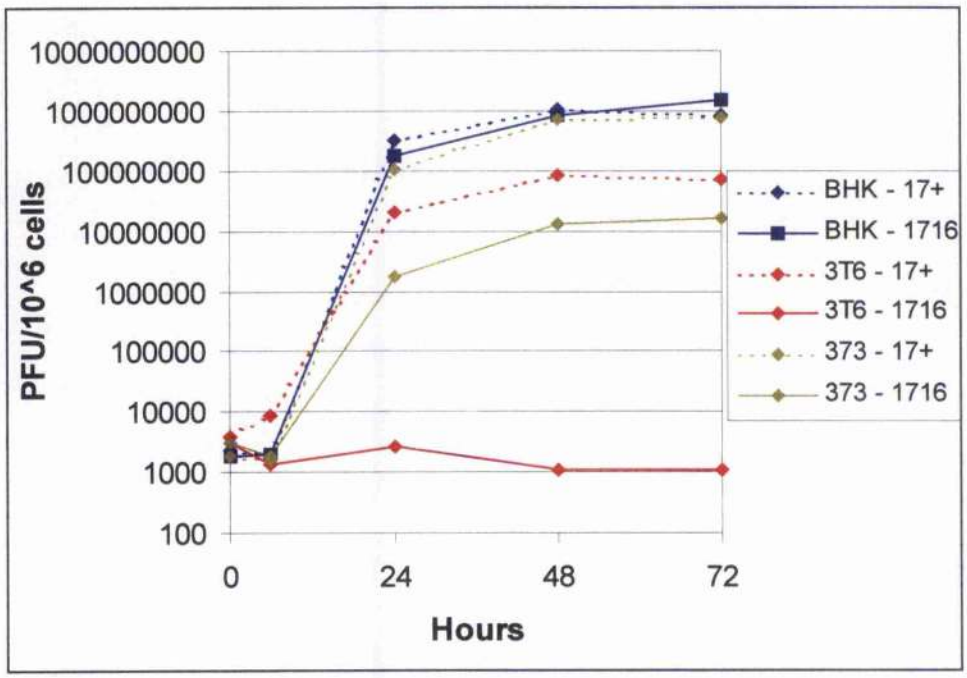


***Figure 14.15**; Multicycle growth experiment of HSV1716 and HSV17$^+$ in 373, BHK and 3T6 cell lines.*

The 72 hour yield of HSV17$^+$ demonstrates that 373 cells are as permissive to the growth of HSV17$^+$ as the fully permissive BHK cells. The yield of HSV1716 obtained from the 373 cells by 72 hours is reduced compared to the yield obtained

from the BHK cells suggesting that the 373 cells are less permissive for the growth of HSV1716 than the known fully permissive cell line BHK. Given that the growth of the wild type strain HSV17^{+} was comparable in the 373 and BHK cell lines then the reduction in the yield of HSV1716 seen in the 373 is likely to be real and not due to a difference in the cell density at the time of HSV1716 inoculation. As has been demonstrated previously the 3T6 cells are permissive for HSV17^{+} and not for HSV1716.

It was therefore decided that by using the 373 cell line in an MTS assay it might be possible to demonstrate less than 100% cell kill by day 6-7 with HSV1716 alone. If this was the case then it was possible that the additional kill afforded by radiation when combined with HSV1716could be detected using the MTS assay.

14.16 Cytotoxicity Assay in 373 and BHK Cells Administering Ionising Radiation Six Hours After HSV1716 and HSV17^{+} Infection

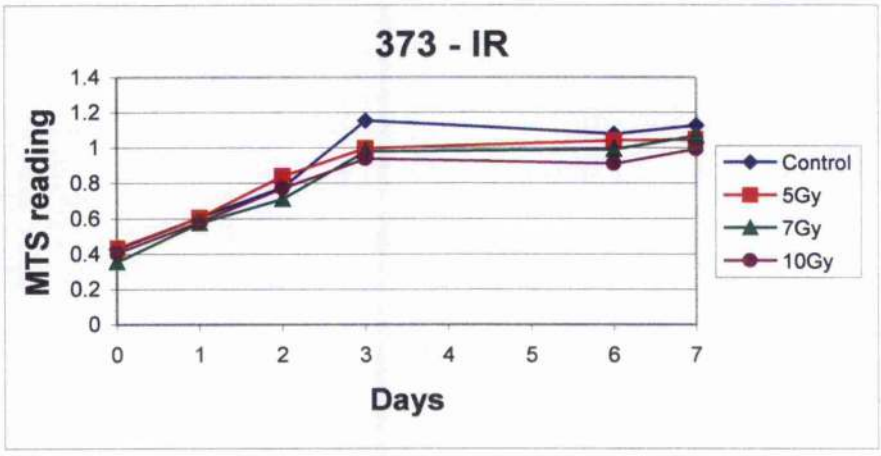
Given that 373 cells appeared less permissive to the growth of HSV1716 than the fully permissive BHK cell line they were used in the following MTS assay when HSV1716 and HSV17^{+} were combined with ionising radiation.

The 373 cell line appears to be relatively radio-resistant with only a minimal decrease between day 3 and day 7 in overall viable cell numbers detected by the MTS assay, compared to the non-irradiated control (figure 14.16.1a). Under the conditions in these experiments (i.e. a MOI of 0.1 and actively replicating cells), the 373 cell line was fully permissive to HSV17^{+} and HSV1716; with both viruses causing complete cell kill independent of the radiation (figure 14.16.1b and figure 14.16.1c).

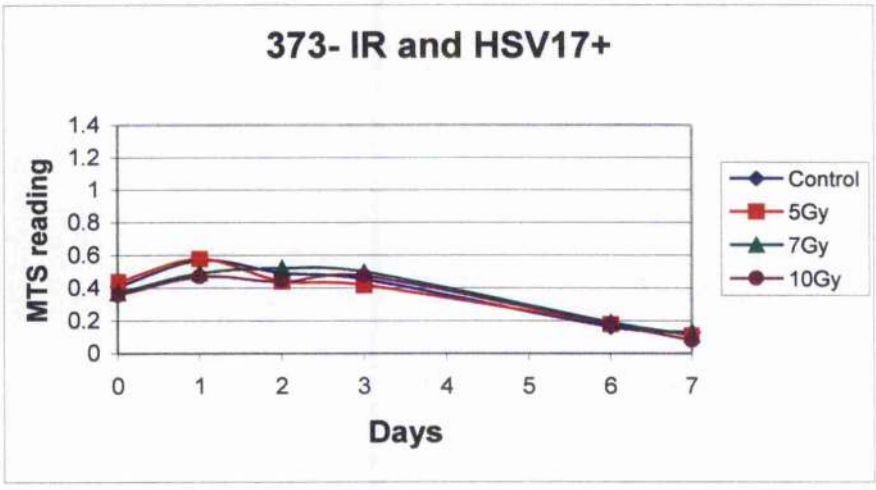
In replicating BHK cells the effect of radiation was most evident following treatment with 10Gy, although by the seventh day the irradiated monolayers were virtually as confluent as the control (figure 14.16.2a). As with the 373 cells, irradiation of BHK cells under these conditions did not provide any additional kill

when combined with HSV17^{+} or HSV1716 as the viruses alone caused complete cell kill (figure 14.16.2b, figure 4.16.2c).

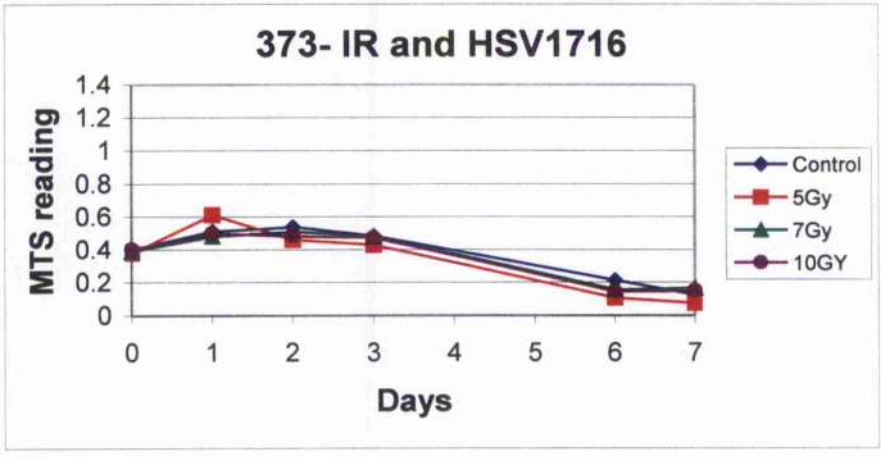
Figure 14.16.1; *MTS analysis of 373 cells following (a) radiation alone, (b) HSV17^{+} and radiation and (c) HSV1716 and radiation.*



(a)

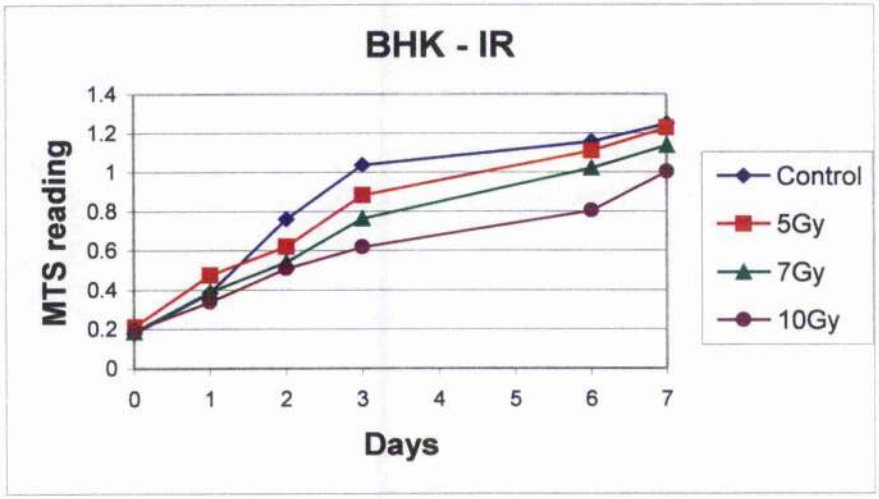


(b)

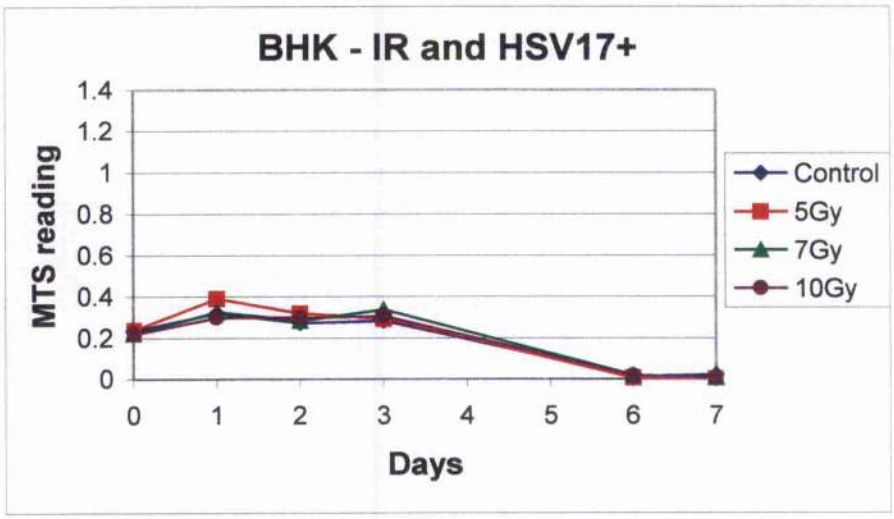


(c)

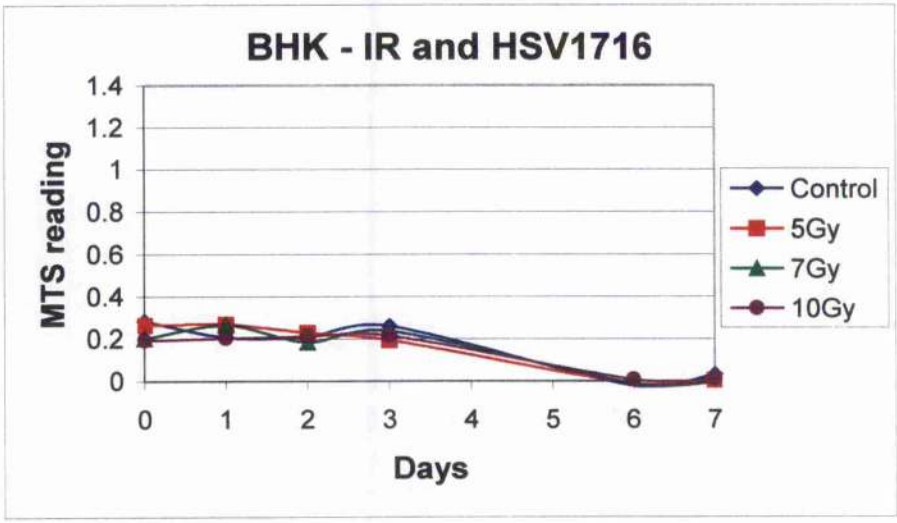
Figure 14.16.2; *MTS analysis of BHK cells following (a) radiation alone, (b) HSV17^{+} and radiation and (c) HSV1716 and radiation.*



(a)



(b)



(c)

14.17 Assessment of Cell Kill when HSV1716 is Delivered 1 Hour After Ionising Radiation

The infection of a replicating cellular monolayer with HSV1716 prior to radiation was employed in light of *in vivo* experiments published prior to commencing the project. During the course of investigations into the combination of HSV1716 followed by ionising radiation, reports were published suggestive of an additive and possibly synergistic relationship between other ICP34.5 null HSV mutants and ionising radiation detected *in vivo* and *in vitro*.

Blank *et al.* (2002) demonstrated enhanced cervical cancer cell kill with low dose (1.5Gy or 3Gy) radiation followed by treatment with ICP34.5 null HSV (G207 or R3616). In light of this, cell viability after irradiation with 1Gy and 5Gy followed one hour later with inoculation was assessed in combination with HSV1716 (MOI 0.1) in 373 and MOG cell lines.

As expected from the previous experiments showing the relative radio-resistance of 373 cells the growth of 373 cells was not altered by low dose (1Gy) radiation. A dose of 5Gy caused minimal growth delay in the first few days but the number of viable cells was soon similar to the control (figure 14.17.1 a).

In this experiment (figure 14.17.1 b) unlike in the previous experiment (figure 14.16.1 c) infection with HSV1716 did not significantly reduce cell viability until day 7 when there did appear to be a decline compared to the control. The apparent anomaly is probably, once again, due to a difference in the multiplicity of infection as the result of a larger number of cells being present in the latter experiment. Under light microscopy it was apparent that over 50% of the cells infected with HSV1716 showed the rounded morphology characteristic of viral infection. Therefore, although these cells were still alive and capable of converting the MTS substrate it is probable that they were destined to die by viral lysis.

The combination of HSV1716 with 1Gy resulted to a similar level of cell kill to HSV1716 alone. However the combination of HSV1716 with 5Gy radiation

resulted in a further reduction in cell viability than was detected with either virus or radiation give in isolation.

When the viable 373 cells were expressed as a percentage of the untreated control, by day six and seven there appeared to be fewer viable cells when HSV1716 and radiation were combined than the sum of reductions in cell viability generated by HSV1716 or radiation alone (figure 14.17.1 c).

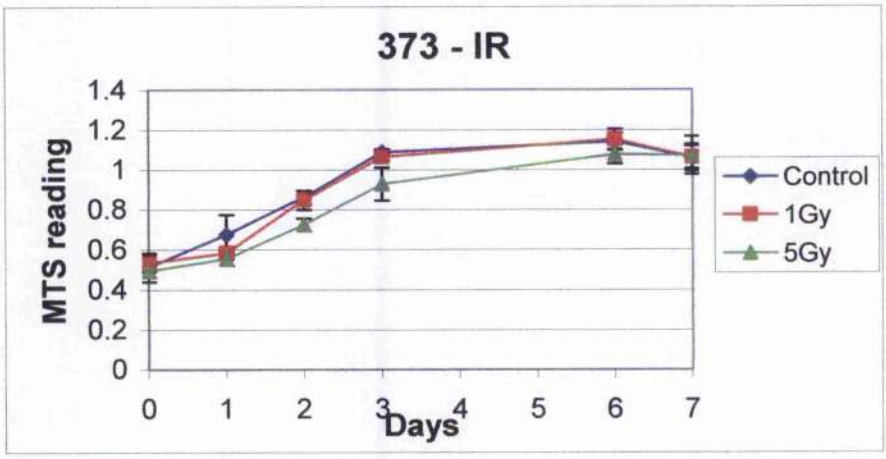
With radiation alone, growth of the MOG cells was not altered by low dose radiation (1Gy) or radiation of 5Gy when compared to the control (figure 14.17.2 a).

As was observed with the 373 cell line, the combination of HSV1716 with radiation of 5Gy resulted in a greater reduction in MOG cell viability by day 6, than was detected with either virus or radiation given in isolation (figure 14.17.2 b).

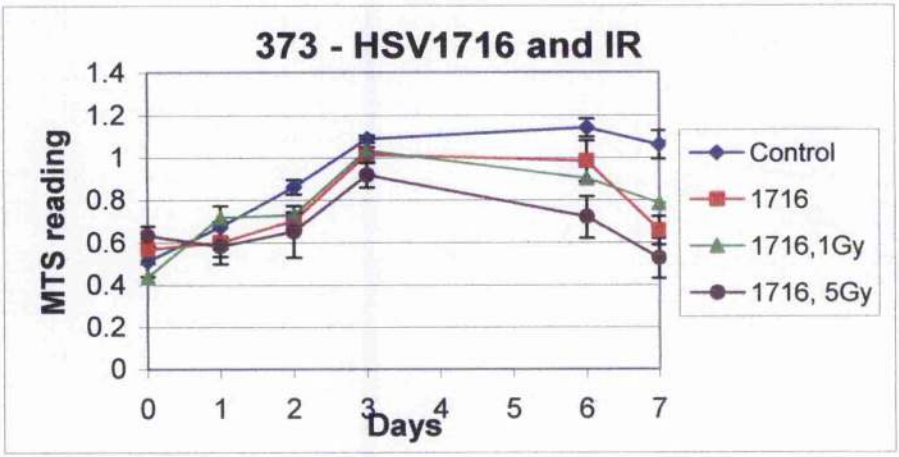
The viable MOG cells following radiation, virus or a combination of the two were expressed as a percentage of the viable cells in the untreated control. When HSV1716 and 5Gy were combined there appeared to be a greater percentage reduction in viability than with either in isolation (figure 14.17.2 c).

Low dose radiation (1Gy) had no impact on the number of viable cells in isolation or when combined with virus infection in either cell line. However a number of independent experiments demonstrated that in both MOG and 373 cells the combination of 5Gy radiation 1 hour prior to infection with 0.1 MOI HSV1716 gave more cell kill than either of the treatments in isolation.

Figure 14.17.1; *MTS analysis of 373 cells following (a) radiation alone, (b) HSV1716 (MOI 0.1) and radiation. (c) The cell viability expressed as percentage of the viable cells in the control. The error bars represent +/-SD in (a) and (b).*



(a)



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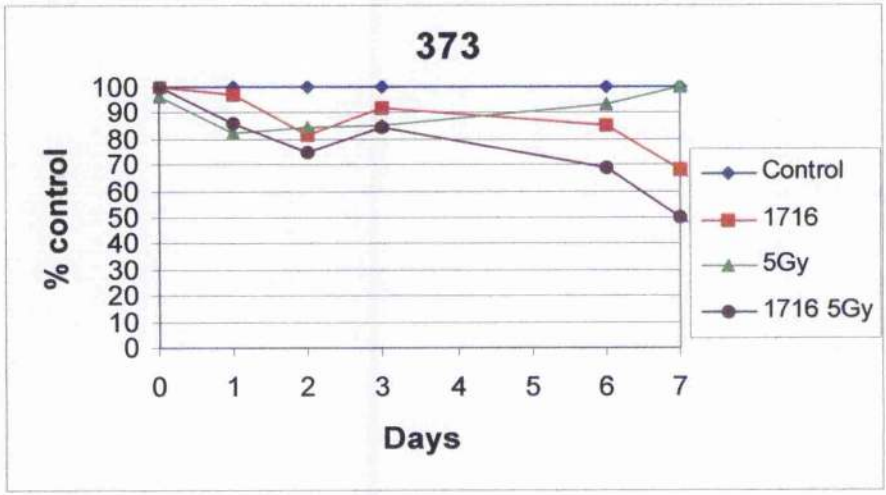
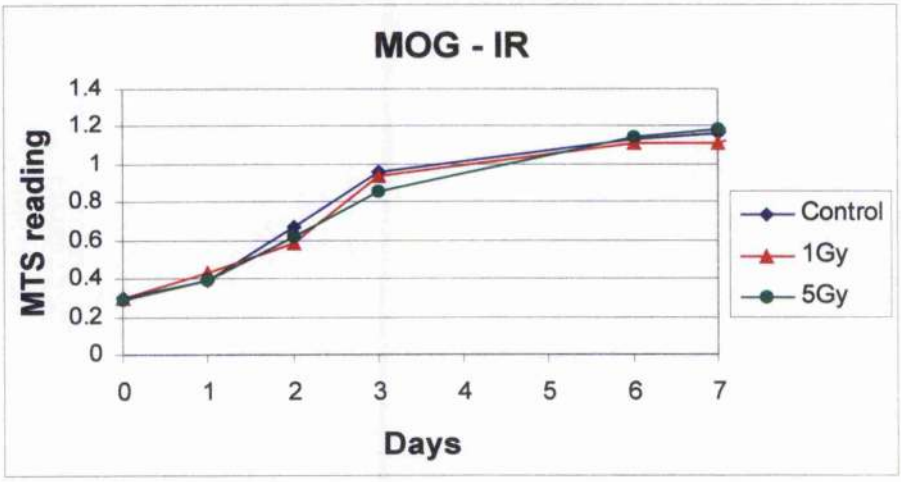
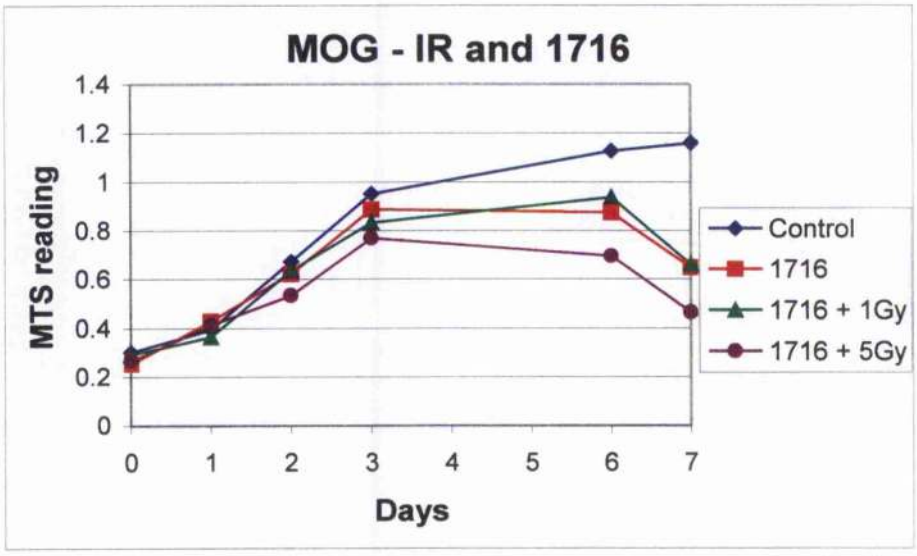


(c)

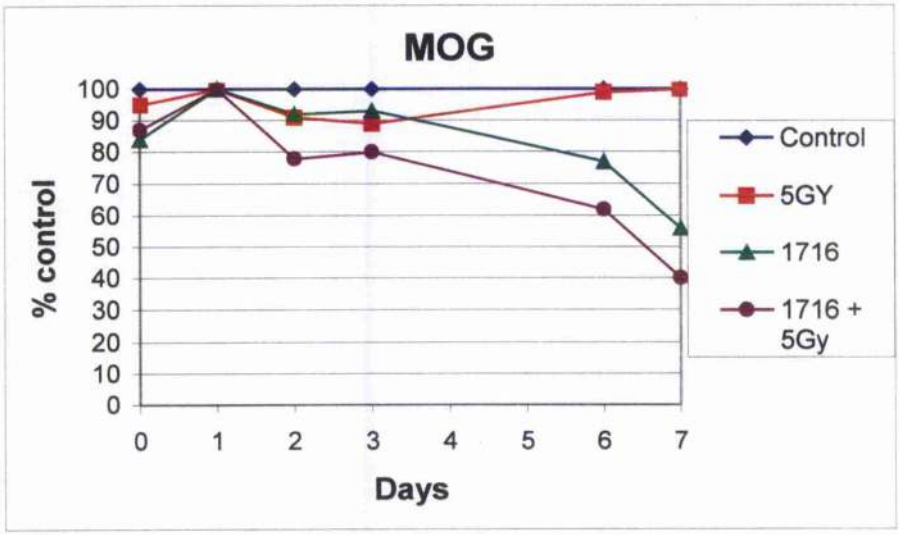
Figure 14.17.2; *MTS analysis of MOG cells following (a) radiation alone, (b) HSV1716 (MOI 0.1) and radiation. Cell viability expressed as percentage of the viable cells in the control is represented in (c).*



(a)



(b)



(c)

14.18 MTS Cytotoxicity Assay in Cell Monolayers Irradiated 1 Hour Prior to HSV1716 Infection

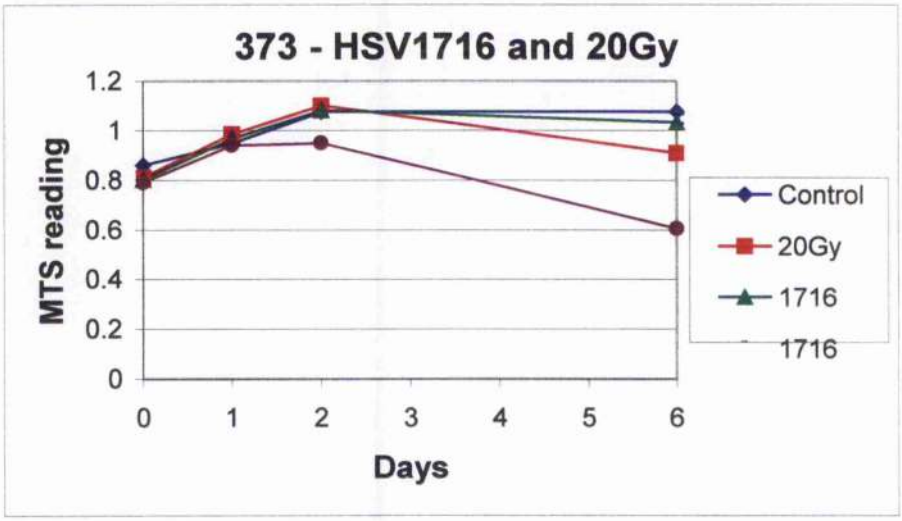
Low dose radiation (1Gy) had been investigated in experiment 14.17 and not shown to enhance the cell kill when combined with HSV1716 although doses of 5Gy appeared to have an additive effect. *In vivo* experiments using the ICP34.5 null HSV mutant R3616 suggested a synergistic relationship with higher radiation fraction sizes of 20-25Gy (Advani SJ *et al.*, 1998). The relationship between HSV1716 and radiation dose of 20Gy was investigated *in vitro* in 373 and MOG cells.

Cell viability was assessed in 373 and MOG cells irradiated with 20Gy one hour prior to infection with HSV1716 at an MOI of 0.1 pfu/cell. Six days later in the 373 cells there was less cell viability detected when HSV1716 and radiation were combined than when either was used in isolation (figure 14.18.1 a,b,c). The percentage reduction in cell viability when HSV1716 and radiation were combined appeared to be greater than the sum of the percentage reduction in cell viability following virus and radiation given alone. These results suggest a supra-additive effect when HSV1716 at an MOI of 0.1 is combined with radiation of 20Gy. It is noted however, that 20Gy radiation on its own resulted in very little cell kill by day 6 (figure 14.18.1 a). Although it was intended that $3x10^3$ cells per well of a 96 well plate were seeded at the outset the initial MTS reading was higher than in the previous experiment and may indicate that the initial cell number per well was higher than intended. This increased cell number may have impeded normal cell replication and consequently reduced the impact of radiation or HSV1716 lytic infection on the monolayer. This said however, when the two modalities were combined the relationship did appear to be supra-additive.

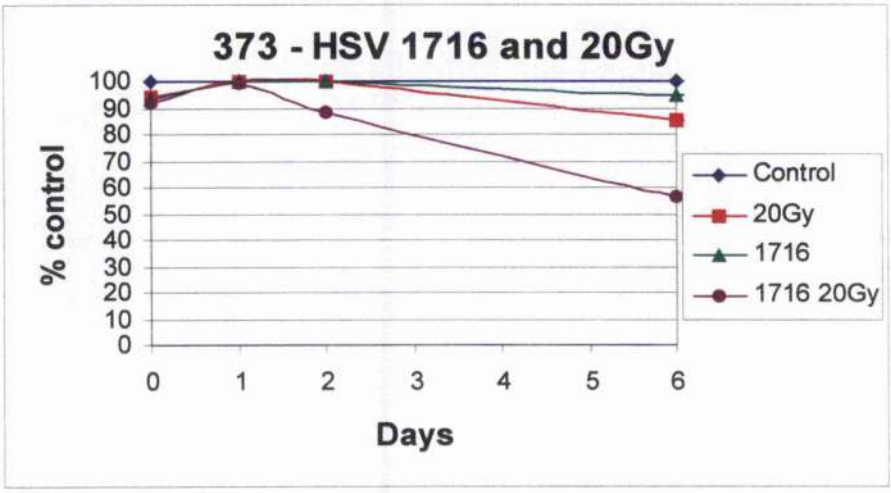
By the sixth day, 20Gy radiation had had a significant effect on the MOG cell viability and HSV1716 had reduced the cell viability by approximately 80% of the control. The combination of 20Gy radiation and 0.1 MOI HSV1716 infection appeared to cause approximately the same percentage reduction in cell viability as the sum of the percentage reductions in viability caused by either radiation or

virus alone (figure 14.18.2 a, b, c). The effect in this cell line when the two modalities are combined appears to be at best additive.

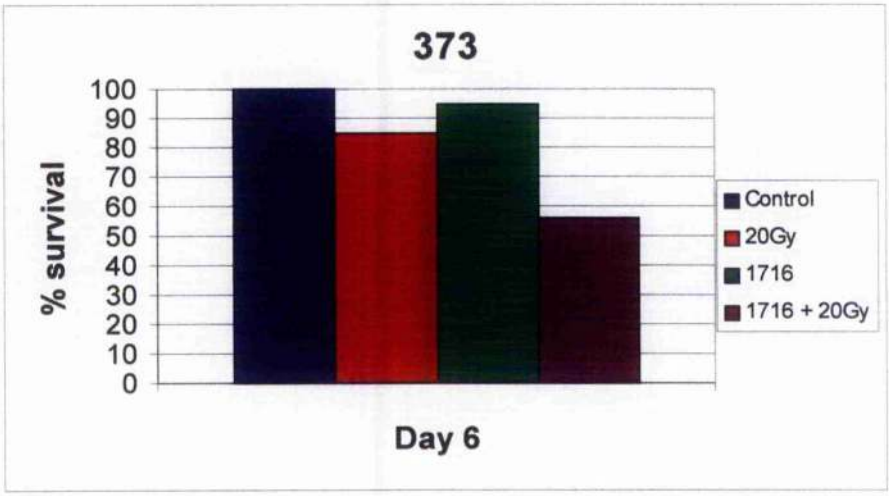
Figure 14.18.1; *MTS analysis of 373 cells following (a) radiation and HSV1716 (MOI 0.1) alone and in combination, (b) Cell viability expressed as percentage of the viable cells in the control (c) bar chart depicting the percentage of viable cells compared to the control on day 6 of the investigation.*



(a)

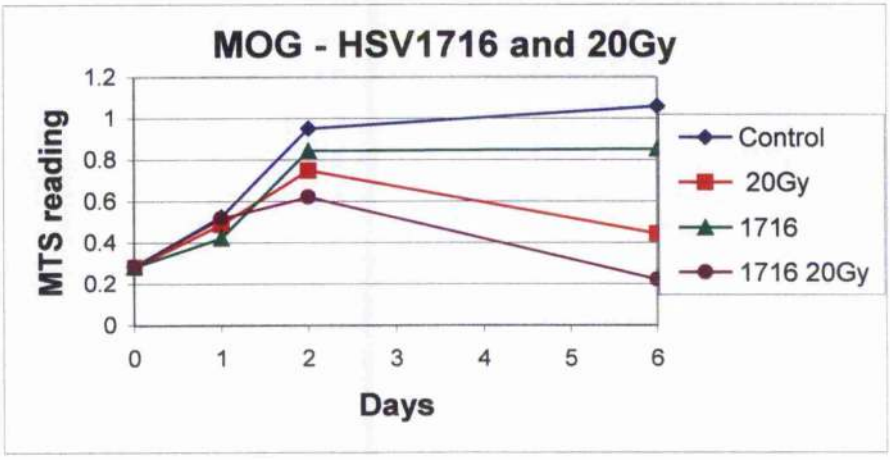


(b)

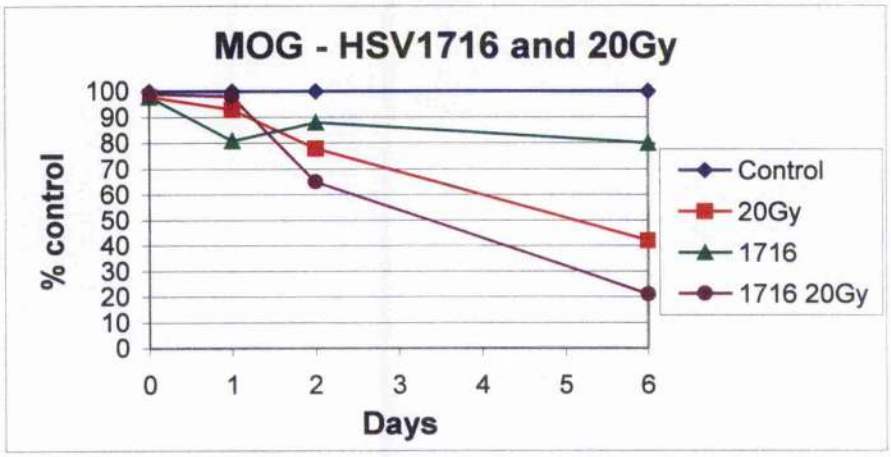


(c)

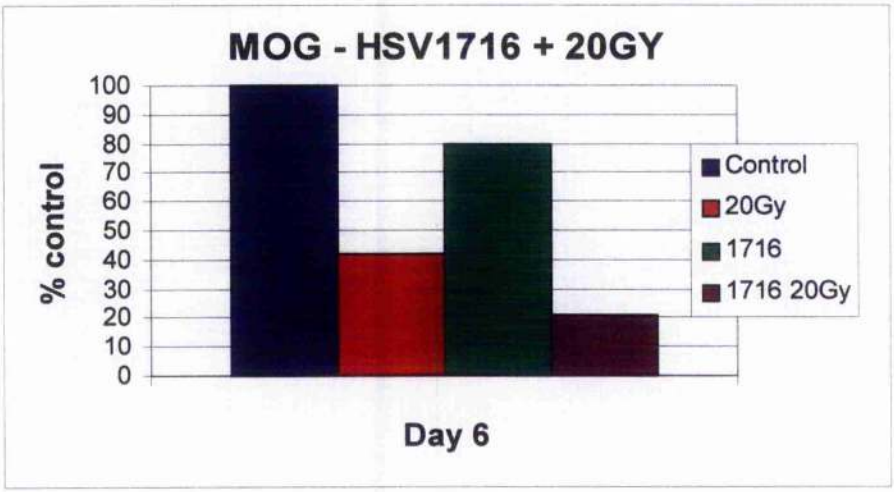
Figure 14.18.2; *MTS analysis of MOG cells following (a) radiation and HSV1716 (MOI 0.1) alone and in combination, (b) Cell viability expressed as percentage of the viable cells in the control (c) bar chart depicting the percentage of viable cells compared to the control on day 6 of the investigation.*



(a)


MOG - HSV1716 and 20Gy
% control
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Days
Control
20Gy
1716
1716 20Gy

(b)


MOG - HSV1716 + 20GY
% control
100
90
80
70
60
50
40
30
20
10
0
Day 6
Control
20Gy
1716
1716 20Gy

(c)

14.19 Cytotoxicity Assay in 373 Cells Combining Single Fraction or Fractionated Ionising Radiation and HSV1716

On delivering radiation one hour prior to HSV1716 infection there were results suggesting that the effect on cell kill was additive in MOG cells and synergistic in 373 cells. The doses of radiation used in the preceding experiments were either; 1Gy, 5Gy or 20Gy. These regimes do not reflect the radiation schedules used in the treatment of malignant disease. In response to this, a clinically relevant fractionated regime of 2Gy fractions was delivered to replicating monolayers of 373 cells. The MTS assay was used to compare cell viability following HSV1716 infection with single dose radiation versus the same total radiation dose delivered in a fractionated schedule.

All cells received a total of 10Gy either as a single fraction or in the case of the fractionated regime, an initial 2Gy dose. One hour after the completion of radiation HSV1716 (MOI of 0.1) was added where indicated. The cells due for fractionated irradiation received further 2Gy doses over the subsequent 4 days until a total of 10Gy over 5 days had been delivered.

By six days the reduction in cell viability detected when radiation and HSV1716 were combined in the 373 cell line was the same when the radiotherapy was fractionated or delivered as single 10Gy dose. The reduction in cell viability when radiation and virus infection were combined appeared to be greater than the sum of the reductions in viability of each treatment in isolation (figure 14.19.1 and 14.19.2).

The delivery of 2Gy radiation doses over the course of five individual fractions showed no significant difference to 10Gy given as a single dose (figure 14.19.2).

On a separate occasion the results were analysed at seven days. Once again the reduction in cell viability when radiation and virus infection were combined appeared to be greater than the sum of the reductions in viability of each treatment in isolation (figure 14.19.3 and 14.19.4).

Figure 14.19.1; *MTS analysis of 373 cells following single dose and fractionated radiation, and HSV1716 (MOI 0.1) alone and in combination.*

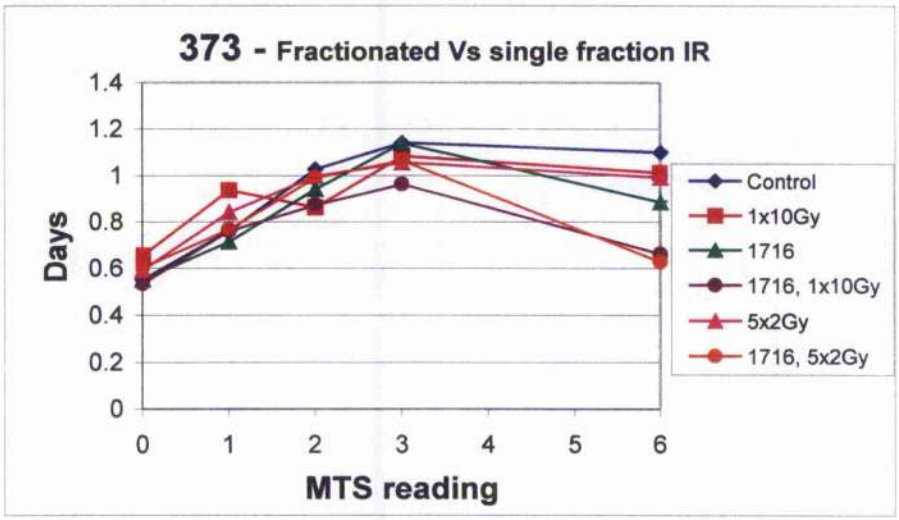
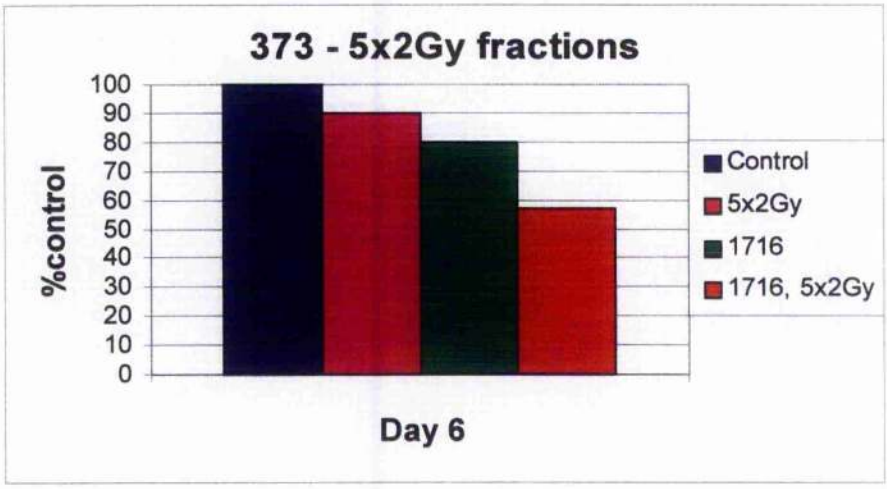
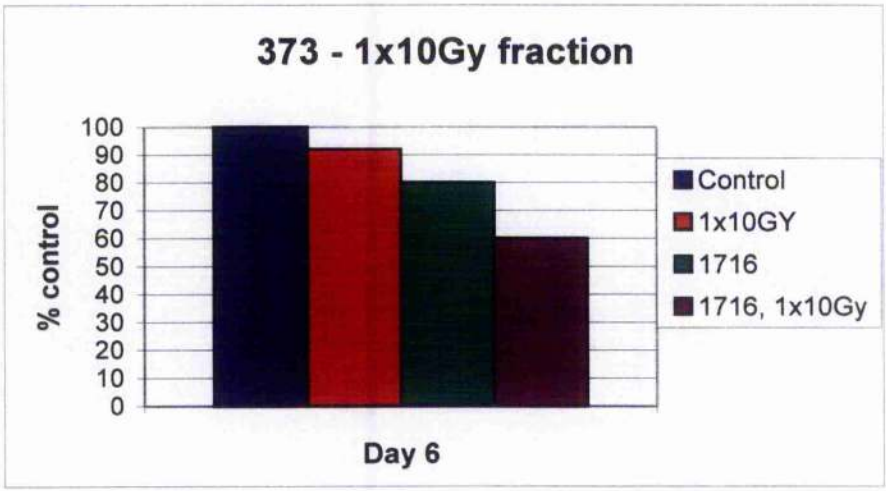


Figure 14.19.2; *Cell viability expressed as a percentage of control cell viability measured at day 6, when (a) 10Gy was delivered as a single fraction and (b) when 10Gy was delivered over 5 fractions.*



(a)



(b)

Figure 14.19.3; *MTS analysis of 373 cells following single dose and fractionated radiation, and HSV1716 (MOI 0.1) alone and in combination.*

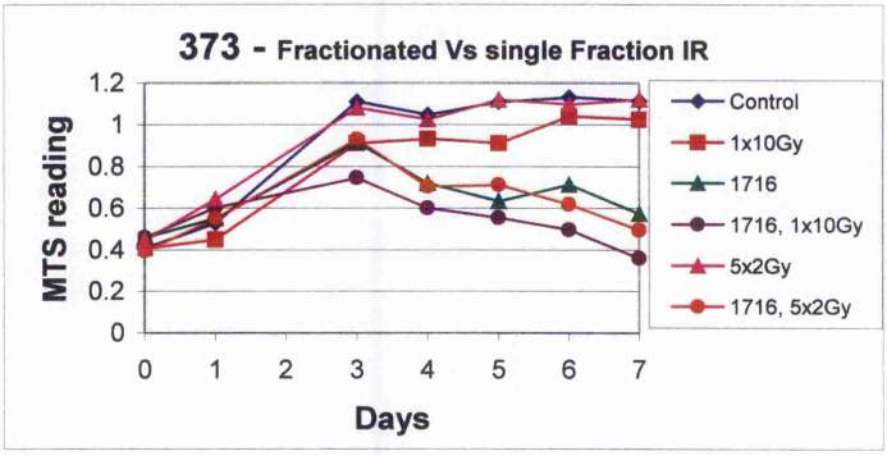
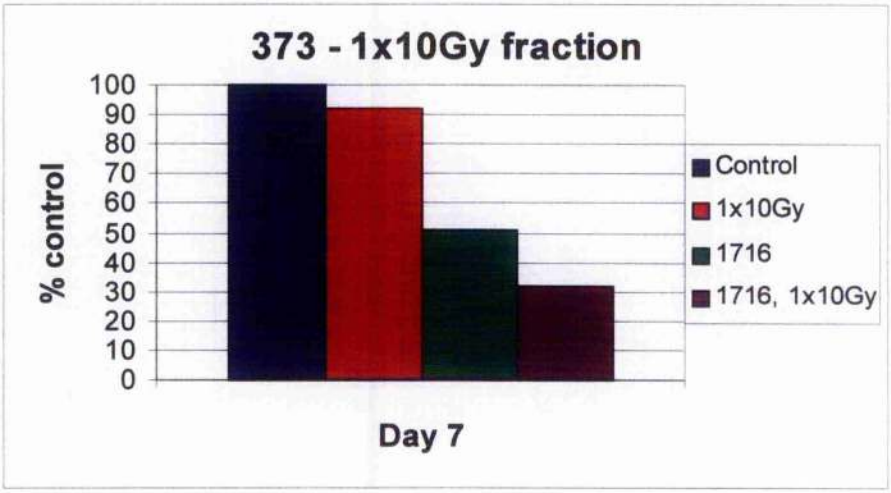
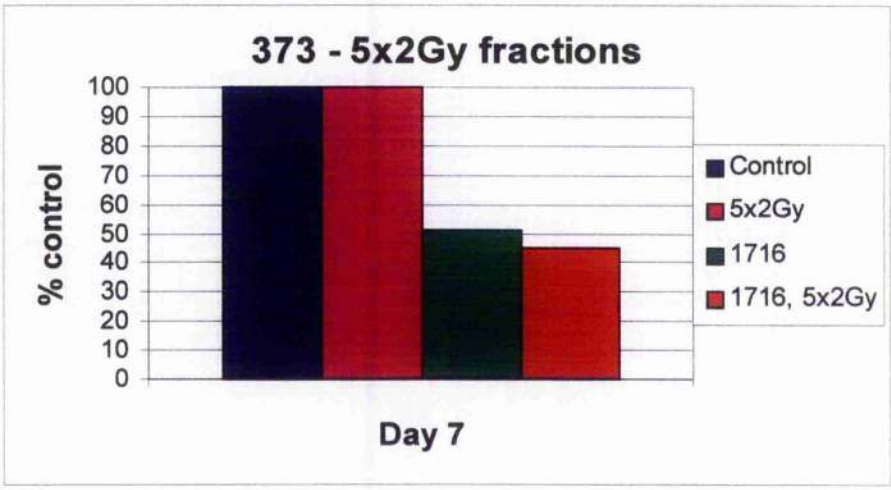


Figure 14.19.4 *- Cell viability expressed as a percentage of control cell viability measured at day 7 when (a) 10Gy was delivered in a single fraction and (b) when 10Gy was delivered over 5 fractions.*



(a)



(b)

14.20 Multi-cycle Virus Growth Experiments with Pre–irradiated 373, MOG and BHK Cells

Many of the papers that demonstrated a synergistic relationship with a ICP34.5 null mutant HSV and ionising radiation showed an increase in the viral titre obtained from pre-irradiated cells in a virus growth experiment. An explanation proposed by a number of the investigators (Spear M *et al.*, 2000; Blank S *et al.*, 2002) is that ionising radiation induces the up regulation of certain proteins, such as mammalian RR or GADD34, that the attenuated virus can utilise to facilitate its lytic replication. This experiment sought to investigate whether irradiating cells prior to the inoculation of HSV1716 would result in an increased titre in a multicycle growth experiment. The cell lines used were BHK, in which HSV1716 is fully replication competent and the human glioma lines 373 and MOG in which replication of HSV1716 is impaired compared to the parental wild type strain $HSV17^{+}$.

BHK

Although at 24 hours the yield of HSV1716 from irradiated cells was marginally lower than in control cells the final titre obtained with the pre-irradiated cells was the same as for the non-irradiated cells.

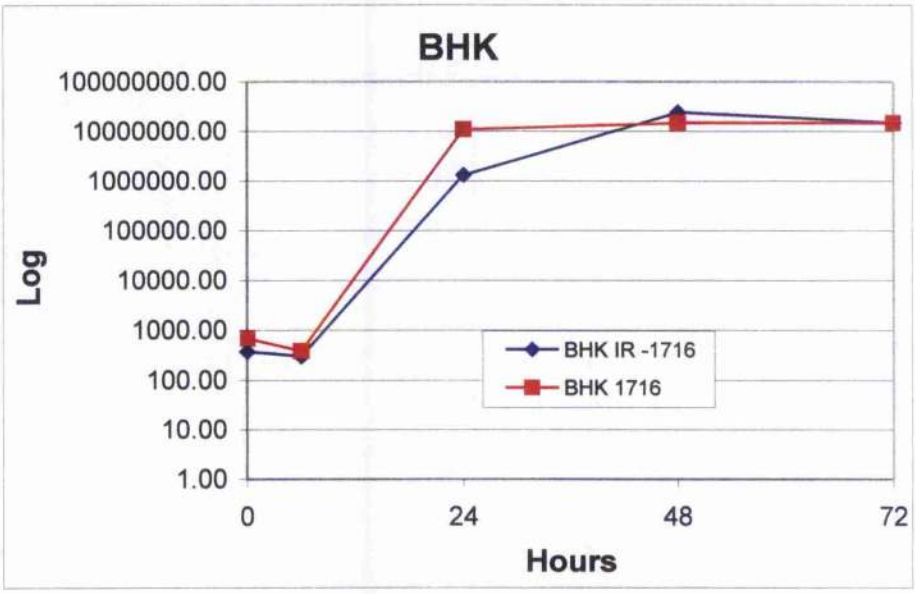


Figure 14.20(a); *Multicycle growth curve BHK cells*

MOG

The replication of HSV1716 was almost identical in irradiated and control MOG cells throughout the experiment and the final titre obtained with the pre-irradiated cells was the same as for the non-irradiated cells.

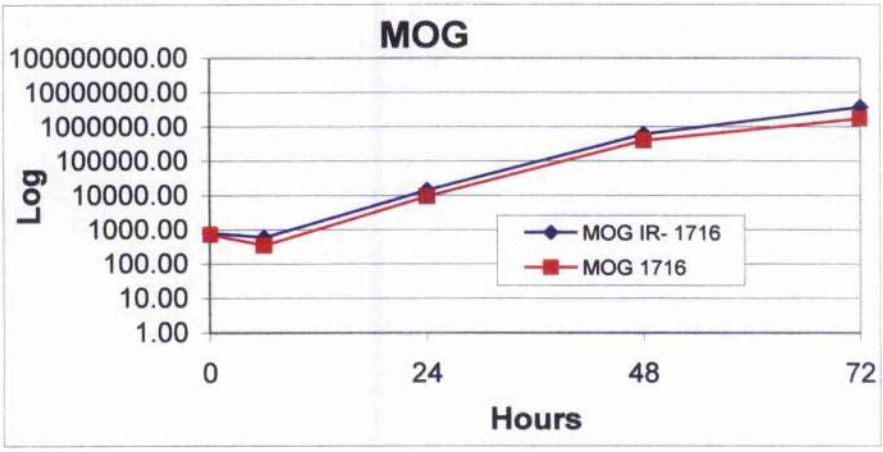


Figure 14.20(b); *Multicycle growth curve MOG cells*

373

The replication of HSV1716 was almost identical in irradiated and control 373 cells throughout the experiment and the final titre obtained with the pre-irradiated cells was the same as for the non-irradiated cells.

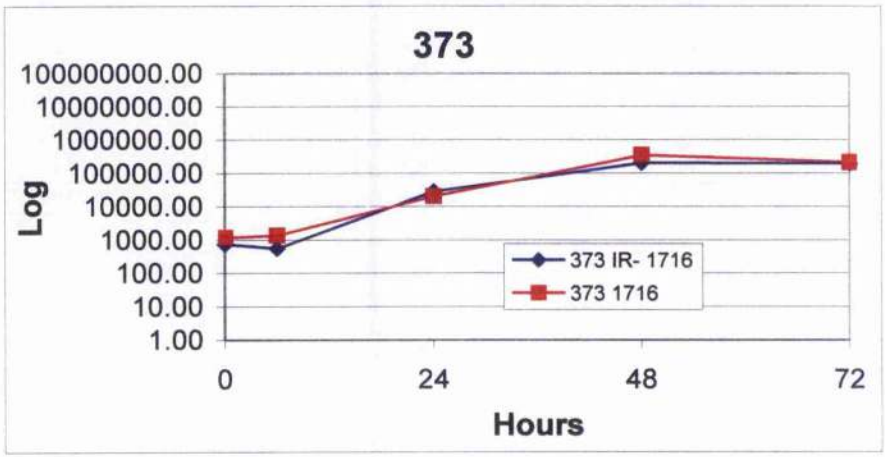


Figure 14.20(c); *Multicycle growth curve 373 cells*

Irradiation of all three cell lines prior to HSV1716 failed to result in enhanced viral replication and resulted in the same viral titre as obtained with the non-irradiated cells.

14.21 Isobologram Analysis of Synergy Between HSV1716 and Ionising Radiation in 373 Cells

Radiation and HSV1716 infection kill cells through different mechanisms. A synergistic relationship it terms of cytotoxicity has been demonstrated between HSV mutants and ionising radiation. In these instances however the cytotoxicity resulting from a combination of radiation and virus was documented as cell kill more than or equal to the sum of the cell kill exerted by each modality individually (Blank S *et al.*, 2002; Stanziale S *et al.*, 2002). This method of assessing if a relationship is synergistic or additive is only acceptable if independent dose-response curves of the two agents follow first order kinctics. The dose response curves for HSV1716 and radiation do not follow first order kinetics and therefore it is inappropriate to analyse data in this way when these agents are combined. The isobologram method used to analyse the interaction between two entities was first described in relation to radiation and chemotherapy interactions with non-linear dose response curves (Steel, GG and Peckham, MJ, 1979, Plumb J *et al.*, 1994).

To analyse the supra-additive effect on 373 cell kill from ionising radiation combined with HSV1716 infection suggested by the previous results, it was necessary initially to ascertain the dose-response in terms of cell survival to various doses of radiation and HSV1716 for this cell type. From these graphs the dose required to kill 50% of the cells (ID_{50}) is estimated as the absorbance value equal to 50% of that of the control well.

The cell viability of 373 cells was assessed using the MTS assay at 6 days following various doses of ionising radiation or HSV1716 at a range of multiplicity of infection.

The cell viability results were graphed and the radiation dose and MOI of HSV1716 that generated 50% cell kill (ID_{50}) compared to the control were calculated.

Radiation:

373 Control MTS reading = 1.09

50% of the MTS reading = 0.55

The ID_{50} for radiation in 373 cells = **15Gy**

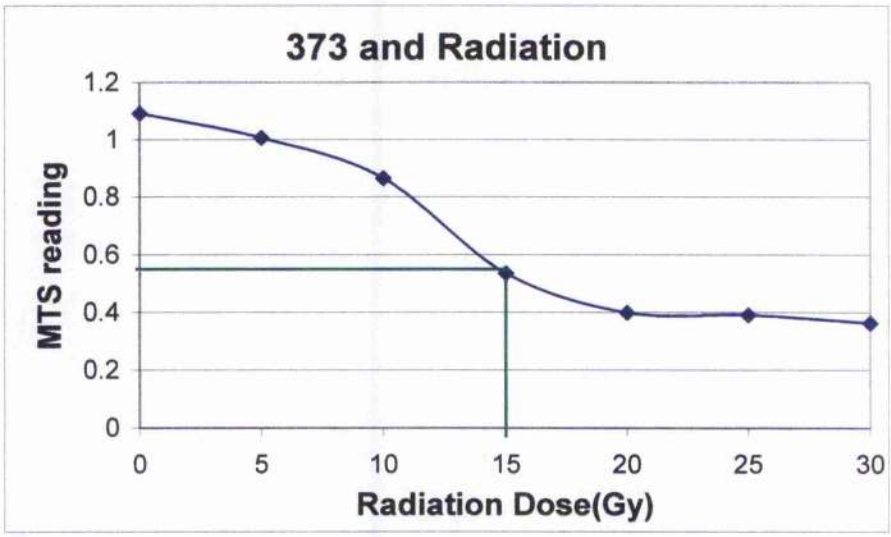


Figure 14.21a; Ionising radiation dose response curve at day 6 in 373 cells demonstrating the ID_{50}.

HSV1716:

373 Control MTS reading = 1.09

50% of the MTS reading = 0.55

The ID_{50} for HSV1716 in 373 cells = **MOI 0.25**

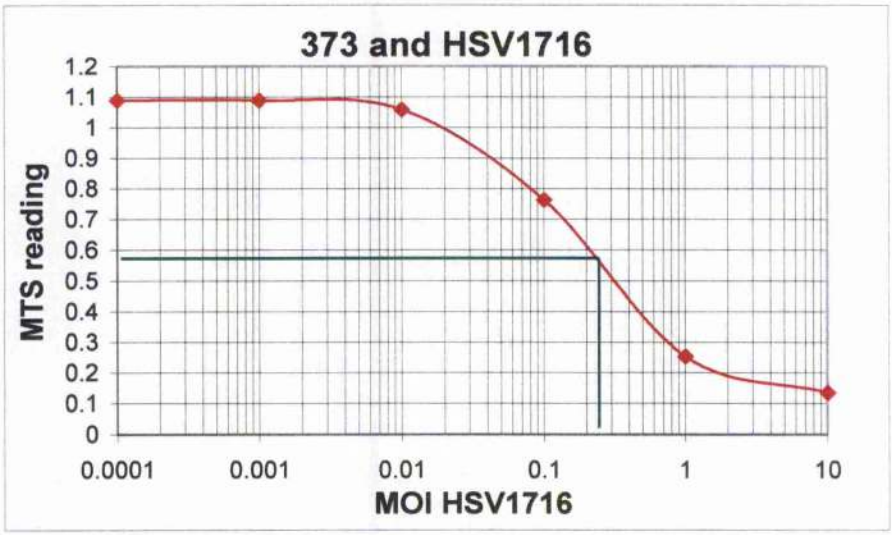


Figure 14.21b; *HSV1716 dose response curve at day 6 in 373 cells demonstrating the* ID_{50}.

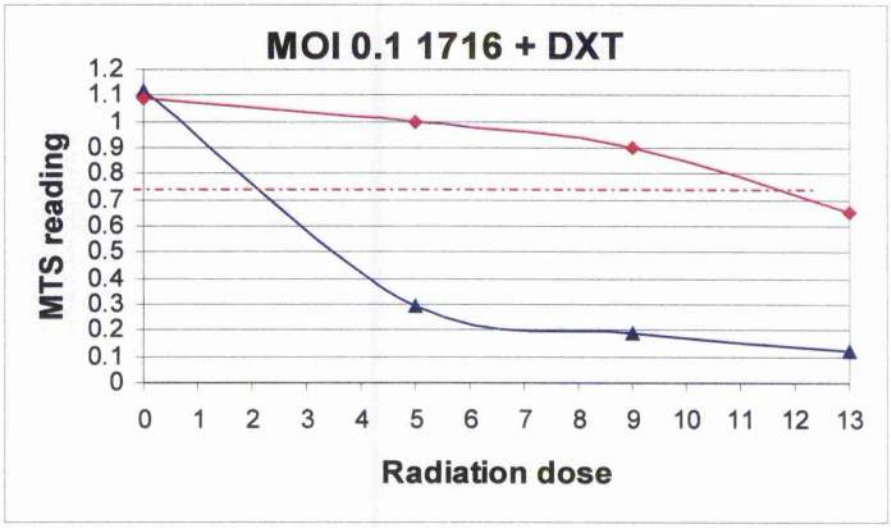
Based on the information obtained from the ID_{50} experiments above, combination experiments with HSV1716 and radiation were designed. HSV1716 and radiation were combined using doses of each modality that caused less than 50% reduction in cell viability by day 6. Each well of a 96 well plate was seeded with $2.5x10^3$ cells in 100μl.

The experimental plates were irradiated one hour prior to HSV1716 inoculation. The combined doses were as follows. The experiment was run for 6 days.

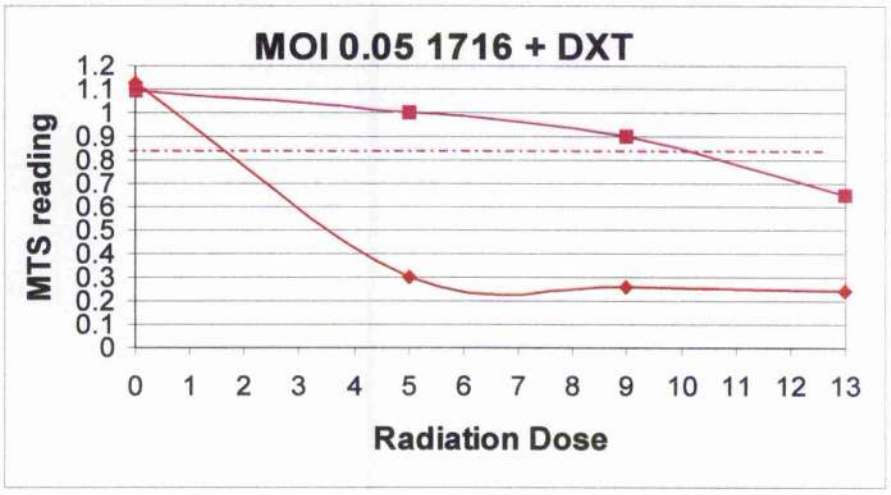
Plate	Radiation Dose	MOI of HSV1716
1	Control	Control
5	5Gy	0.1
6	5Gy	0.05
7	5Gy	0.01
8	9Gy	0.1
9	9Gy	0.05
10	9Gy	0.01
11	13Gy	0.1
12	13Gy	0.05
13	13Gy	0.01

Table 14.21(i); *Dose combinations of IR and MOI HSV1716*

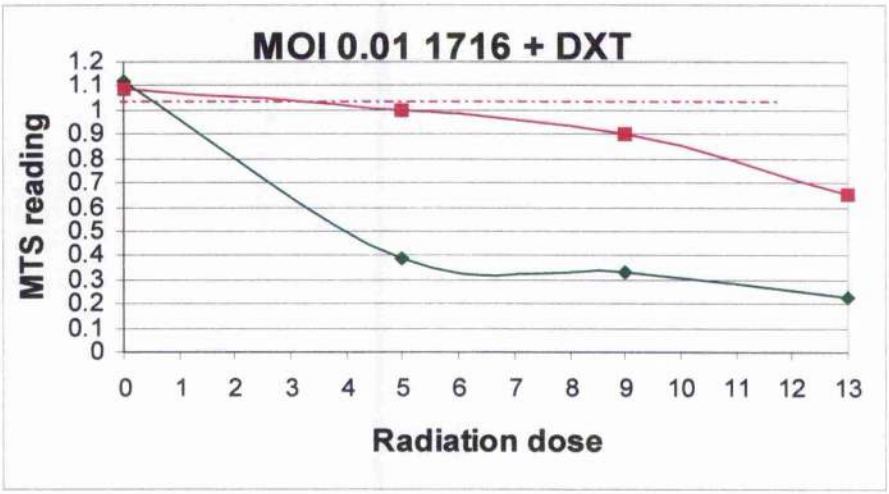
Figure 14.21 c, d , e - *MTS assay dose response curve (solid blue, solid red and solid green lines) at day 6 when (c) MOI 0.1 HSV1716 is combined with various doses of radiation, (d) MOI 0.05 HSV1716 is combined with various doses of radiation, (e) MOI 0.01 HSV1716 is combined with various doses of radiation. The dotted pink line represents the MTS reading at day 6 obtained following infection of the 373 monolayer with HSV1716 alone at either 0.1, 0.05 or 0.01 MOI. The solid pink represents the dose response curve of 373 cells obtained at day 6 following various doses of radiation independently.*



(c)



(d)



(e)

Using the information from the graphs in figure 14.21 c, d, and e the MTS reading representing a 50% reduction in cell viability compared to the control is calculated for each of the 3 combinations.

Fifty percent of the MTS reading for the control in each of the three graphs gives a value of 0.55. A straight line is drawn from point 0.55 on the *y*-axis to the point that it intersects with the graph. From this point another straight vertical line is drawn down to intersect the *x*-axis. The point on the *x*-axis that is intersected gives a value of radiation that will give 50% cell kill by day 6 in 373 cells if combined with the initial MOI of HSV1716.

The ID_{50} calculated from figure 14.21 (c)(d)(e) are represented in the following table.

HSV1716 MOI	Radiation dose required for 50% kill
0.1	3Gy
0.05	3.2Gy
0.01	3.5Gy

Table 14.21(ii); *ID_{50} values from combination experiments*

Construction of the ID_{50} isobologram for HSV1716 and radiation

Data from the dose-response curves following HSV1716 and radiation obtained at day 6 enables the construction of an isobologram plot. Different regions within the plot indicate if the combination of HSV1716 and ionising radiation is infra-additive, additive or supra-additive. The ID_{50} isobologram indicates the dose of radiation alone, HSV1716 alone and the various combinations of each that is required to cause a 50% reduction in 373 cell viability. To construct the isobologram the protocol outlined by Kano *et al* was followed (Kano Y *et al.*, 1992; Kano Y *et al.*, 1988). Three different plots Mode I, Mode IIa and Mode IIb are derived from the previous experiments that indicate the relationship between HSV1716 and ionising radiation.

Mode I line.

For a given dose of radiation the fraction of cell growth that was inhibited compared to the control is Fa (A_1). A dose of (x) is thus required by HSV1716 to generate a total reduction in cell viability of 50% when the radiation dose of Fa(A1) is given in combination with HSV1716.

For example -

Taking the radiation dose response curve for 373 at day 6, 15Gy results in 50% growth inhibition (red arrow - figure 14.21f). 13Gy would result in 39% [Fa(A_1)] of the required 50% being killed (turquoise arrow). To reach 50% cell growth inhibition 11% additional kill (x) would have to be achieved by HSV1716 (pink arrow).

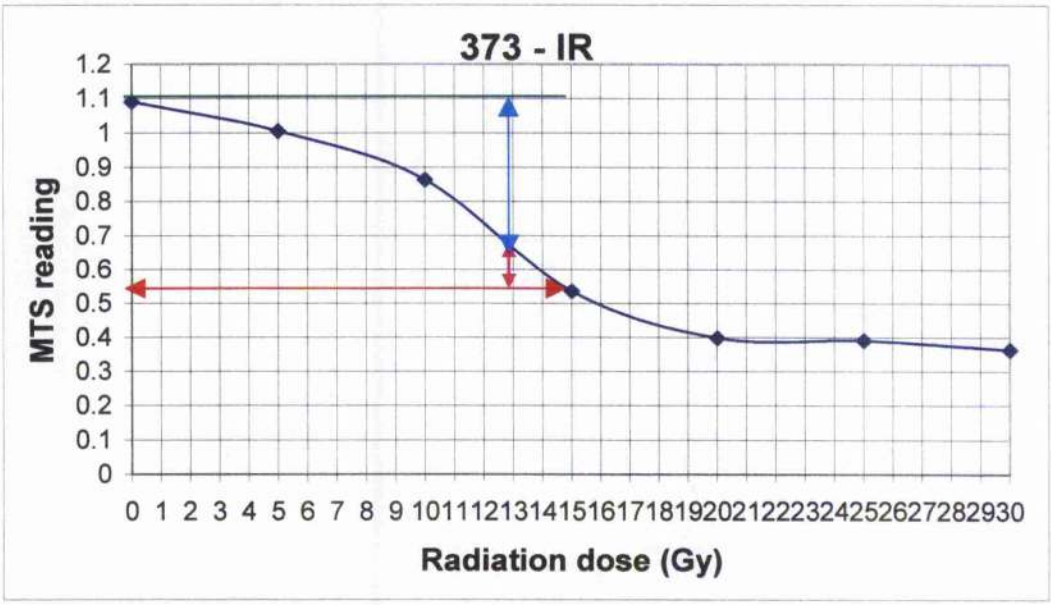


***Figure 14.21(f)**; Dose response curve for IR in 373 cells*

A number of points are calculated in a similar manner and are detailed in the table below.

Radiation dose	**% kill achieved [Fa(A_1)]**	**% kill required by HSV1716 (x)**
13Gy	39	11
11Gy	26	24
10Gy	21	29
8Gy	14	36
5Gy	8	42
2Gy	4	46

***Table 14.21(iii);** Values of Fa(A_1) and (x) obtained from IR dose response curve*

Using the dose response curve at day 6 obtained for HSV1716 (figure 14.21b) the percentages of HSV1716 (x) indicated in table 14.21(iii) required to produce a 50% reduction in cell viability when combined with radiation can be converted into an exact MOI of HSV1716.

For example -

From the radiation dose response curve, 8Gy radiation dose caused 14% [Fa(A_1)] inhibition of the cells. Therefore 36% (x) further reduction in cell viability is required by HSV1716 to produce a 50% reduction in the cell kill. This is indicated in figure 3.24(g) where [Fa(A1)] is represented as a red arrow and (x) is represented as a turquoise arrow. The pink arrow is the actual MOI of HSV1716 that is required to produce the 36% cell kill that when combined with 8Gy will cause an overall reduction in cell viability of 50%.

This is taken as B1 = MOI 0.15 which is now a point on the Mode 1 line.

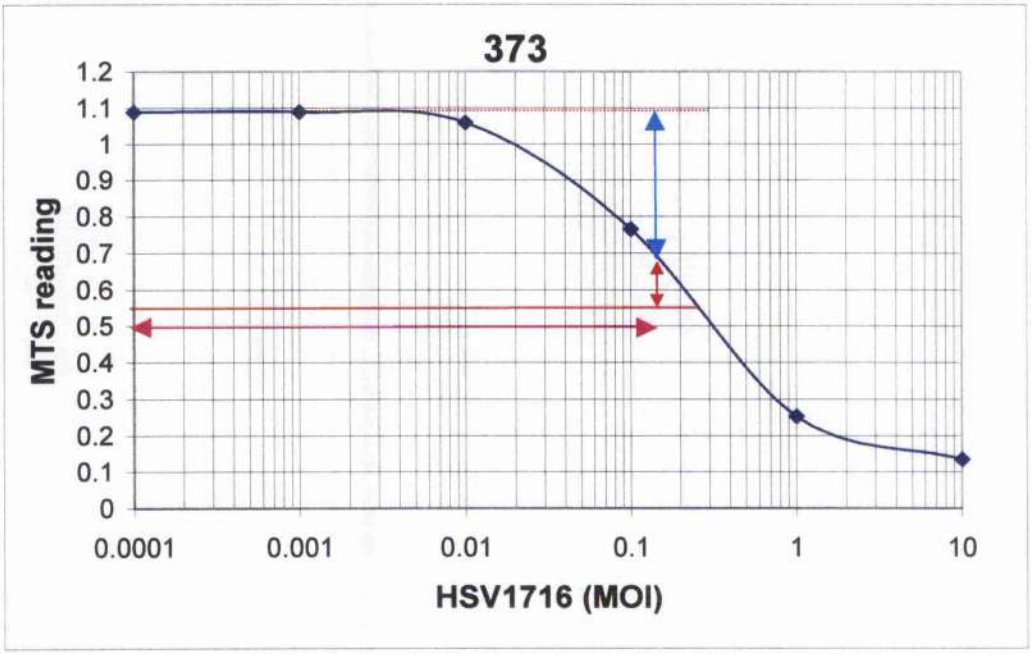


Figure 14.21(g); *Dose response curve of HSV1716 in 373 cells*

This is repeated for all the points taken from the radiation curve detailed in the table 14.21(iii).

***Mode IIa line*:**

The doses of HSV1716 expressed as a percentage in table 14.21(iii), required to result in 50% cell kill when added to radiation can be plotted on the HSV1716 dose response curve in a different way. This iso-effect line is calculated as indicated below.

For example;

For 8Gy of radiation the percentage of cell kill Fa(A1) = 14% (brown arrow). The amount of cell kill required from HSV1716 to reach 50% is x = 36% (green arrow). The pink arrow below is the dose of virus required to produce 50% reduction in cell viability starting from the point on the dose response curve that the effect of radiation had ended.

The dose of HSV1716 is B2 = **MOI 0.22** and is a point on the Mode II a line.

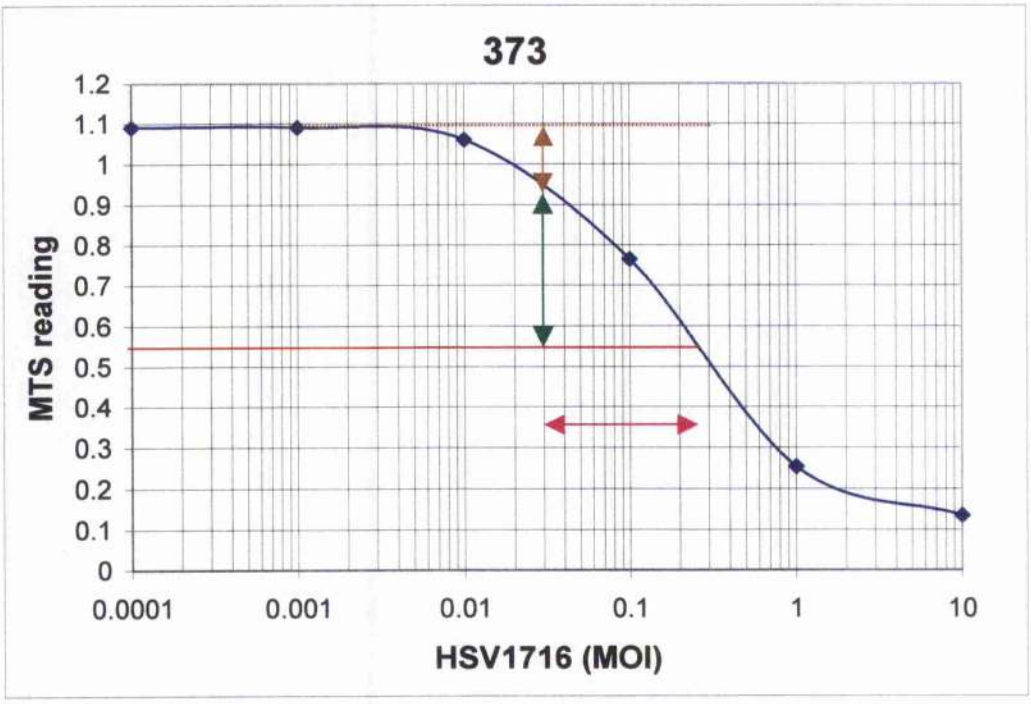


***Figure 14.21(h);** Dose response curve of HSV1716 in 373 cells*

The Mode I and Mode IIa line, data points B1 and B2 respectively, were calculated to give the percentages of HSV1716 required to produce a 50% reduction in cell viability when combined with known doses of radiation as indicated in table 14.21(iii).

Radiation Dose	B1 (MOI)	B2 (MOI)
13Gy	0.025	0.1
11Gy	0.065	0.17
10Gy	0.07	0.195
8Gy	0.15	0.22
5Gy	0.2	0.23
2Gy	0.23	0.235

Table 14.21(iv); *Radiation dose, B1 and B2 calculations*

The absolute HSV MOI values detailed in table 14.21(iv) are converted to a percentage of the HSV1716 MOI value that generated a reduction in 50% cell viability independently, i.e HSV1716 ID_{50}, derived from figure 14.21(b) as MOI=0.25.
For example; (0.1 / 0.25) MOI x 100 = 40%.

The radiation doses in table 14.21(iv) are also expressed as a percentage of the Radiation ID_{50} value, derived from figure 14.21(a) as 15Gy.
For example; (13 / 15) Gy x 100 = 86.7%.

The calculations for all the values in table 14.21(iv) are detailed as percentages in table 14.21(v) below.

% Radiation Dose 15Gy	B1 - % of MOI 0.25	B2 - % of MOI 0.25
86.7	10	40
73.3	26	68
66.7	28	78
53.3.	60	88
33.3	80	92
13.3	92	94

Table 14.21(v); *Values from Table 14.21(iv) as a percentage of the* ID_{50}

This is then graphed to give the **mode I** and **mode IIa** lines

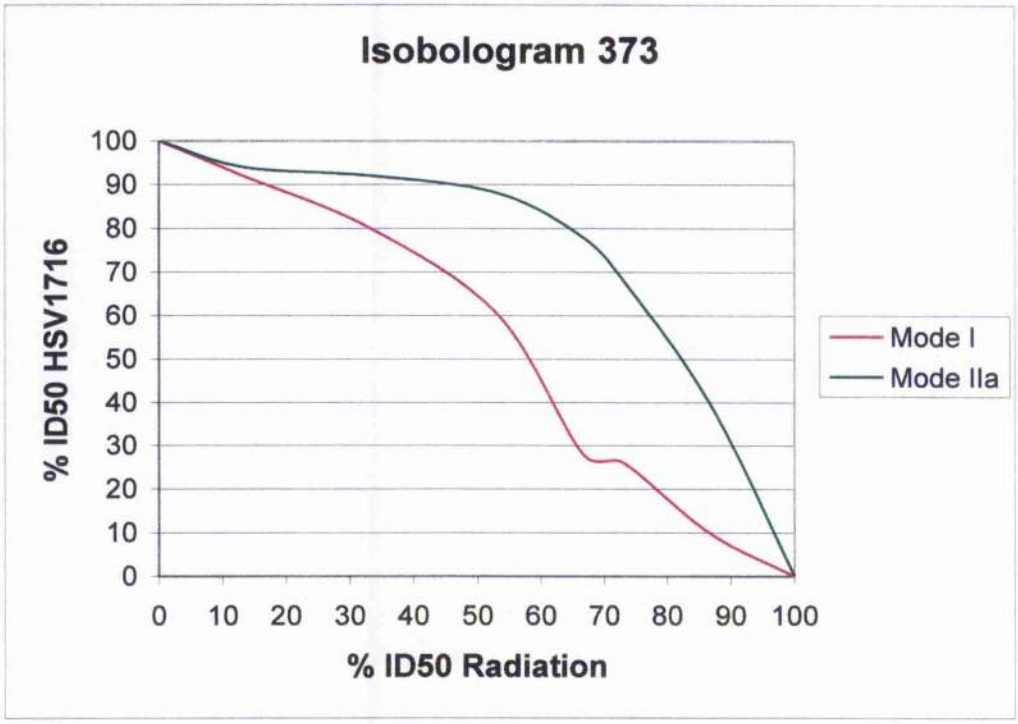


Figure 14.21(j); *Mode I and Mode IIa lines as part of the Isobologram*

***Mode IIb*;**

When a dose of HSV1716 causes a reduction in cell viability there remains an increment in effect to be produced by radiation to produce a total 50% reduction in cell viability. Points on this iso-effect line are calculated in the manner described below.

For example – As indicated in the figure 14.21(k) below, HSV1716 MOI 0.1, results in a 30% cell kill by day 6 (green arrow). Therefore radiation would have to generate a further reduction of 20% cell viability (purple arrow) to reach ID_{50}.

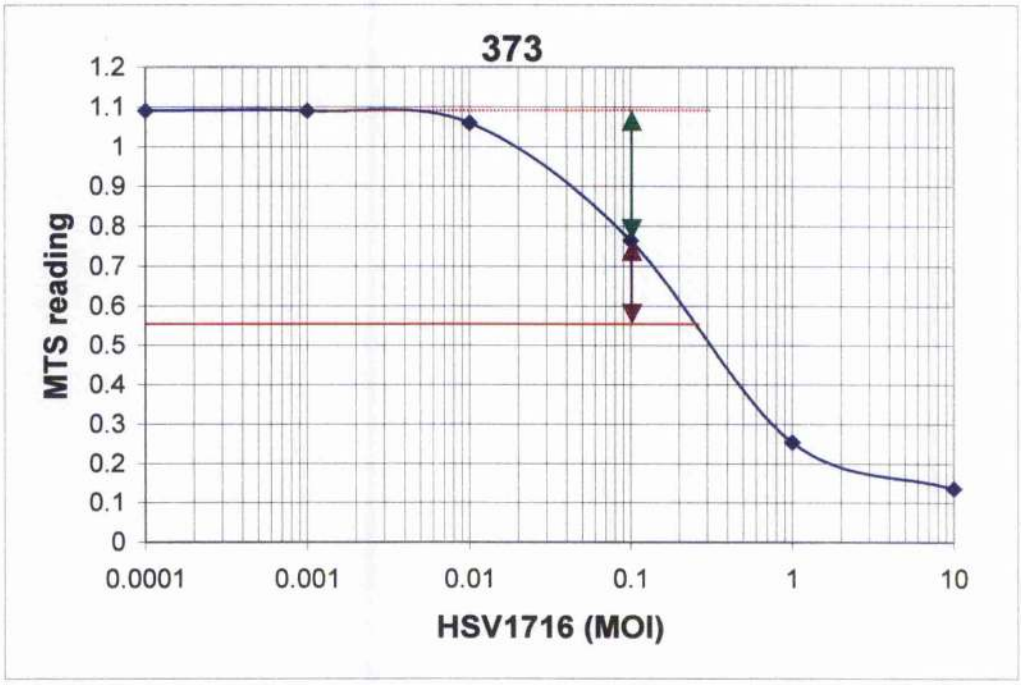


Figure 14.21(k); *Does response curve of HSV1716 in 373 cells*

This was repeated using various multiplicity of infection of HSV1716. The results are detailed in table 14.21(vi) below.

MOI HSV1716	% kill from HSV1716	% required from DXT
0.01	3	47
0.04	17	33
0.07	25	25
0.1	30	20
0.2	43	7

Table 14.21(vi); *Generation of Mode IIb points*

Using the dose response curve obtained for radiation the above percentages required to reach 50% cell viability when combined with HSV1716 can be converted into an absolute dose of radiation (Gy).

For example;

From the HSV1716 dose response curve an MOI of 0.1 causes a 30% reduction in cell viability. Radiation therefore is required to generate a further 20% reduction (green arrow) in cell viability to reach an ID_{50}.

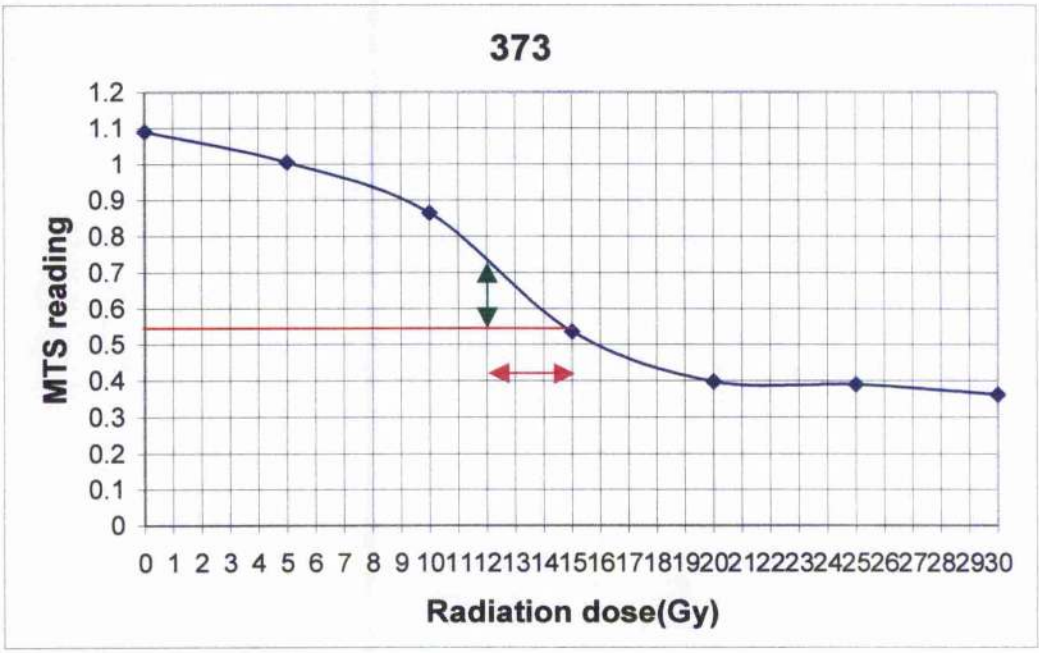


Figure 14.21(l); *Dose response curve of IR in 373 cells*

The pink arrow represents the dose of radiation required to make up the short fall, which in this instance is 3Gy. This is a point on the mode IIb line. This calculation was repeated for each of the multiplicities of infection. The absolute value measured in Gy is calculated as a percentage of the radiation dose that generated 50% reduction in cell viability, ID_{50} calculated as 15Gy. The MOI of HSV1716 is also expressed as a percentage of the MOI of HSV1716 required to generate 50% reduction in cell viability. The calculations are detailed in table 14.21(vii) below.

MOI HSV1716	**Virus dose as as % of ID_{50}**	**Required radiation dose Gy to achieve 50% kill**	**Radiation dose expressed as % of ID_{50}**
0.01	4	13.5	90
0.04	16	6	40
0.07	28	4	26.7
0.1	40	3	20
0.2	80	1.5	10

Table 14.21(vii); *Mode IIb line data*

The mode IIb line is then plotted on the isobologram together with the Mode I and Mode IIa data shown in figure 14.21(j).

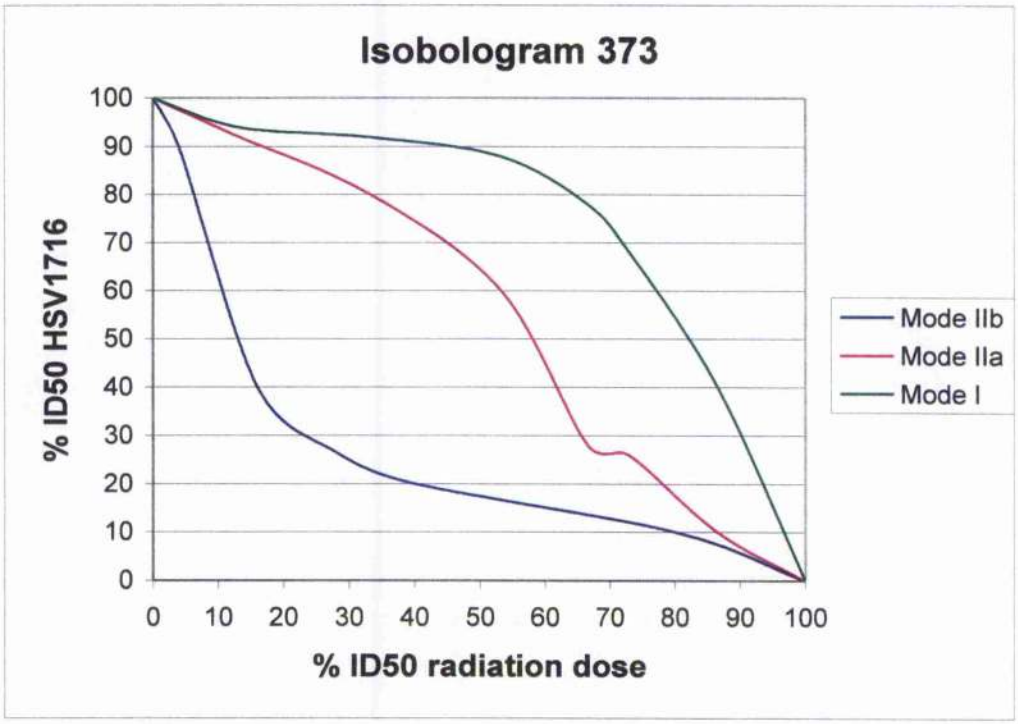


Figure 14.21(m); *Isobologram plot with Mode I, IIa and IIb lines plotted*

It is worth noting that if radiation followed first order kinetics then the mode IIa line would be identical to the mode I line. If HSV1716 infection and ionising radiation both followed first order kinetics then all three iso-effect lines would be straight lines between the two 100% points on the graph above.

When the iso-effect curves diverge as above (figure 14.21m) the area between the solid blue line and the green line is the envelope of additivity. An experimental plot in between the blue and green line would suggest that the combination is non-interactive (additive). An experimental plot above the green line would indicate an infra-additive relationship between HSV1716 and radiation and a plot below the blue line indicates a supra-additive relationship.

The results obtained from the combination experiments, detailed in table 14.21(ii), are plotted onto the isobologram graph on figure 14.21(n) as red squares.

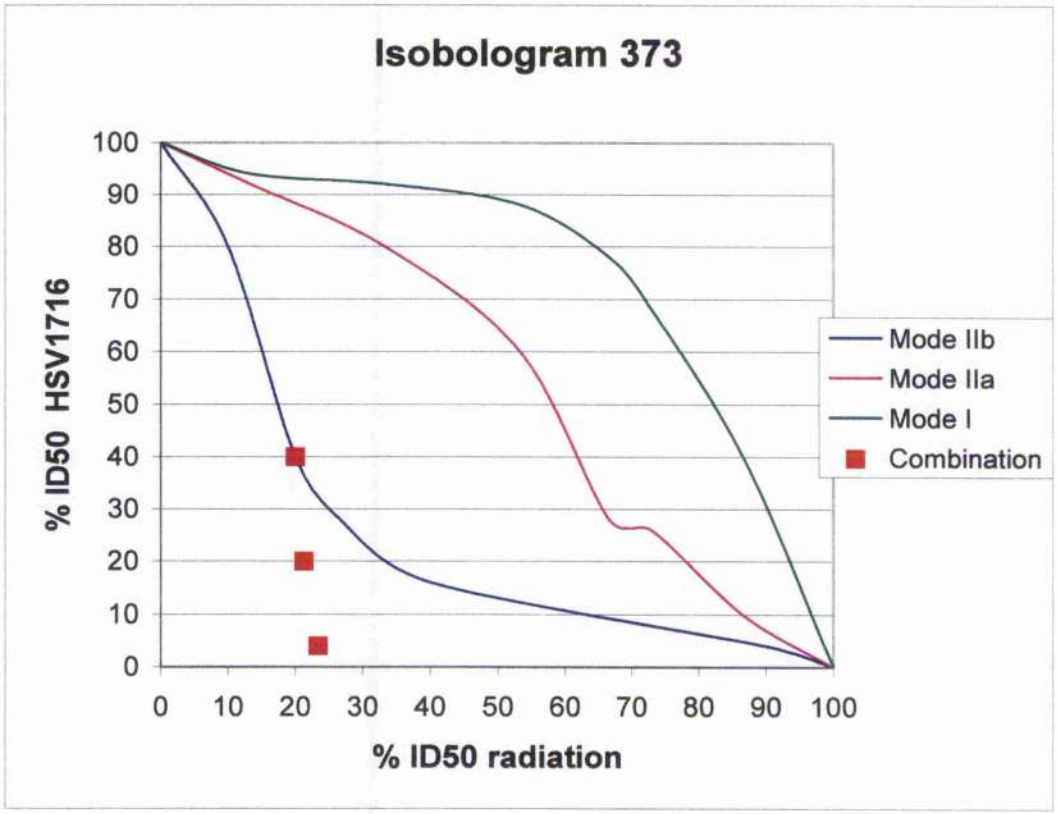


Figure 14.21(n); *Isobologram plot with results of combination experiments*

In the case of HSV1716 infection and radiation in 373 cells at day 6 there appears to be a supra-additive relationship. One point does lie on the Mode IIb line of the envelope of additivity whilst two of the points lie clearly within the region of supra-additivity. These suggest that the cell kill from the radiation and HSV1716 infection is at least additive if not synergistic.

14.22 Isobologram Analysis of the Relationship Between HSV1716 and Ionising Radiation in MOG Cells

The relationship between ionising radiation and HSV1716 lytic infection with respect to cell kill in MOG cells at 6 days was investigated using the isobologram analysis. Firstly it was necessary to demonstrate the dose response curves for IR and HSV1716 independently in MOG cells. The cells were seeded at a density of $2.5x10^3$ cells in 100µl of medium per well of a 96 well plate. The six day ionising radiation and HSV1716 dose response curves are detailed below in figures 14.22(a) and 14.22(b).

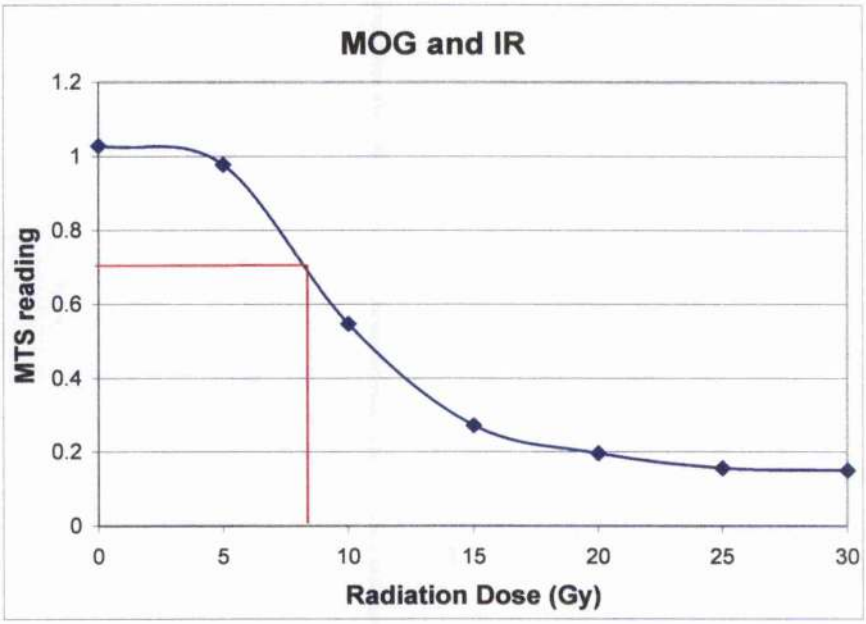


Figure 14.22(a); *Dose response curve of ionising radiation in MOG cells*

***Figure 14.22(b)**; Dose response curve of HSV1716 in MOG cells*

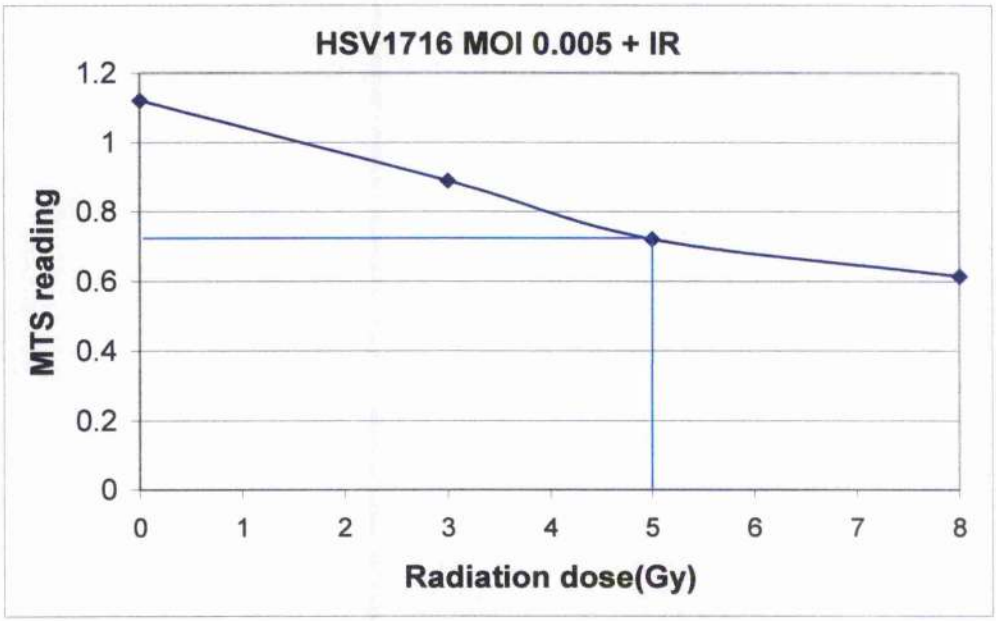
From these figures the IR and HSV1716 ID_{30} values were determined. ID30 values were taken because the results of the preliminary experiments where HSV1716 and IR were combined in MOG cells did not achieve a 50% reduction in cell survival by day 6.

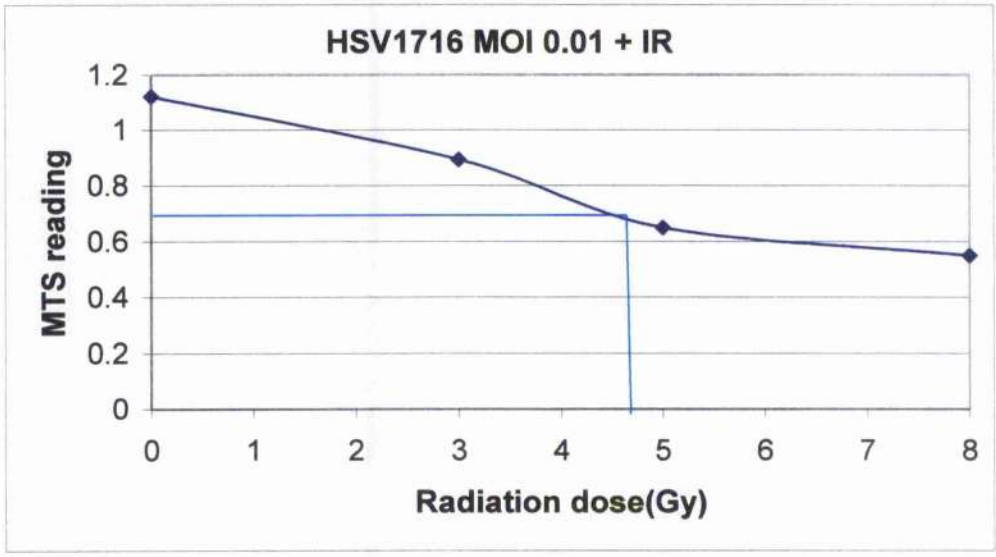
The control MTS reading for MOG cells at day 6 was 1.04.
The 30% reduction in the control MTS value is 0.7.
From figure 14.22(a) the value of IR that results in 30% reduction in cell kill compared to the control is 8Gy.
From figure 14.22(b) the value of IR that results in 30% reduction in cell kill compared to the control is MOI 0.03.

Combination experiments were designed that combined doses of each modality that caused less than 30% reduction in cell viability by day 6. Each well of a 96 well plate was seeded with $2.5x10^3$ cells in 100µl of medium. The cells were irradiated and 1 hour later inoculated with HSV1716.

From these experiments the point of 30% reduction in cell viability is calculated. This is demonstrated in figures 14.22 (c) and (d).

Figure 14.22(c) and (d) *– The solid blue line indicates the MOG cell response to the various concentrations of HSV1716 and radiation. The turquoise line indicates the dose of radiation when combined with the MOI that generated 30% cell kill.*





The results are indicated in table 14.22(i) and expressed as a percentage of the ID30 calculated for IR (8Gy) from figure 14.22(a) and for HSV1716 (MOI 0.03)

from figure 14.22(b). These results are converted into a percentage of the radiation and HSV1716 ID_{30} value calculated from figures 14.22(a) and (b) and are presented in table 14.22(i) below.

MOI HSV1716	% of HSV1716 ID_{30}	Radiation Dose required for 30% cell kill	% of Radiation ID_{30}
0.005	17	5	62.5
0.01	33	4.6	57.5

Table 14.22(i); *Results of combination experiments also expressed as a percentage of the* ID_{30}

The mode I, IIa and IIb lines were constructed in the same way as described in Experiment 14.21. The isobologram analysis of combination experiments combining HSV1716 and ionising radiation is indicated below in figure 14.22(e).

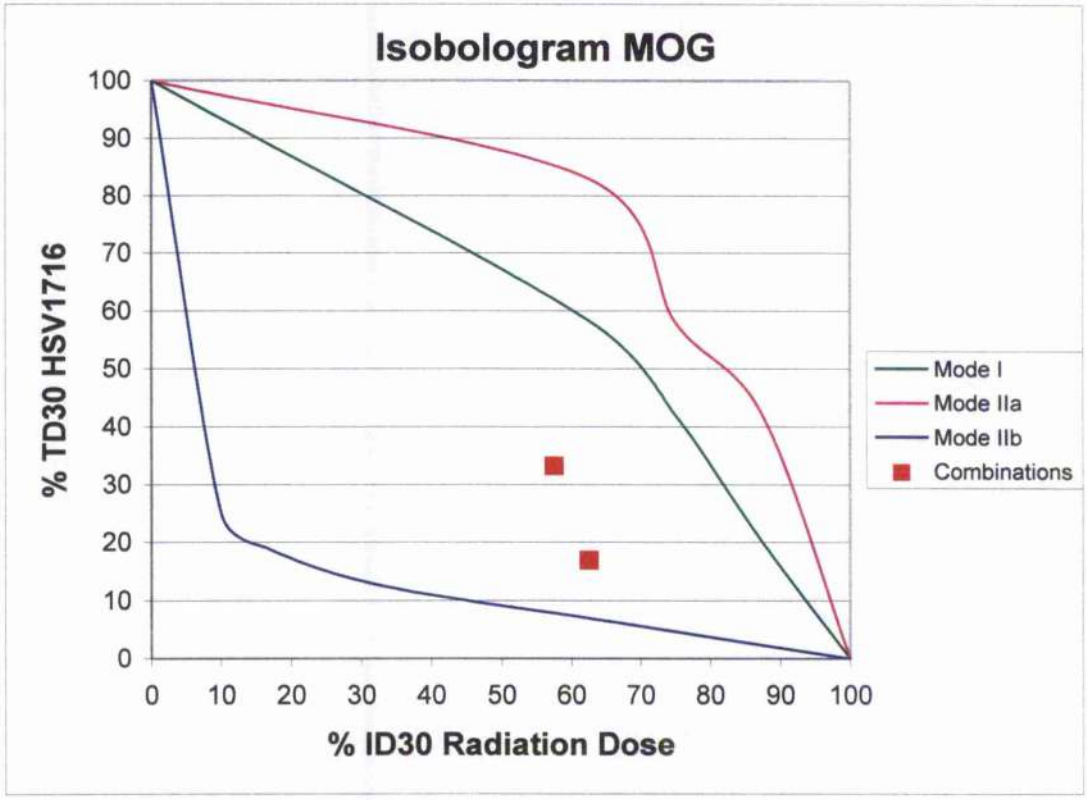


Figure 14.22(e); *Isobologram plot with results of combination experiments*

The area between the mode IIa and mode IIb lines indicate the envelope of additivity. In the case of HSV1716 lytic infection and ionising radiation in MOG cells at day 6 there appears to be an additive relationship with both plots (red squares) from combination experiments lying with in the envelope of additivity.

14.23 Summary of Results

Preliminary experiments using the Promega MTS cytotoxicity assay indicated that it was suitable to detect cell kill generated by ionising radiation and HSV1716. It is demonstrated that there is a linear relationship between the actual cell number and the MTS colorimetric reading. Subsequent experiments used cell densities within this linear range.

BHK and 3T6 cells, used extensively in the laboratory, were characterised with respect to the lytic replication of HSV1716. A multicycle growth experiment investigating HSV1716 replication in the human high-grade glioma cell line, MOG, demonstrated less efficient replication of HSV1716 compared to wild type $HSV17^{+}$ under the same experimental conditions. It was proposed that by using the MTS cytotoxicity assay additional cell kill could be realised when HSV1716 was combined with ionising radiation, in MOG cell line cultures.

The density of the cells seeded was crucial to allow exponential cell replication over the course of the experiment. Densities were required which enabled the cells to grow exponentially for the 6-7 day duration of the experiment, allowing cell kill caused by ionising radiation to be detected by the MTS assay.

Initial experiments established that seeding densities of $2\text{-}3x10^{3}$ cells in 100µl of medium per well of a 96 well plate was optimal to allow cell viability experiments following exposure to various doses of ionising radiation. Higher densities, in the order of $1x10^{4}$ cells in 100µl per well of a 96 well plate, resulted in BHK and 3T6 cells becoming confluent and quiescent too early in the course of the experiment to allow the demonstration of cell kill generated by ionising radiation. In the case of the MOG cells, such high density cultures resulted in a reduction in cell

viability, attributed to the reduction in essential nutrients within the growth medium, which would have masked any cell kill due to ionisation.

Some cytotoxicity was noted following the irradiation of various cell lines in culture, though it was minimal. It appeared that the cells *in vitro* were radio-resistant. Hall and Brenner. (1994) demonstrated that lower dose rates generated reduced cell kill due to sub-lethal damage being repaired during protracted delivery of the radiation. By reducing the distance between the source and our tissue culture cells the dose rate was increased by a factor of four. The maximum dose rate possible using the available Alcyon II Teletherapy Unit did not however generate a reduction in cell viability when compared to the standard dose rate.

Experimental conditions were required that would enable HSV1716 to exert an *in vitro* reduction in cell viability without eradicating all the cells in the monolayer before ionising radiation could exert an influence. Using initial cell densities of between $2\text{-}3x10^3$ cells per 100µl, previously shown to be optimal for detection of radiation cell kill at 5-7 days, various MOIs of HSV1716 and $HSV17^+$ were investigated. This cell density ensured that the cells remained in a replicative state, and permissive to HSV1716. It was demonstrated that any infection greater than an MOI of 0.1 would quickly generate lytic replication, thus preventing exponential cell growth. An MOI of 0.1 was adopted for subsequent experiments.

It was necessary to investigate if the treatment of HSV1716 with ionising radiation would diminish its ability to infect cells and undergo lytic replication *in vitro*. Multicycle growth experiments using irradiated HSV1716 did not show a reduction in oncolytic cell kill compared to non-irradiated HSV1716.

Experiments combining ionising radiation and HSV1716 were then embarked upon. Previous experiments had demonstrated that the MOG and BHK cells were permissive to HSV1716 and additional cell kill generated by ionising radiation would be difficult to detect. Initial combination experiments sought to investigate if ionising radiation would promote cell kill due to HSV1716 lytic replication in non-permissive 3T6 cells. Ionising radiation did generate some delay in

exponential cell growth but did not appear to increase the permissivity of HSV1716 in replicating 3T6 cells and promote viral lytic replication.

Further experimentation demonstrated that the human malignant glioma cell line 373 was less permissive to HSV1716 infection than MOG cells. This was confirmed by a multicycle growth experiment where the yield of HSV1716 obtained from the 373 cells by 72 hours was reduced compared to the yield of wild type 17^{+}. By using 373 cells it was hoped that there would be incomplete cell kill due to HSV1716 alone by day 6-7, thereby enabling additional cell kill from ionising radiation to be detected by the MTS assay. Combination experiments delivering ionising radiation 6 hours after virus inoculation failed to demonstrate additional cell kill by day 7. HSV1716 and $HSV17^{+}$ alone, were capable of generating complete cell kill.

In light of the demonstration by Blank *et al.* (2002) of enhanced cell kill when ICP34.5 null HSV was delivered 1 hour after monolayer irradiation a modified protocol was adopted. Five Gray radiation 1 hour prior to virus infection (MOI 0.1), in both 373 and MOG cell lines, resulted in additional cell kill at day 7, when compared to either modality given in isolation. This additional cell kill was mirrored at day 6 in experiments combining HSV1716 and a higher dose of radiation, 20Gy, in MOG and 373 cells at day 6.

Large fraction sizes are rarely used in clinical radiotherapy practice, and as ionising radiation did not appear to compromise the replication and lytic capability of HSV1716, experiments were conducted to investigate any additional cell kill when the dose of radiation was fractionated. Ten Gray of radiation was either delivered as a single dose or fractionated into 2Gy fractions over 5 days. Additional cell kill was achieved by combining the radiation 1 hour prior to virus inoculation but there was no difference in the reduction in cell viability when 10Gy was delivered as a single fraction or delivered over the course of 5 days in 2Gy fractions.

A multicycle growth experiment was performed to investigate if irradiating cells prior to infection with HSV1716 would result in an increased titre, which may account for the additional cell kill when the two modalities are combined. Radiation of the investigated cell lines did not result in enhanced viral replication with the same viral titre obtained as with the non-irradiated cells.

Following the detection of additional cell kill of cells *in vitro* when HSV1716 and ionising radiation were combined analysis was performed to show if the increase was additive or synergistic. As the dose response curves of these two modalities did not follow first order kinetics then it was not possible to state if the observed reduction in cell viability when the two modalities were combined was greater than the sum of the two modalities used independently. Isobologram analysis was used to confirm the relationship between HSV1716 and ionising radiation.

The isobologram of 373 cells demonstrated a region of additive cell kill between the Mode I and Mode IIb lines. Plots below the Mode IIb line indicate a synergistic relationship between HSV1716 and ionising radiation. Three MTS assay experiments, combining HSV1716 one hour after ionising radiation at doses known to generate less than 50% cell kill independently, were then plotted onto the isobologram. Two plots from these experiments were seen to lie below the Mode IIb line, whilst the remaining plot fell exactly on the Mode IIb line. This would suggest that the cell kill relationship between HSV1716 and ionising radiation when 373 cells are irradiated 1 hour prior to inoculation with HSV1716 is synergistic.

The isobologram of MOG cells also demonstrates an envelope of additivity, which lies within the Mode IIa and Mode IIb line. Two MTS experiments, combining HSV1716 one hour after ionising radiation at doses known to generate less that 30% cell kill independently were plotted onto the isobologram. Both plots lie within the region between the Mode IIa and Mode IIb lines and hence the relationship between HSV1716 and ionising radiation under these conditions is additive.

Chapter 5

15 Generation and Characterisation of Stably Transfected Cell Lines Expressing ICP34.5 and ICP34.5-GFP

15.1 Introduction

The reasons for the selective replication phenotype of HSV ICP34.5 null mutants in different cell types and cell states are still under investigation. A better understanding of the complicated processes involved should allow better use of ICP34.5 null mutants in clinical practice. In this section of laboratory research, the strategy was to develop stable cell lines expressing ICP34.5 with the aim of elucidating the mechanism of action of the ICP34.5 in the replication cycle of HSV.

It has been demonstrated (Aranda-Anzaldo *et al.*, 1992) that infection with HSV causes single-strand breaks in the host DNA comparable to those produced by moderate doses (2-5J/m^2) of UV radiation. It has also been shown (Harland *et al.*, 2003) that following HSV infection there is rapid influx of PCNA to the cell nucleus, which the authors postulated is recruited to repair the DNA damage. They showed that at early times in infection, ICP34.5 and PCNA co-localise at discrete sites within the nucleus, and hypothesised that ICP34.5 interacts with the recruited PCNA, switching its function from repair to DNA replication.

In this project the purpose was to engineer cells to express ICP34.5 in order to investigate if this would interact with PCNA in the cell nucleus to allow initiation of DNA replication. It was proposed to study, in the absence of a viral infection, what effect ICP34.5 has on cell cycle regulation. However, in non-dividing cells, in the absence of viral infection, there would be no recruitment of PCNA to sites of DNA damage, and therefore possibly very little PCNA bound to cellular DNA. It was intended therefore to investigate the effect of ionising radiation (IR) on these ICP34.5 expressing cells. The hypothesis being that IR would cause DNA

damage to which PCNA would be recruited and that, in resting cells, the expressed ICP34.5 could interact with this PCNA to allow DNA replication. It was postulated that any aberrant DNA replication following the IR would be manifest as continued cell division when normally cell division ceases until any DNA damage had been repaired.

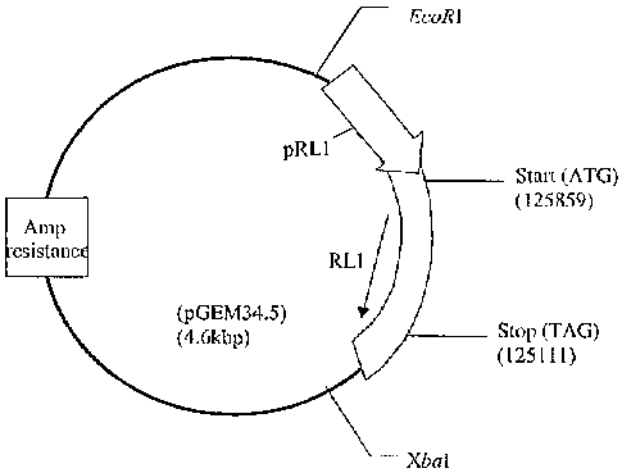
It was proposed to engineer plasmids with a Zeocin resistance gene to introduce ICP34.5 with or without a GFP marker and to use these plasmids to transfect cells. Transfected cells (expressing ICP34.5 or ICP34.5-GFP) could be selected by exposing them to Zeocin. It was intended to study the effect of stable expression of ICP34.5 on the phenotype of the cells in different cell cycle states. It was hoped that if a stably expressing cell line could be generated it should be possible to overcome (complement) the block in replication of HSV1716 in normally non-permissive 3T6 cells. It was also intended to study co-localisation of PCNA and ICP34.5 at different stages in the cell cycle and, if possible, the effect of radiation on ICP34.5 expressing cells compared to control cells.

The strategy was to construct a plasmid containing the Zeocin resistance gene and the RL1 gene, expressing ICP34.5, under the RL1 promoter and a similar plasmid with RL1 and a reporter gene, to express ICP34.5 fused to the green fluorescent protein. Using the constructed plasmids, the aim was to generate 3T6 and BHK cell lines that were stably transfected and expressing ICP34.5 or ICP34.5-GFP. Following the generation of stably transfected 3T6 and BHK cell lines the aim was to characterise them and to investigate if the expression of ICP34.5 or ICP34.5-GFP in normally non-permissive 3T6 cells could support a full replication cycle of the ICP34.5 null mutant HSV1716. In addition it was proposed to investigate whether 3T6 cells expressing ICP34.5 could be put into a state of growth arrest and, in the growth arrested state, determine the distribution of PCNA.

15.2 Construction of Zeocin Resistant Plasmids pc-34.5 and pc-34.5-GFP

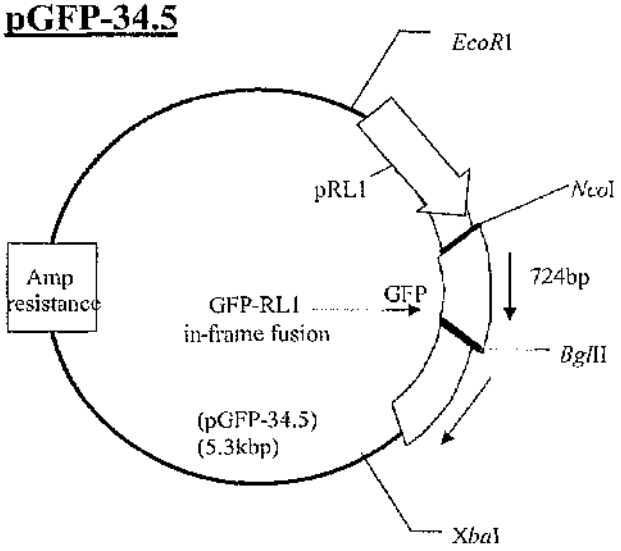
The initial aim of this piece of research was to construct plasmids containing a Zeocin resistance gene expressing ICP34.5 or ICP34.5-GFP under the control of RL1 promoter. The reagents used to do this were pGEM34.5 (McKie E *et al.*, 1994), pGPF-34.5 (Paul Dunn, Ph.D. Thesis, 2004) and pcDNA4/*myc*-His (Invitrogen) illustrated below.

pGEM34.5



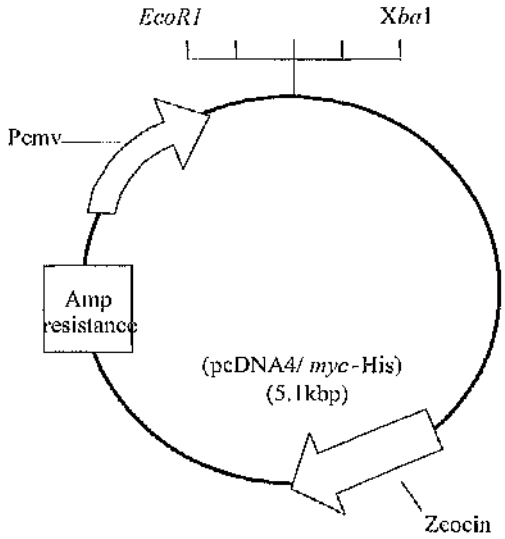
The plasmid pGEM34.5 (McKie E *et al.*, 1994) consists of the 1.46kb *Ahu*I/*Rsa*I restriction enzyme sub-fragment of HSV-1 *Bam*III k, (nucleotides 125074 to 126530; Perry LJ & McGeoch DJ, 1998) which contains the entire RL1 ORF, cloned into the *Sma*I site of pGEM-3Z(-) (Promega).

pGFP-34.5



The plasmid pGFP-34.5 (PaulDunn Ph.D. thesis 2004) was generated by introducing the GFP gene excised from the pGEFP-C3 plasmid (Clonetech) cloned in-frame between the RL1 promoter and the RL1 gene in pGEM34.5 (McKie E *et al.*, 1994).

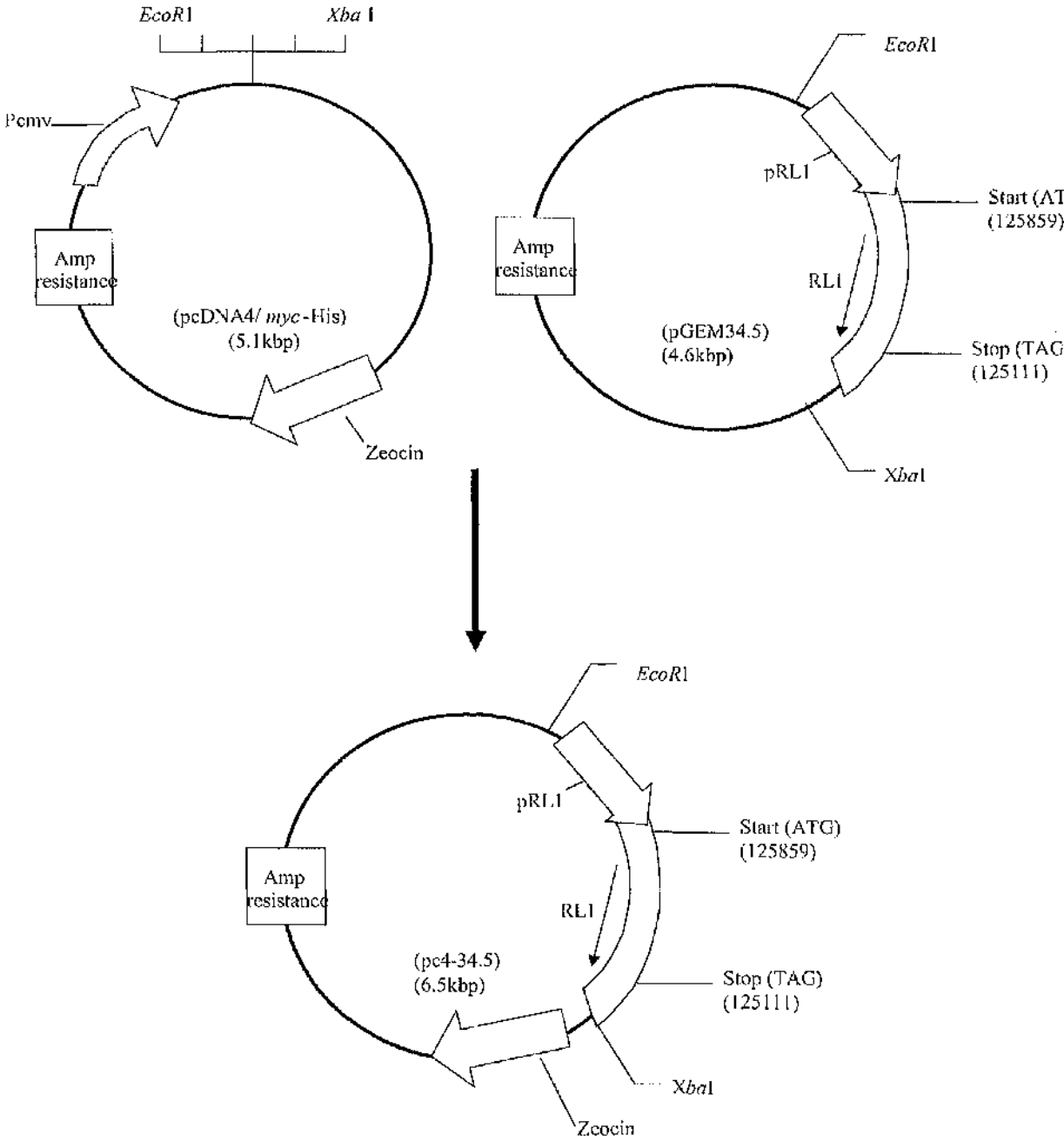
pcDNA4/*myc*-His



The plasmid pcDNA4/*myc*-His was supplied by Invitrogen.

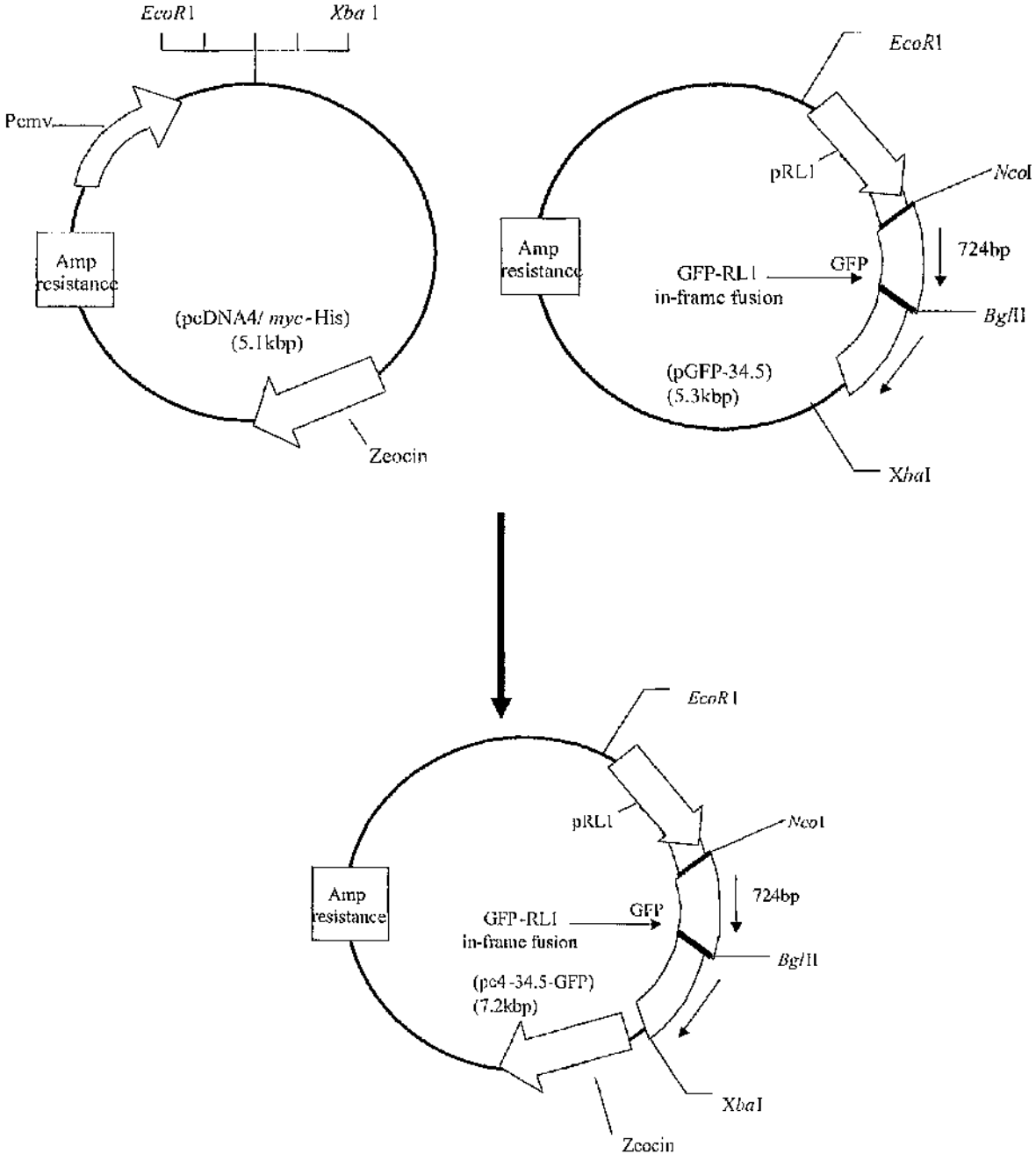
The cloning strategy involved excising the RL1 gene encoding ICP34.5 from the plasmid pGEM34.5, or GFP-RL1 in-frame fusion gene from pGFP-34.5 and inserting it into the backbone of plasmid pcDNA4/*myc*-His containing a Zeocin resistance gene.

Cloning strategy to generate pc4-34.5:



1. pGEM34.5 digested with *EcoR*I and *Xba*I, run on an agarose gel and the ~1.5kb fragment, containing the RL1 gene and promoter sequences, purified.
2. pcDNA4/Myc-His digested with *EcoR*I and *Xba*I.
3. RL1 gene fragment ligated into the pcDNA4/Myc-His backbone to yield pc4-34.5.

Cloning strategy to generate pc4-34.5-GFP



1. pGFP-34.5 digested with EcoRI and XbaI, run on an agarose gel and the (~2.2kb) fragment, containing the RLI promoter the EGFP and the RL1 sequences, purified.
2. pcDNA4/Myc-His digested with EcoRI and XbaI.
3. RL1-GFP fusion gene fragment ligated into the pcDNA/Myc-His backbone to yield pc4-34.5-GFP.

15.3 Western Blot Characterisation of Stably Transfected Cell Lines

Plasmid DNA (pc4-34.5 and pc4-34.5-GFP) was extracted, linearised and used to transfect 3T6 and BHK cells. Various quantities of plasmid and lipofectamine 2000 reagent (Invitrogen) were added to actively dividing cell cultures. The cell lines were grown and serially split. After the first passage the cells were incubated in media containing Zeocin. This allowed only those cells successfully transfected with plasmid expressing the Zeocin resistance gene to survive.

Notwithstanding the Zeocin resistance of the transfected cells, it was necessary to demonstrate expression of ICP34.5. Published data from other groups (Mao H *et al*,. 2003) suggested that constitutive expression of ICP34.5 may be toxic to certain cell lines, favouring the selection of cells with continued Zeocin resistance but loss of ICP34.5 expression. Although the levels of protein expression induced by the weak RL1 promoter are generally considered to be low it was hoped that sufficient ICP34.5 might be expressed to allow detection by Western blotting. Additionally, it was hoped that in confluent 3T6 cells, which are normally non-permissive for HSV1716 replication, the constitutive expression of ICP34.5 might complement the virus mutation. If this could be demonstrated it would show that the plasmid expressed ICP34.5 was functionally equivalent to the virally expressed protein.

A Western blot to detect ICP34.5 (figure 15.3) was performed with extracts of ICP34.5 and ICP34.5-GFP expressing BHK and 3T6 cells compared to extracts from normal 3T6 and BHK cells. Additionally, to allow comparison of plasmid-expressed and virally-expressed ICP34.5, extracts of the same cells infected with HSV17^{+} and HSV1716 were also included in the blot.

3T6

Extracts of 3T6 cells, following a 16 hour infection with HSV17^{+} (figure 15.3, lane 3), show a typical Western blot pattern with the 137 rabbit polyclonal antibody against ICP34.5. This polyclonal serum, in addition to detecting the 36kD ICP34.5, also consistently binds to a 70kD species, thought to be a virally encoded protein and a 21kD protein thought to be the product of the HSV late

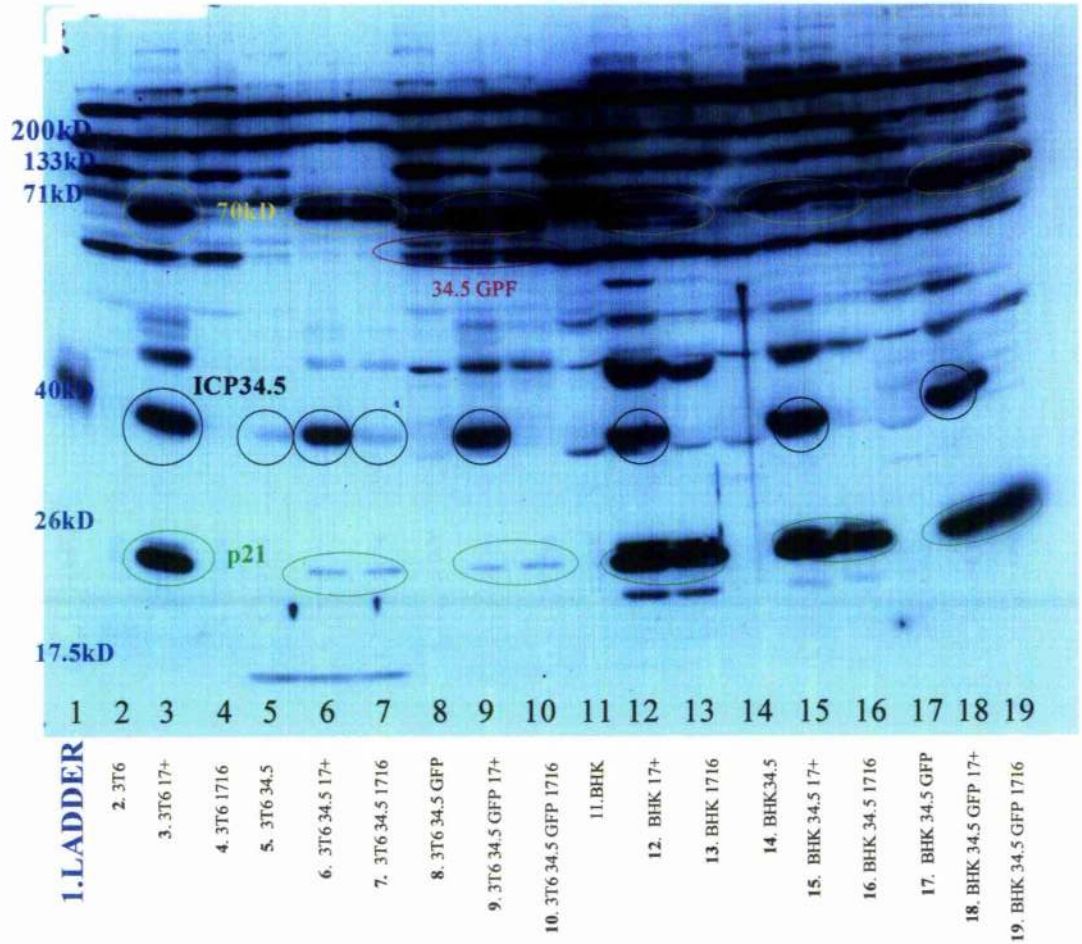
gene, U_S11 (Harland J *et al.* 2003). None of these bands are seen in HSV1716 infected 3T6 cells (lane 4), where lack of ICP34.5 expression results in blockage of viral DNA replication and failure to express late proteins. The location of the ICP34.5 band in the $HSV17^+$ infected track provides a marker to enable identification of ICP34.5 protein expression in the stably transfected cell line 3T6-34.5 (lane 5). The ICP34.5, 70kD and 21kD bands in 3T6-34.5 cells following infection with $HSV17^+$ (lane 6) or HSV1716 (lane 7) are also present. The ICP34.5 bands due to viral expression (lanes 3 and 6) are much more intense than from the stably transfected cells (lanes 5 and 7). There is clear evidence in lanes 8, 9 and 10, of ICP34.5-GFP expression from the stably transfected 3T6 cells containing the plasmid. The expression of the 70kD protein and the late 21kD protein in HSV1716 infected 3T6-34.5 cells (lane 7) and 3T6-34.5-GFP (lane 10) but not in normal 3T6 cells (lane 4) indicates that the ICP34.5 expressed by the plasmids has overcome the normal block in HSV1716 replication seen in 3T6 cells.

BHK

There is no evidence that the stably transfected BHK cells were expressing ICP34.5 or ICP34.5-GFP. The only BHK samples with ICP34.5 bands were those infected with $HSV17^+$ (lanes 12, 15 and 18) and there was no sign of an ICP34.5-GFP band in the BHK-34.5-GFP cell extracts (lanes 17, 18 and 19). Also, because BHK cells are fully permissive for HSV1716 and consequently express the 70kD and 21kD proteins, it is not possible to infer expression of ICP34.5 from the presence of these proteins in the HSV1716 extracts. Subsequent Western blot analysis of the BHK-34.5 or BHK-34.5-GFP cell lines also failed to demonstrate expression of ICP34.5 or ICP34.5-GFP.

Figure 15.3; *Western blot using the rabbit polyclonal antibody serum 137 against ICP34.5*

Western blot using the rabbit polyclonal antibody serum 137 against ICP34.5 blotted against cells extracts harvested after 16hour infection with HSV17+ or mock infection. The protein marker in lane 1 was Bio-Rad Laboratories Kaleidoscope prestained standards



15.4 Multicycle Growth Experiment Using the 3T6 and BHK Cells Lines Transfected with ICP34.5 and ICP34.5-GFP

Normally, confluent 3T6 cells are unable to support the replication of ICP34.5-null HSV such as HSV1716. The Western blot data indicated that plasmid-expressed ICP34.5 and ICP34.5-GFP were capable of overcoming this block to ICP34.5-null HSV replication in 3T6 cells. To confirm that this was the case and to give an indication of the level of complementation achieved, multicycle growth experiments were carried out. Although BHK cells are known to be fully permissive for ICP34.5-null HSV replication , similar growth experiments were performed to study the growth of $HSV17^+$ and HSV1716 in non-transfected BHK cells.

3T6

The results of a multicycle growth experiment with 3T6, 3T6-34.5 and 3T6-34.5-GFP following infection with $HSV17^+$ and HSV1716 are shown in figure 15.4(a). Characteristically for the 3T6 cells, there was very little evidence of HSV1716 replication (dotted blue line), the 72 hour yield being similar to the input virus dose, whereas they are fully permissive for wild type $HSV17^+$(solid blue line) with a 72 hour yield more than four logs greater than the input virus. HSV1716 replicated more efficiently in the 3T6-34.5 (dotted red line) and the 3T6-34.5-GFP (dotted green line) cells than in the non-transfected 3T6 cells. In 3T6-34.5 and 3T6-34.5-GFP cells the 72-hour yield of HSV1716 was one to two logs higher than the yield of HSV1716 from 3T6 cells. The 3T6-34.5 and 3T6-34.5-GFP cell lines gave lower yields of $HSV17^+$ (solid red and green lines) than 3T6 cells. The 3T6-34.5 and 3T6-GFP cell lines gave approximately 1 log lower yields of $HSV17^+$ (solid red and green lines) than 3T6 cells. The reason why the transformed cells might support wild type HSV infection less well than control 3T6 cells is unclear but is supported by the western blot data showing lower quantities of the late 21K viral protein by 16 hours post infection in the transfected 3T6 cells compared to control 3T6 cells.

The demonstration that 3T6-34.5 and 3T6-34.5-GFP can support HSV1716 replication resulting in elevated virus yields by 24 hours, supports the Western blot data indicating that the 3T6 cells expressing the transfected genes were producing protein capable of complementing the lack of viral expression of ICP34.5. The Western blot data (Figure 15.3) indicating lower levels of ICP34.5 in the transfected cells correlates with the multicycle growth curve data showing that although the replication of HSV1716 was improved it did not reach wild type HSV levels.

BHK

The results of a multicycle growth experiment with BHK, BHK-34.5 and BHK-34.5-GFP following infection with $HSV17^{+}$ and HSV1716 are shown in figure 15.4(b). BHK cells are fully permissive to HSV1716 and the 72hour yields were the same for both transfected and non-transfected BHK cells. Unfortunately, neither the Western blot data nor the virus replication experiment gave any evidence that the BHK cells have been successfully stably transfected.

Figure 15.4(a); *Multicycle virus growth experiment of HSV17^{+} or HSV1716 in 3T6, 3T6-34.5 and 3T6-34.5-GFP cells.*

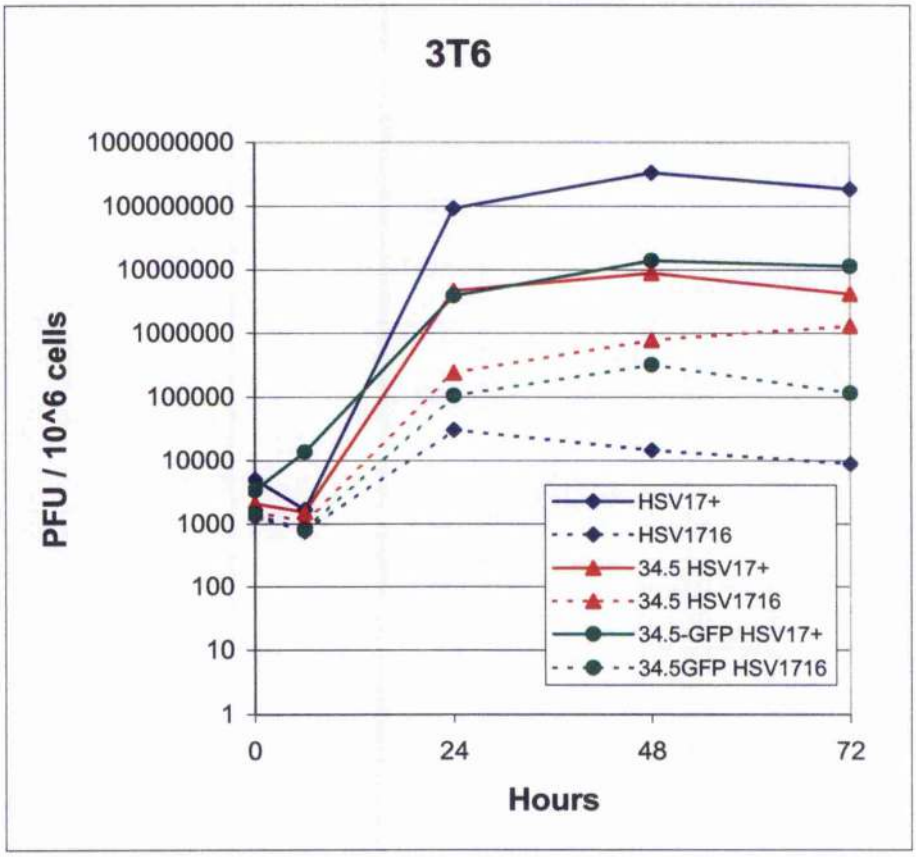
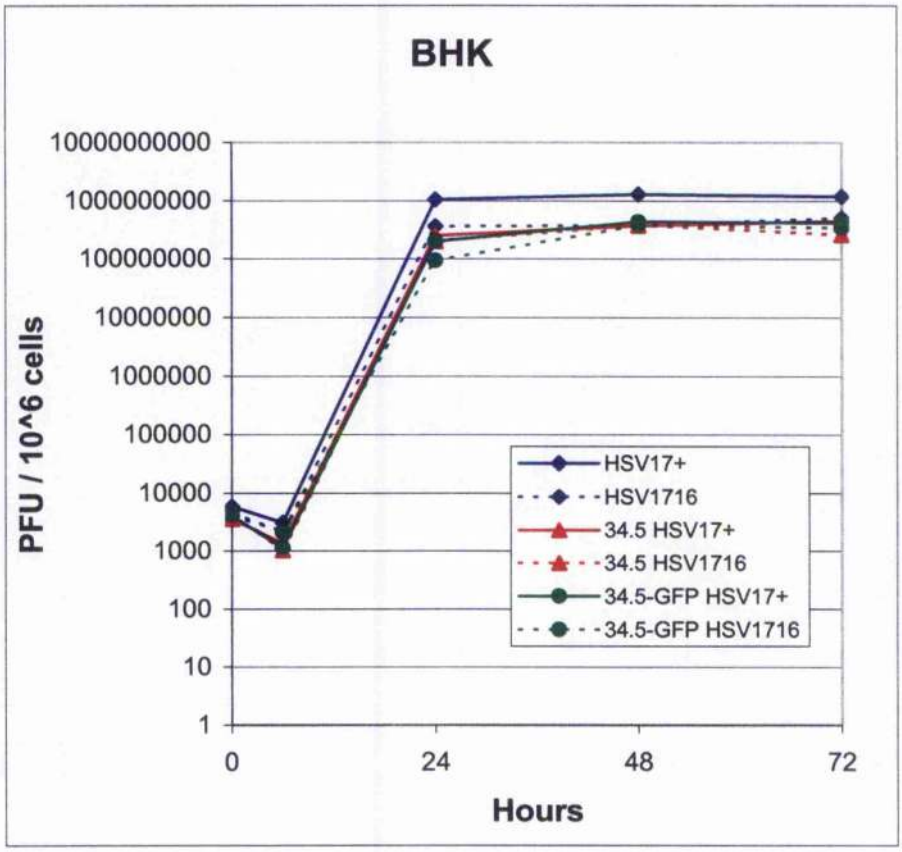


Figure 15.4(b); *Multicycle virus growth experiment of HSV17^{+} or HSV1716 in BHK, BHK-34.5 and BHK34.5-GFP cells.*



15.5 HSV1716 and HSV17^{+} Infection of Semi-Confluent Replicating 3T6, 3T6-34.5 and 3T6-34.5-GFP Cells

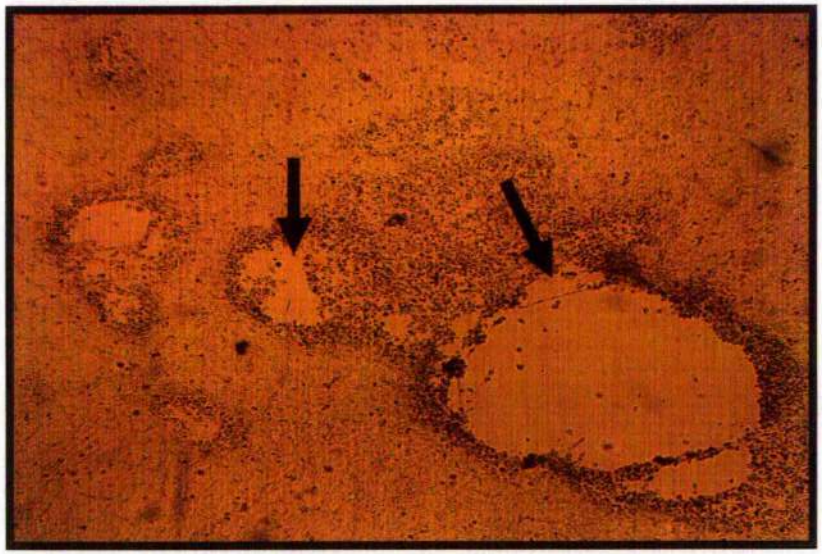
In experiment 15.4 it was shown that, unlike wild type HSV17^{+}, HSV1716 did not replicate in confluent 3T6 cells. It is believed that this is because, when confluent, 3T6 cell division is very efficiently halted by contact inhibition, requiring ICP34.5 to switch the cellular PCNA function to initiate viral DNA replication. When 3T6 cells are actively dividing, however, they are able to support a productive HSV infection in the absence of ICP34.5. In this experiment, approximately 80% confluent 3T6 monolayers were infected. At this density a proportion of the cells would be actively dividing and hence would be permissive to infection with HSV1716.

It has been demonstrated by Western blot analysis that the transfected 3T6 cell lines expressed ICP34.5 and ICP34.5-GFP. The multi-cycle growth curve in experiment 15.4 demonstrated that 3T6 cells expressing either ICP34.5 or ICP34.5-GFP, support the replication of HSV1716 and produce a virus yield higher than that achieved from the non-transfected 3T6 cells.

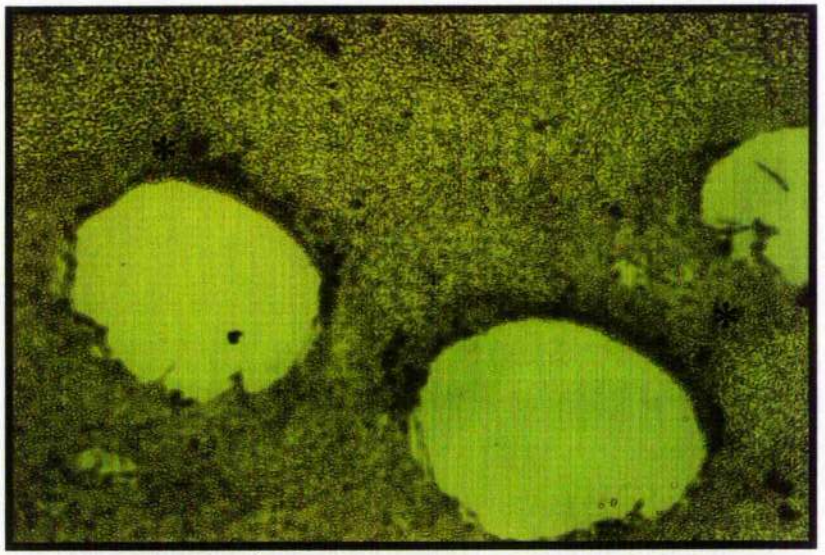
In this experiment, cell monolayers following infection with either HSV17^{+} or HSV1716 were assessed to detect a functional change resulting from the expression of ICP34.5 or ICP34.5-GFP. The intention was to show that the expression of ICP34.5 or ICP34.5-GFP in 3T6 cells could support a lytic HSV1716 infection. To provide a control, untransfected 3T6 monolayers were also investigated following infection with HSV17^{+}. 3T6 and the transfected 3T6 cell lines should be fully permissive to wild type HSV infection.

The results are indicated in figures 15.5.1, 15.5.2 and 15.5.3. Each cell line following either HSV1716 or HSV17^{+} infection is discussed in turn.

Figure 15.5.1; 3T6

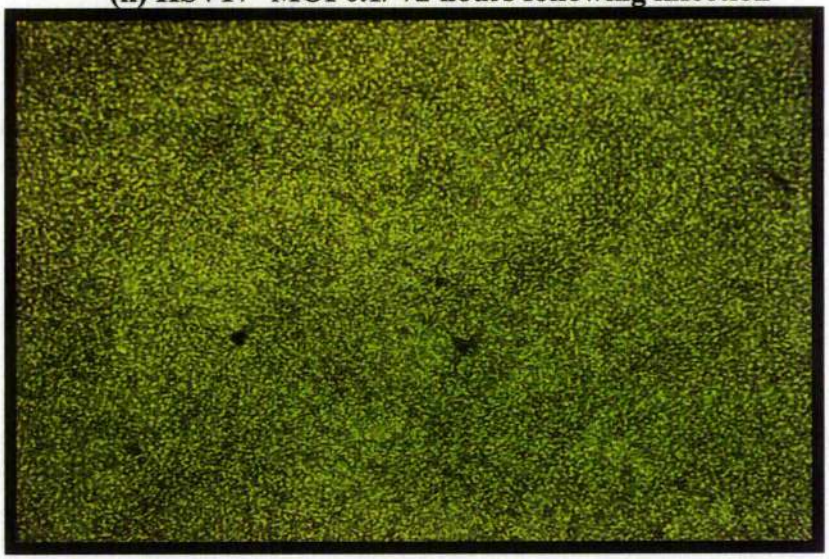


(i) HSV17^{+} MOI 0.1/ 48 hours following infection



(ii) HSV17^{+} MOI 0.1/ 72 hours following infection

(iii) HSV1716 MOI 0.1/ 48 hours following infection



(iv) HSV1716 MOI 0.1/ 72 hours following infection

3T6 cell line

HSV17^{+}

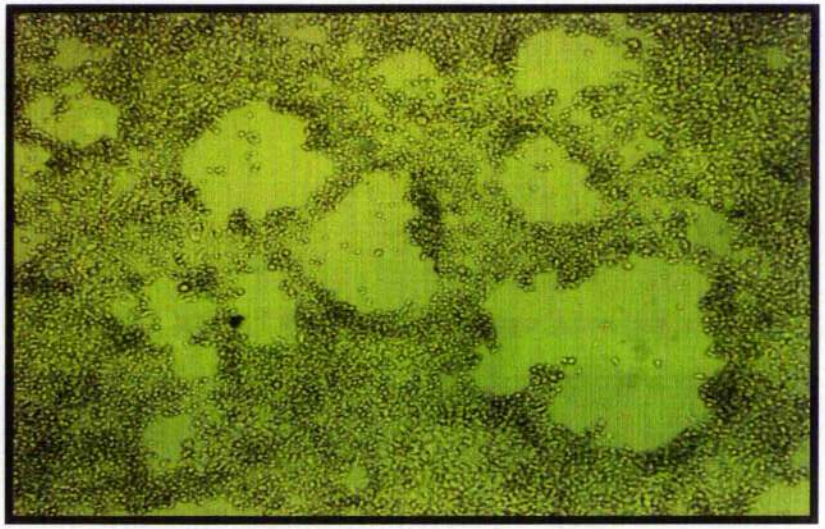
The arrows in figure 15.5.1(i) indicate viral plaques that have formed as a result of lytic replication by 48 hours post virus infection. Figure 15.5.1(ii) shows that the viral infection is more advanced by 72 hours following infection. The cells surrounding the plaques in figure 15.5.1(ii), indicated by *, appear to be infected with virus with lysis imminent.

HSV1716

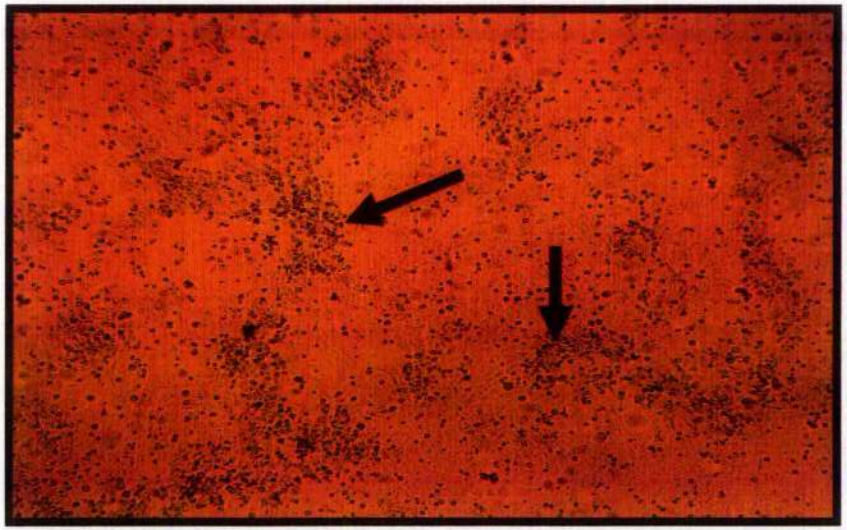
By 48 hours after infection of the semi-confluent 3T6 monolayers with HSV1716 there are apparent foci of infection - figure 15.5.1(iii). These areas are indicated by *. However, possibly as the result of increased contact inhibition of cell division as the monolayers become more confluent, by 72 hours figure 15.5.1(iv) these abortive plaques have disappeared and the cell monolayer appears completely uninfected.

Figure 15.5.2; *3T6-34.5 cell line*

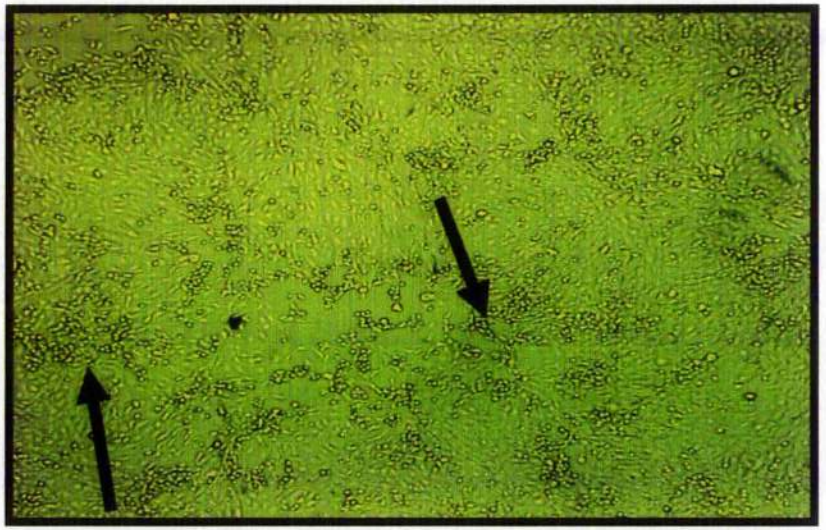
(i) HSV17^{+} MOI 0.1/ 48h hours following infection



(ii) HSV17^{+} MOI 0.1/ 72 hours following infection



(iii) HSV1716 MOI 0.1/ 48 hours following infection



(iv) HSV1716 MOI 0.1/ 72 hours following infection

3T6-34.5 cell line

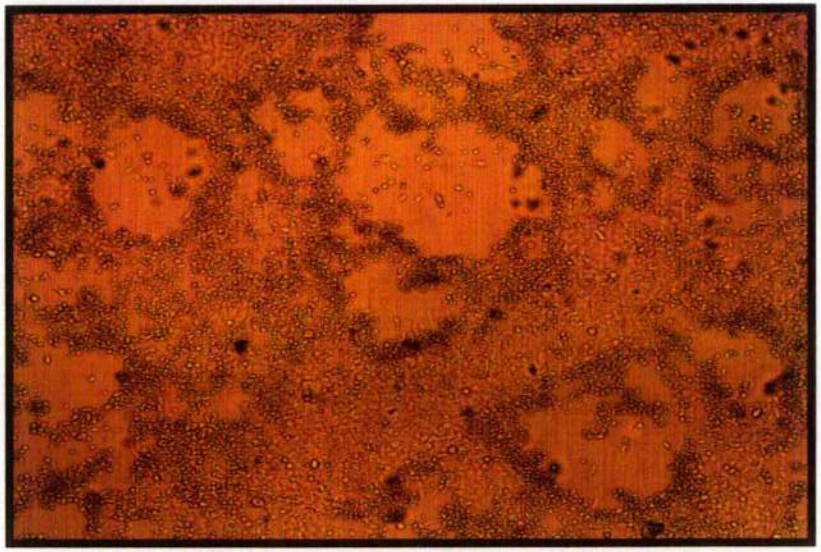
HSV17^{+}

Lytic replication was apparent by 48 hours with large plaques indicative of cell lysis demonstrated in figure 15.5.2(i). By 72 hours the infection had become more extensive with all the cells showing evidence of infection demonstrated in figure 15.5.2(ii).

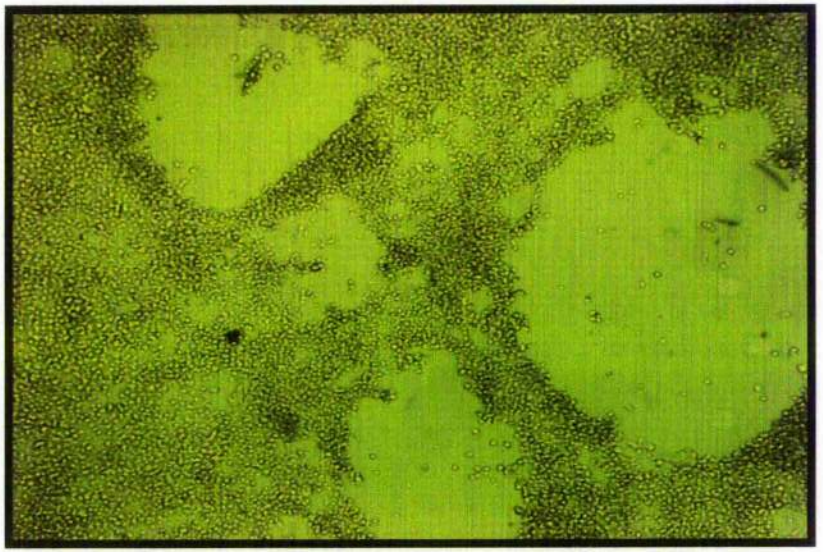
HSV1716

There was evidence of virally infected cells by 48 hours, indicated by the arrows in figure 15.5.2(iii) although there was no obvious plaque morphology demonstrated. By 72 hours there was clear evidence that HSV1716 is replicating in the 3T6-34.5 cells and plaques had begun to develop, indicated by the arrows in figure 15.5.2(iv).

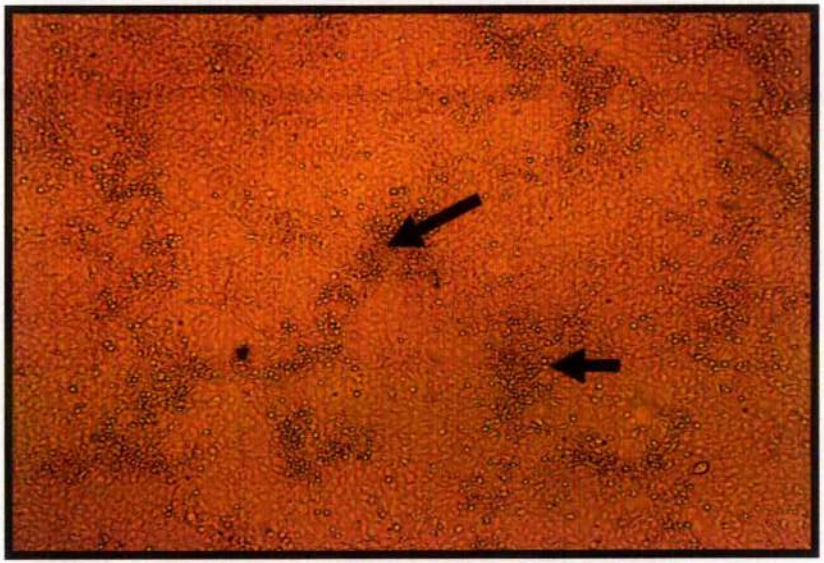
Figure 15.5.3; *3T6-34.5-GFP cell line*



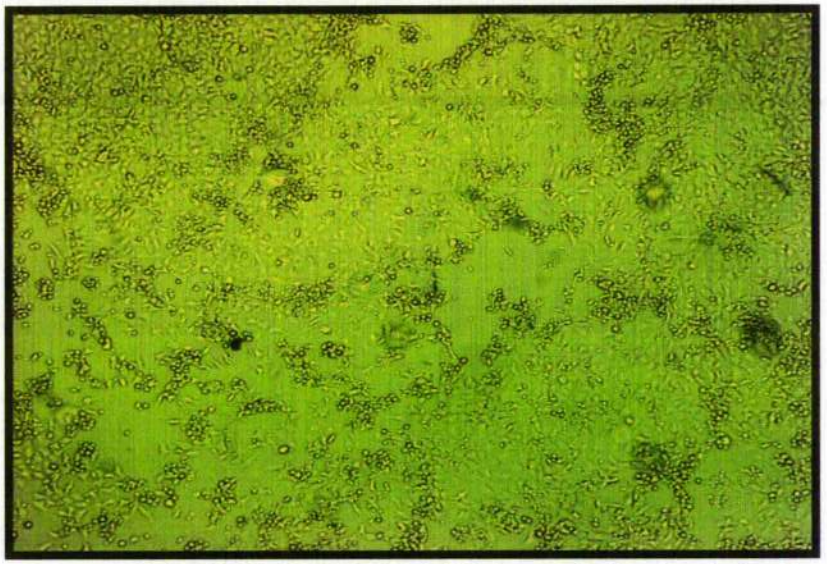
(i) HSV17^{+} MOI 0.1 / 48 hours following infection



(ii) HSV17^{+} MOI 0.1/ 72 hours following infection



(iii) HSV1716 MOI 0.1/ 48 hours following infection



(iv) HSV1716 MOI 0.1 / 72 hours following infection

3T6-34.5-GFP cell line

HSV17^{+}

By 48 hours post virus infection there was clear evidence of lytic viral infection with obvious plaques shown in figure 15.5.3(i). These plaques were more extensive and beginning to coalesce by 72 hours when all of the cells appeared to be infected as demonstrated in figure 15.5.3(ii).

HSV1716

By 48 hours following virus infection although there were no plaques, the cells showed characteristic signs consistent with HSV infection, indicated by the arrow in figure 15.5.3(iii). By 72 hours post infection plaques were present and the majority of the cells had an appearance consistent with virus infection.

The appearance of foci of rounded 3T6 cells seen 48 hours after infection with HSV1716 supports the view that a proportion of the cells, possibly because they were actively dividing, were capable of supporting a productive infection. However, by 72 hours when the cells were fully confluent, there was no further sign of these foci or any other evidence of HSV1716 lytic replication. In contrast, HSV1716 underwent full lytic replication in 3T6-34.5 and 3T6-34.5-GFP cells, producing the characteristic plaque morphology which progressed between 48 and 72 hours. Therefore, it is possible to conclude that the expression of ICP34.5 or ICP34.5-GFP enabled HSV1716 to replicate in and lyse the transfected 3T6 cells. The appearance of the infected monolayers supported the multicycle growth experiment evidence (figure 15.4a) of enhanced viral replication in 3T6 cells expressing ICP34.5.

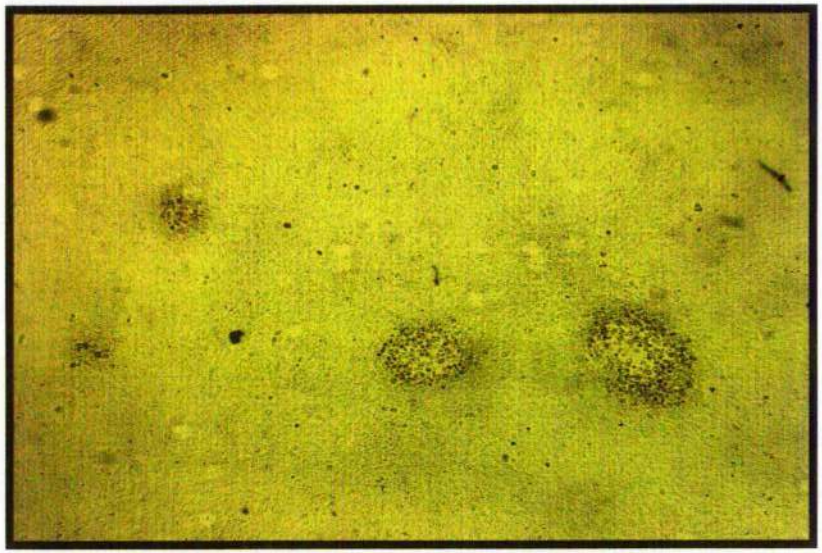
Because the ICP34.5 plasmids complemented the deleted RL1 gene, HSV1716 was capable of replication in the transformed cells, however, it did not replicate as well as $HSV17^{+}$. It is hypothesized that the failure of the plasmid-expressed ICP34.5 to enable HSV1716 to replicate as well as wild type HSV may result from lower levels of ICP34.5 being present; a suggestion supported by the finding in the Western blot that the level of ICP34.5 was lower in the expressing cells than in $HSV17^{+}$ infected extracts.

15.6 HSV1716 and HSV17^{+} Infection of Non-Replicating 3T6, 3T6-34.5 and 3T6-34.5-GFP Cells

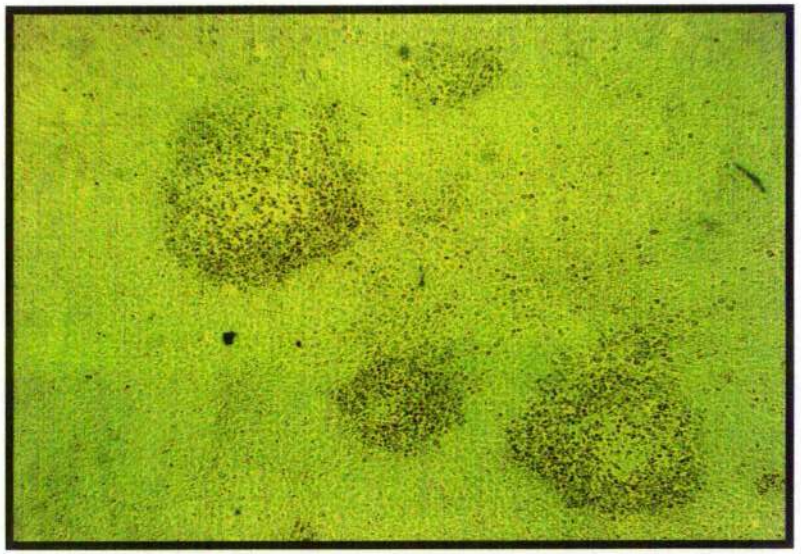
In experiment 15.5, the cell monolayers were 80% confluent and the cells were still actively dividing. It is known that HSV1716 can replicate in dividing 3T6 cells but that it will not replicate in non-dividing 3T6 cells. In this experiment HSV1716 and HSV17^{+} were used to infect 3T6, 3T6-34.5 and 3T6-34.5GFP monolayers that were 100% confluent and had entered a resting, non-replicative state.

The results are indicated in figures 15.6.1, 15.6.2 and 15.6.3. Each cell line following infection with HSV1716 and HSV17^{+} is discussed in turn.

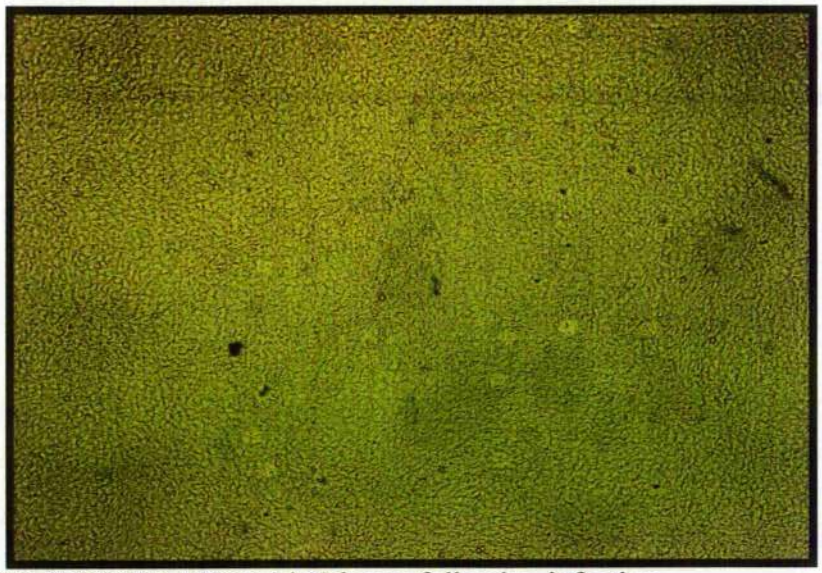
Figure 15.6.1; *3T6 cell line*



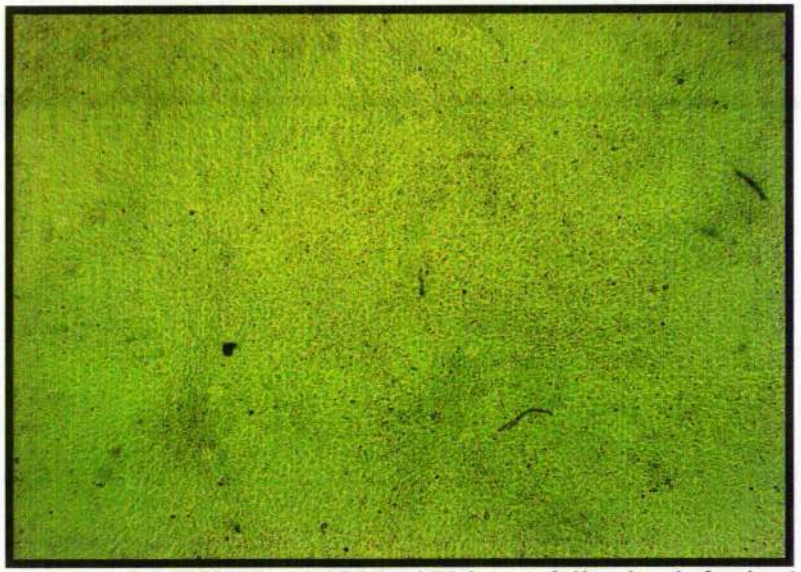
(i) HSV17^{+} MOI 0.1/ 48 hours following infection



(ii) HSV17^{+} MOI 0.1/ 72 hours following infection



(iii) HSV1716 MOI 0.1/ 48 hours following infection



(iv) HSV1716 MOI 0.1/ 72 hours following infection3T6 cell line

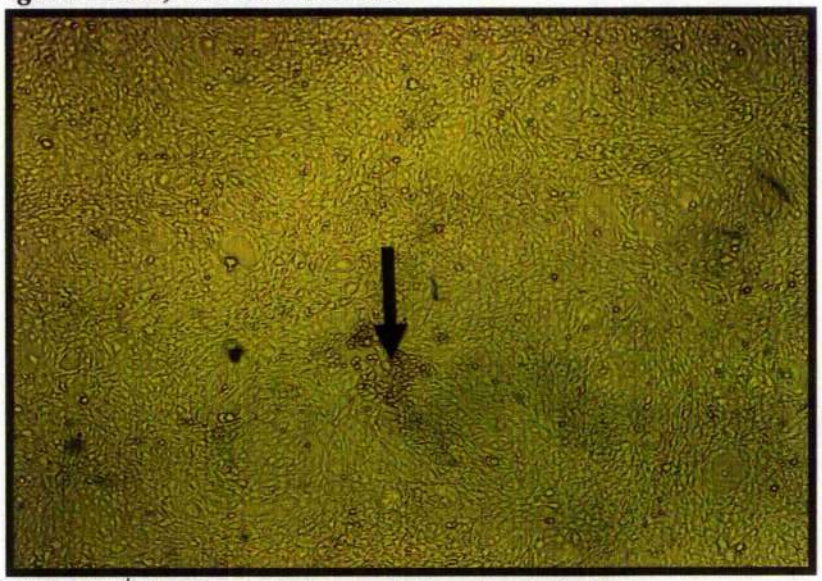
HSV17^{+}

By 48 hours plaques had begun to develop in the HSV17^{+} infected confluent 3T6 cells demonstrated in figure 15.6.1(i). The HSV17^{+} infection is more advanced by 72 hours, indicated by larger plaques shown in figure 15.6.1(ii).

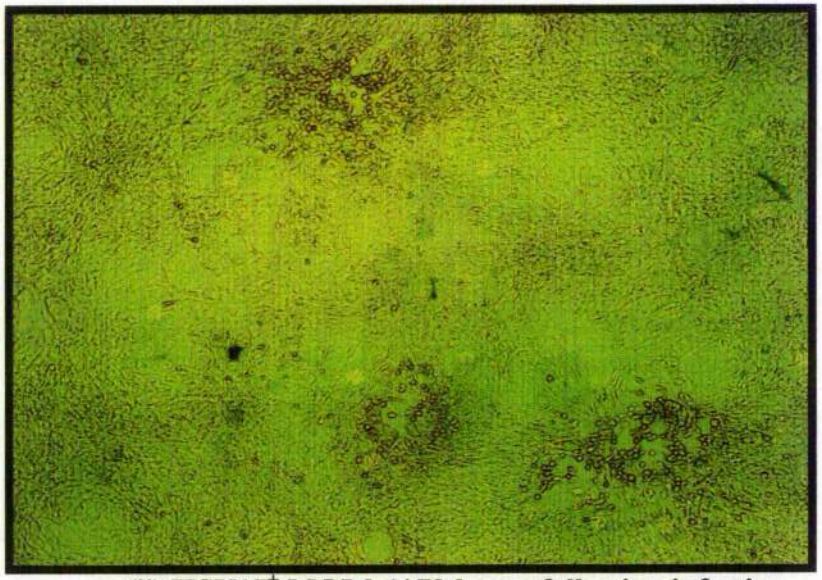
HSV1716

There was no evidence of HSV1716 replication at 48 or 72 hours post infection demonstrated in figure 15.6.1 (iii). There were complete intact monolayers with no evidence of plaques at both of the time points demonstrated in figure 15.6.1(iv).

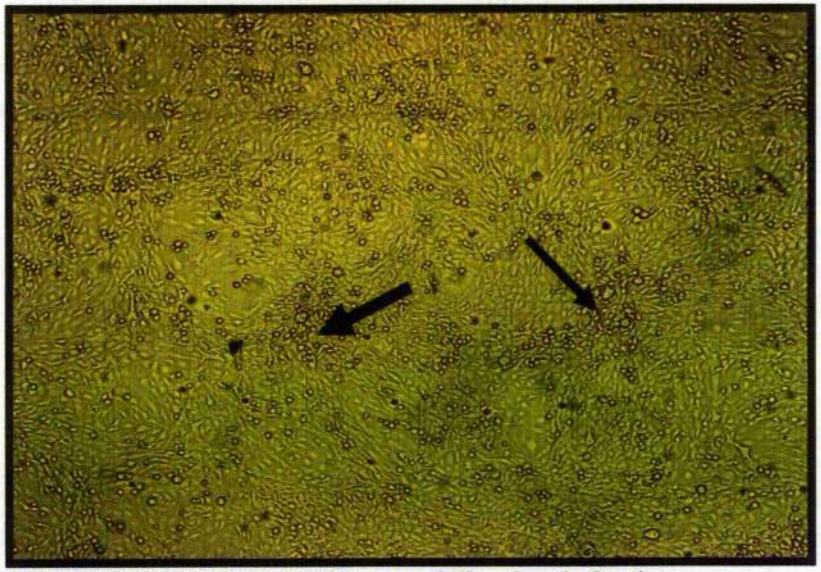
Figure 15.6.2; *3T6-34.5 cell line*



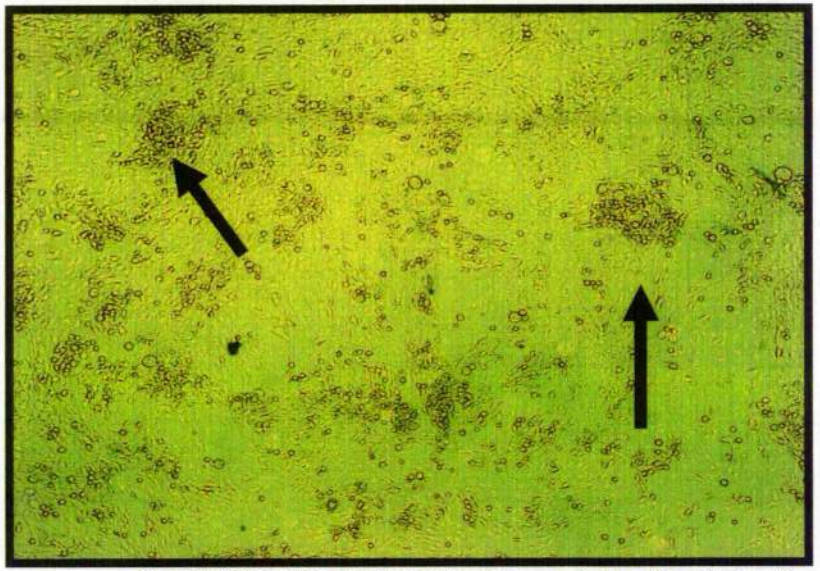
(i) HSV17^{+} MOI 0.1/ 48 hours following infection



(ii) HSV17^{+} MOI 0.1/ 72 hours following infection



(iii) HSV1716 MOI 0.1/ 48 hours following infection



(iv) HSV1716 MOI 0.1/ 72 hours following infection

3T6-34.5 cell line

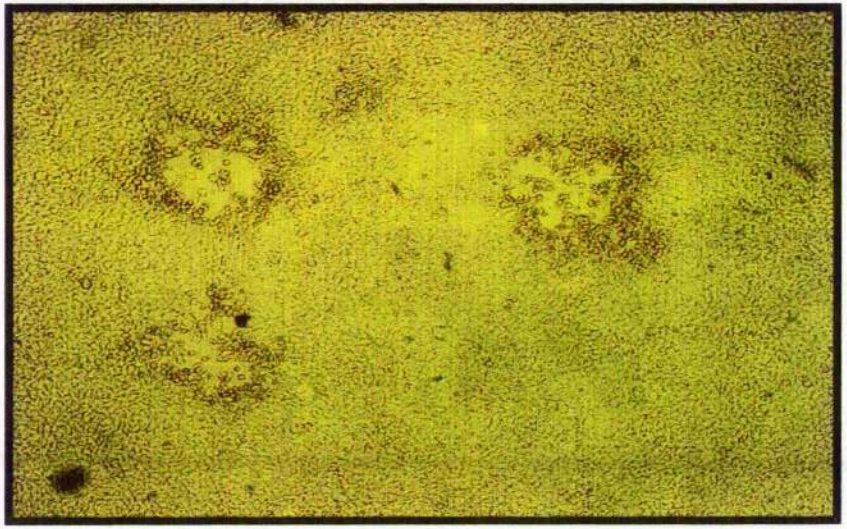
HSV17^{+}

By 48 hours after infection of 3T6-34.5 cells with HSV17^{+} there was some evidence of virus activity indicated by the arrow in figure 15.6.2(i) however no plaques had developed. There were clear plaques by 72 hours demonstrated in figure 15.6.2(ii).

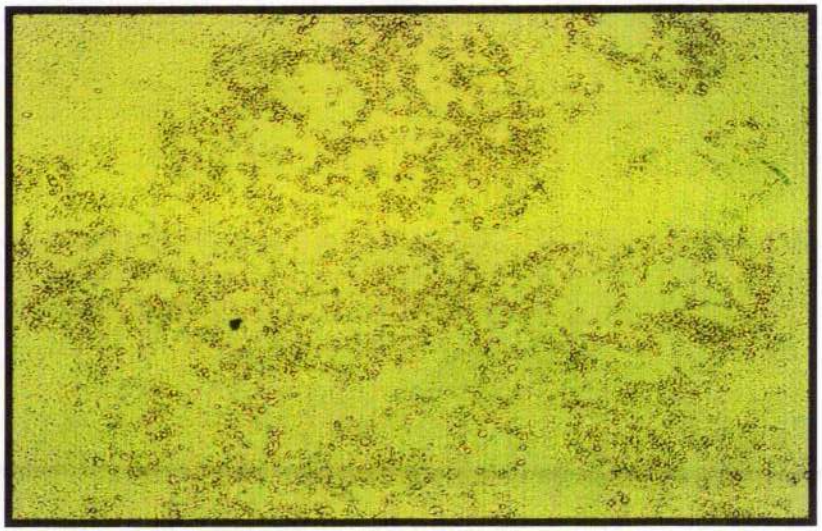
IISV1716

By 48 hours there was some evidence of HSV1716 infected cells as indicated by the arrows in figure 15.6.2(iii). The infected cells were rounded and appeared to be in clumps, however there is no evidence of plaques forming. By 72 hour the viral infections was more extensive and aggregates of virus infected cells were more apparent. In this field there is the evidence of small plaques developing, indicated by the arrows in figure 15.6.2(iv)

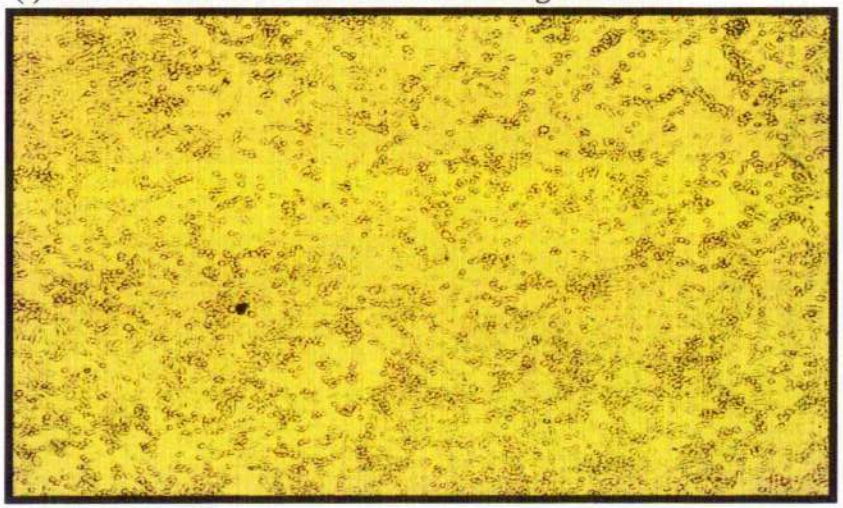
Figure 15.6.3; *3T6-34.5-GFP cell line*



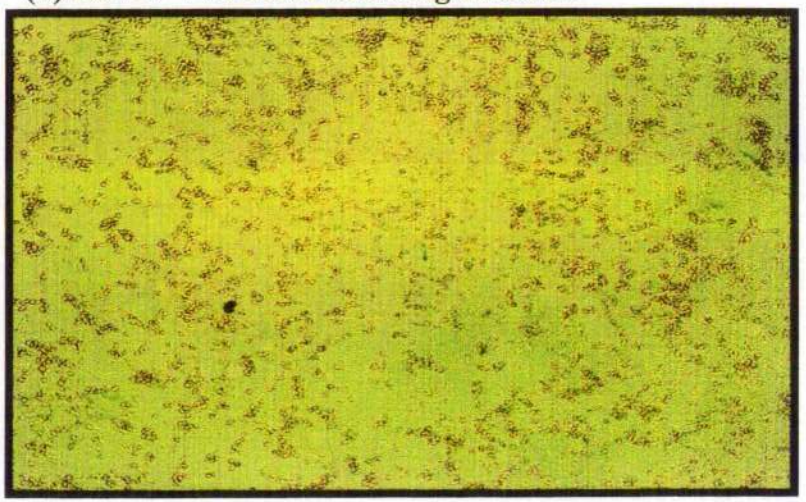
(i) HSV17^{+} MOI 0.1 / 48 hours following infection.



(ii) HSV17^{+} / 72 hours following infection.



(iii) HSV1716 MOI 0.1/ 48 hours following infection.



(iv)HSV1716 MOI 0.1/ 72 hours following infection.

3T6-34.5-GFP cell line

HSV17^{+}

By 48 hours after infection of the 3T6-34.5-GFP cells with HSV17^{+} there was evidence of plaque formation and by 72 hours the infection was extensive, with the plaques beginning to coalesce demonstrate in figure 15.6.3 (i) and (ii).

HSV1716

By 48 hours after infection with HSV1716 there had been extensive viral infection with the vast majority of the cells showing evidence of infection. By 72 hours there were fewer cells present, as the lytic replication had progressed, demonstrated in figure 15.6.3 (iii) and (iv).

The data from the confluent monolayers supported the data obtained from the semi-confluent monolayers showing that in 3T6 cells, expressing either ICP345, or ICP34.5-GFP, HSV1716 is able to replicate to generate a full lytic cycle resulting cell death. There is evidence by 48 hours of an HSV1716 infection, that by 72 hours had become more extensive in both the 3T6-34.5 and 3T6-34.5-GFP cells. The expression of either ICP34.5 or ICP34.5-GFP had changed the phenotype of 3T6 cells making them permissive to ICP34.5 null HSV replication.

15.7 PCNA Localisation in Replicating and Growth Arrested 3T6 cells Expressing ICP34.5 or ICP34.5-GFP

The following experiments were conducted to determine the localisation of PCNA in replicating and growth arrested 3T6 cells expressing ICP34.5 or ICP34.5-GFP.

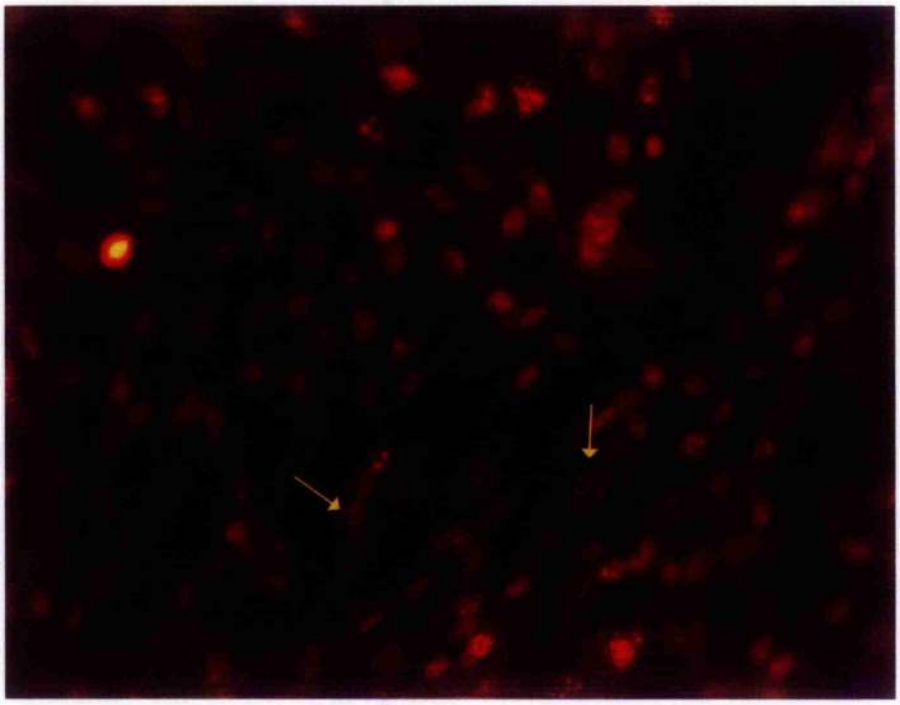
In quiescent cells PCNA exists as a soluble form, whereas in actively replicating cells or those treated with DNA damaging agents, it occurs as a detergent-insoluble form stably associated with DNA. Therefore, to study the localisation of PCNA involved in DNA replication, cells grown on cover-slips in medium with or without serum were treated with a hypotonic solution in order to make them more permeable, and a mild detergent to solubilise any PCNA not bound to DNA. The PCNA was detected by a monoclonal antibody and visualised by immuno-fluorescence.

The results are indicated in figure 15.7.1, 15.7.2 and 15.7.3.

In actively dividing 3T6, 3T6-34.5 and 3T6-34.5-GFP cells (figure 15.7.1(a), 15.7.2(a), 15.7.3(a) respectively), the DNA-bound PCNA is clearly identified as speckling in the nucleus (a few of the nuclei staining for PCNA are identified by yellow arrows). There is no apparent difference in the level of PCNA present in any of the three cell lines. In growth arrested 3T6, 3T6-34.5 and 3T6-34.5-GFP cells (figure 15.7.1(b), 15.7.2(b), 15.7.3(b) respectively), there is virtually no PCNA immuno-fluorescence. It appears that incubation for 48 hours in serum free medium has successfully growth arrested all of the cell lines. The expression of ICP34.5 did not prevent the transfected 3T6 cells from entering a state of growth arrest.

Figure 15.7.1

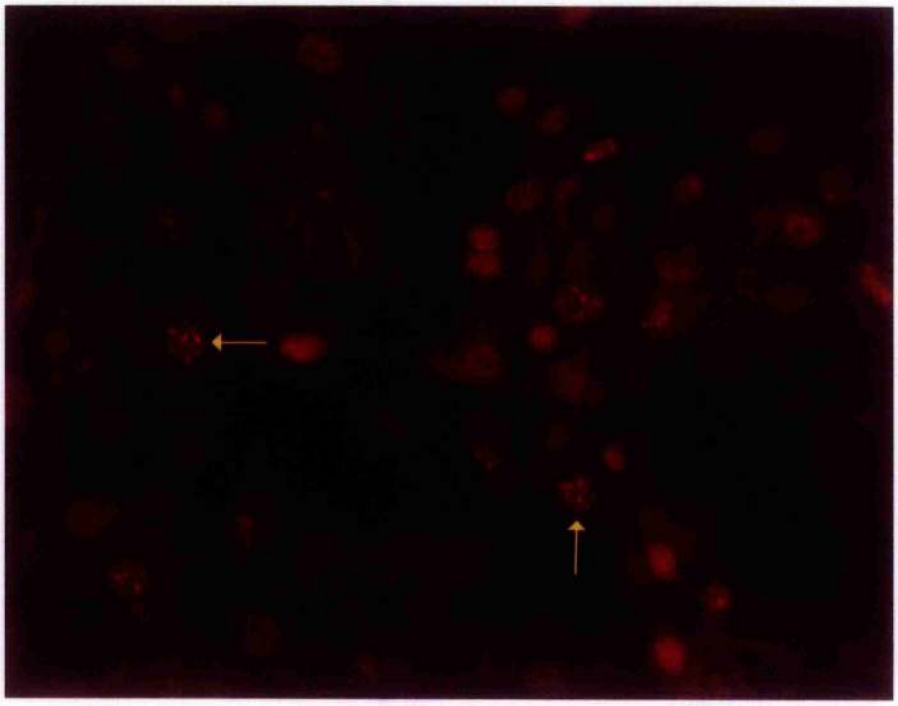
(a) PCNA immunofluorescence of actively dividing 3T6 cells.



(b) PCNA immunofluorescence of growth arrested 3T6 cells.

Figure 15.7.2

(a) PCNA immunofluorescence of actively dividing 3T6-34.5 cells.



(b) PCNA immunofluorescence of growth arrested 3T6-34.5 cells.

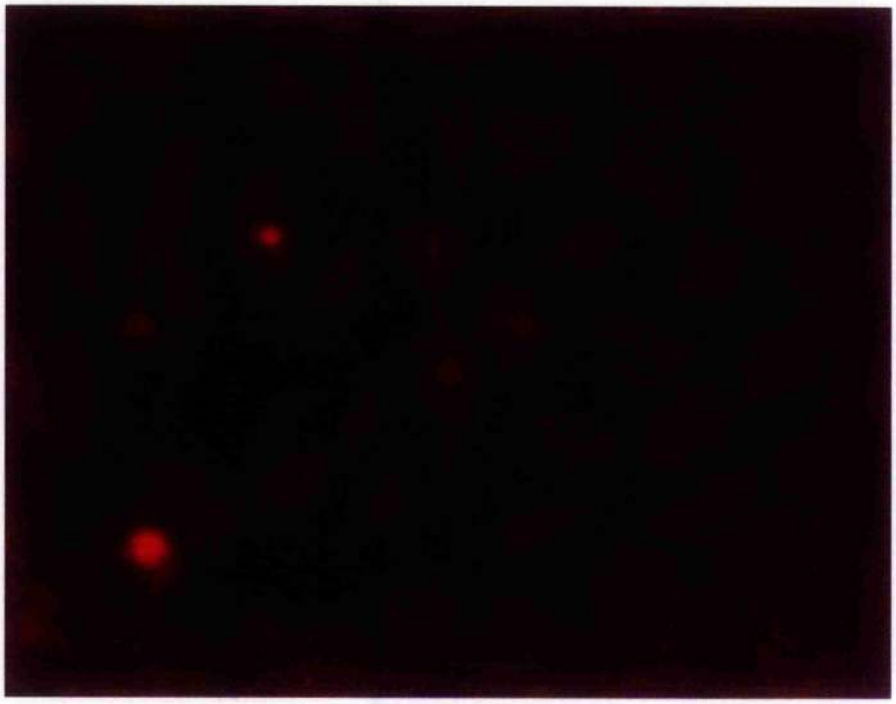
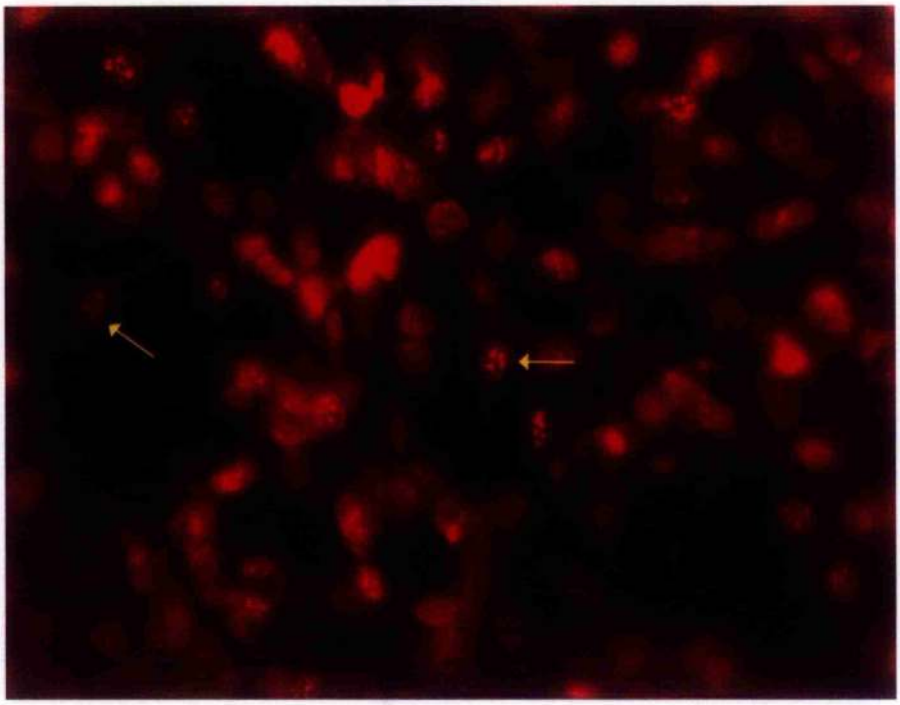
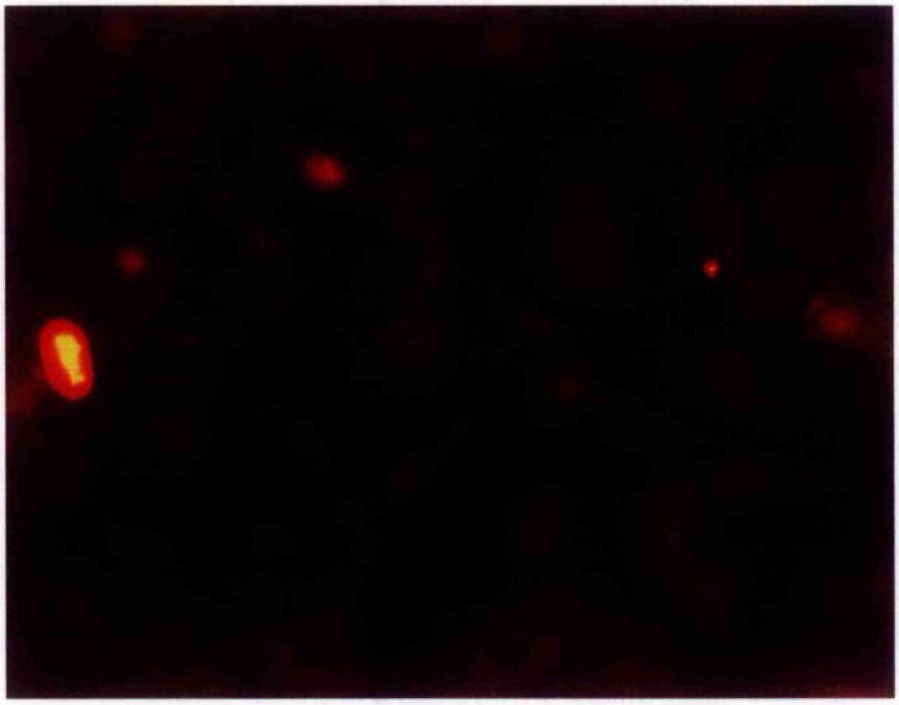


Figure 15.7.3

(a) PCNA immunofluorescence of actively dividing 3T6-34.5-GFP cells.



(b) PCNA immunofluorescence of growth arrested 3T6-34.5-GFP cells.



15.8 Summary of Results

Initially plasmids expressing ICP34.5 or ICP34.5-GFP under the control of the RL1 promoter in addition to a Zeocin resistance gene were constructed. The plasmids pc4-34.5 and pc4-34.5-GFP, were linearised and used to transfect actively dividing 3T6 and BHK cells. Transfected cells were selected by growth medium containing Zeocin.

Confirmation of stable expression by the transfected 3T6 cells was obtained from Western blot analysis, using an antiserum against ICP34.5, which showed protein bands indicative of ICP34.5 and ICP34.5-GFP. In addition, expression of the 70kD and 21kD viral proteins in HSV1716 infected 3T6-34.5 and 3T6-34.5-GFP cells, but not in the non transfected 3T6 cells, indicated that expression of ICP34.5 had overcome the block in HSV1716 replication normally seen in 3T6 cells. Further evidence of the expression of functional ICP34.5 in the transfected 3T6 cells was demonstrated by their ability to support HSV1716 replication giving a higher 72 hour yield than the control 3T6 cells.

Western blot analysis demonstrated that there was expression of ICP34.5-GFP in the 3T6-34.5-GFP cell lines. It was hoped that detection of green fluorescence in the transfected cell line would indicate the localisation within the cell of the fused ICP34.5. Green fluorescence was unfortunately not detected. This may be due to low levels of GFP expression produced by the weak RL1 promoter.

The phenotypes of the transfected 3T6 cells were analysed with respect to HSV1716 infection. It has been shown (Brown SM *et al.*, 1994a) that HSV1716 can replicate in dividing but not in fully confluent, contact inhibited 3T6 cells whereas $HSV17^{+}$ replicates in both dividing and non-dividing 3T6 cells. Actively replicating 3T6 cells expressing ICP34.5 or ICP34.5-GFP were compared to non-transfected 3T6 cells with respect to HSV1716 and $HSV17^{+}$ infection. As expected $HSV17^{+}$ replicated efficiently in all of the cell lines. In the replicating 3T6 cell lines infected with HSV1716 the appearance of 'abortive plaques' indicated early viral replication. In the ICP34.5 and ICP34.5-GFP expressing 3T6 cell lines there was evidence of

HSV1716 lytic replication with the appearance of characteristic plaque formation. Confluent, non-replicating 3T6 cells infected with HSV1716 failed to show any sign of infection, whereas a characteristic HSV plaque morphology was observed in HSV1716 infected confluent 3T6 cells expressing either ICP34.5 or ICP34.5-GFP. Therefore expression of either ICP34.5 or ICP34.5-GFP successfully changed the phenotype of the cell making them permissive to ICP34.5 null HSV replication.

The localisation of PCNA and whether or not it is engaged in cellular DNA replication is thought to determine the ability of HSV1716 to replicate. A series of immunofluorescence experiments was performed to investigate the localisation of PCNA within replicating and growth arrested 3T6 cells. The experiments were designed to study the distribution of DNA bound to PCNA in dividing and growth arrested 3T6 cells. In serum starved, growth arrested 3T6, 3T6-34.5 and 3T6-34.5-GFP cells, there was no evidence of DNA bound to PCNA. In the growth arrested cells the PCNA was presumably present in the cytoplasm in the detergent soluble form. The expression of ICP34.5 did not prevent the transfected 3T6 cells from entering a state of growth arrest.

Chapter 6

Discussion and Conclusions

16 HSV1716 Injection into the Resection Cavity Rim Following Surgical Resection of High Grade Glioma in Patients with Recurrent and *De Novo* Tumours

Formal follow up in this study was completed in February 2003, 22 months after initiation and 14 months after enrolment of the last patient. There was no evidence of local or systemic toxicity following the injection of HSV1716 into the brain adjacent to tumour in any of the twelve patients in this study. At study completion, ten patients had shown evidence of tumour progression; one (patient 12) died from a non-tumour related cause and one (patient 7) was still alive. Patient 1 and 10 were progression free and clinically well at study completion.

Patient 12 developed evidence of tumour recurrence at 8 months and received two cycle of Temozolomide, but died at 11 months post HSV1716 injection from a presumed myocardial infarction.

Patient 7 showed clinical and radiological signs of progression on completion of radical radiotherapy, twelve weeks after surgical resection and HSV1716 treatment. Following further debulking surgery he received six cycles of CCNU chemotherapy. His disease then remained stable until the end of the follow up period 18 months from surgery and 15 months since re-resection and chemotherapy.

Patient 1 showed remarkable improvement over the 22 month follow-up period with no clinical or radiological evidence of disease progression. His Karnofsky status improved from 60, preoperatively, to 90 in February 2003 and his Barthel score from 8/20 to 18/20. His response is of particular note given his poor preoperative condition and the fact that he declined any chemotherapy.

Patient 10 remained progression free and well at 15 months post resection and HSV1716 injection.

As expected in a population such as this, some patients experienced adverse events. Although all of these events were thought to be unconnected with HSV1716 administration, three somewhat unusual events are discussed in some detail.

At 78 days after surgery and HSV1716 injection, patient 1 suffered a seizure and required ventilation and anti-epileptic medication. He had no prior history of seizures and was not receiving anticonvulsant medication. The first blood sample to be analysed by PCR at that time was taken five days later (83 days post injection) and was positive for HSV DNA. The patient recovered well and was discharged from hospital on a maintenance dose of Phenytoin and had no subsequent serious adverse events. Any connection between this adverse event and the HSV1716 treatment remains unproven. Epileptic seizures are by no means uncommon in this group of patients and, given that this patient was seropositive for HSV when enrolled into this trial, the detection of HSV DNA in his blood although of interest it is not conclusive proof of HSV1716 replication. It is unfortunate that the PCR assay developed at the time of this trial could not distinguish HSV1716 from wild type HSV. Therefore it is not possible to say whether the HSV DNA detected in the blood sample was the result of HSV1716 replication causing a transient viraemia or of a possible reactivation of the patient's latent HSV infection. There was no indication that the patient exhibited recrudescence of his latent infection at that time, but this too dose not prove that there was not reactivation of latent HSV, controlled by his immune system. Therefore, whilst a connection between this adverse event and the HSV1716 therapy cannot be ruled out, the evidence is inconclusive.

Two months after virus injection and shortly after completing a standard course of radiotherapy, patient 5 suffered a complex partial seizure and was commenced on Carbamazepine. He was subsequently readmitted to hospital with pyrexia of unknown origin and a generalised rash, which did not respond to broad spectrum antibiotics. Although no HSV DNA was detected in blood and cerebrospinal fluid

by PCR analysis (sensitive at levels equivalent to 10^2 pfu/ml) and whilst there was no evidence that HSV1716 was implicated, Acyclovir was nevertheless administered as a precautionary measure. Despite the Acyclovir, he remained unwell with a spiking temperature. Two weeks following admission, a positive monoclonal antibody ELISA test for Candida antigen confirmed invasive candidosis for which he was treated successfully with intravenous Fluconazole. In this case, it seems highly probable that the adverse event was entirely unconnected with the HSV1716 therapy.

Three weeks after surgery and HSV1716 injection, patient 12, a type II, insulin dependent diabetic presented with a herpes zoster rash in a C5/6 distribution. There was no evidence of HSV obtained by PCR or virus shedding assays on numerous serological and vesicular fluid samples and the clinical diagnosis of Varicella-Zoster reactivation was considered to be unrelated to the HSV1716 therapy.

To the present day, October 2005, of the three patients who were still alive at the end of study follow up (February 2003) two have since died. Patient 1 demonstrated evidence of tumour progression on imaging in May 2003. This was accompanied by deterioration in his neurological state. Given the apparent reduction in residual tumour demonstrated by imaging following HSV1716 administration alone, further HSV1716 was injected directly into the recurrent tumour on receiving approval for the procedure from the Gene Therapy Advisory Committee. In August 2003 there was evidence of recurrent tumour progression and this was resected. Further HSV1716 was injected into the resection cavity rim. Patient 1 then went on to receive 3 cycles of combination chemotherapy (PCV) at his local hospital. He died in June 2004, 46 months following diagnosis and 37 months following the first delivery of HSV1716.

By February 2003 patient 7 had demonstrated evidence of tumour progression, which was excised. He then received a course of CCNU chemotherapy. He remained well until October 2003 when his clinical condition deteriorated and imaging demonstrated tumour regrowth. In November 2003 he underwent further tumour resection and insertion of Gliadel wafers into the resection cavity. His

condition did not improve greatly and he subsequently died in April 2004, 32 months following diagnosis and administration of HSV1716.

To date (October 2005) patient 10 is still alive, 47 months since diagnosis and administration of HSV1716. She was last reviewed in March 2005 and remained well with no clinical or radiological evidence of disease progression.

Patient serum samples were analysed in line with the protocol, for routine biochemistry and haematology. In addition, blood samples were analysed in our laboratory for infectious HSV, HSV DNA by PCR and HSV IgG and IgM by ELISA.

Infectious HSV was not detected in any patient's blood sample. In the case of HSV seropositive individuals, the presence of neutralising antibody makes it extremely unlikely that infectious HSV would ever be detected by this assay. However, given the limitation of detection of the assay, even in HSV seronegative subjects it is probable that only an acute viraemia would be detectable.

As stated previously, the PCR assay, developed to detect the presence of HSV DNA in the clinical samples, was not specific for HSV1716. Due to the obstacles imposed by the high G-C content of the DNA in the long repeat (R_L) region of the HSV-1 genome, a PCR assay to reliably distinguish HSV1716 DNA had not been developed in time for use in analysing the samples in this trial. The probes used, hybridised to a highly conserved region of the genome and had been shown previously to reliably detect diverse HSV-1 wild type isolates. This assay was used in a previous phase I trial in which tumour tissue was injected with HSV1716 and later resected and analysed for the presence of HSV (Papanastassiou V *et al.*, 2002). In that trial it was assumed that any HSV DNA present must be HSV1716, as unattenuated wild type HSV would have caused encephalitis. This argument, however, does not apply to blood samples. As HSV-1 infection results in lifelong latent infection in the sensory ganglia the detection of anti-HSV-1 antibodies in the blood stream indicated the presence of latent virus in a host. Therefore particularly in blood samples from HSV seropositive patients,

it is impossible with this assay to distinguish between HSV1716 DNA and HSV DNA from the reactivation of a latent infection.

Using the PCR assay, serum samples from four patients were positive for HSV DNA. Of these, patients 1, 5 and 10 were HSV seropositive and had a prior history of HSV infection (cold sores). They were positive by PCR assay some time after injection of HSV1716 and it cannot be ruled out that the HSV DNA detected in these patients was the result of a sub-clinical viraemia associated with reactivation of endogenous latent HSV-1. Patient 3 was sero-negative prior to injection of HSV1716 and seroconverted at day 24, when HSV DNA was detected in his serum. Therefore, although not conclusive, the result in this case is indicative of the release of HSV1716 into the bloodstream more than three weeks after virus injection.

The ELISA assay quantified the levels of HSV specific immunoglobulins, IgM and IgG. The commercially available kit, measured the presence of antibody on a colourimetric scale with a designated cut off point above which the result was considered positive. The IgG and IgM responses for each patient throughout the clinical trial are shown in the graphs in section 13.2 – 13.13 (Chapter 3). Patients 2, 3 and 4 were found to be seronegative at the time of enrolment. Of these, patients 3 and 4 mounted an immune response and seroconverted between the third and fourth weeks post virus injection with no adverse symptoms reported. Usually after a naïve individual is exposed to an antigen, the level of IgM increases over the course of a few days and then drops after approximately ten days when there is a concurrent increase in IgG. The timing of the immune response in these patients is, therefore, somewhat slower than might be expected if the response was mounted against the initial inoculum, lending some credence to the possibility of viral replication in the tumours following injection. However, as these patients died at 9 and 15 months respectively of disease progression, any viral replication does not appear to have resulted into an obvious survival advantage given the expected survival for a cohort such as this. The remaining seronegative individual, patient 2, who, unlike the other two, remained on high dose steroids, did not mount an immune response and died six months after injection of virus.

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Patients were monitored throughout the study with regular imaging, primarily to detect any evidence of disease recurrence. Imaging involved MRI (or CT if the patient could not tolerate MRI due to claustrophobia), Thallium-201 SPECT and ^{99m}Tc-HMPAO SPECT. The MRI (or CT) and Thallium-201 SPECT scans were used to detect any evidence of tumour recurrence. The images were later reviewed to look for evidence of tumour response following injection of HSV1716.

In the majority of cases, following surgical resection and HSV1716 injection followed by standard therapy there was some temporary tumour stabilisation, most probably as a result of the adjuvant radiotherapy or chemotherapy. In only one of the twelve patients (patient 1) was there imaging evidence detected of tumour regression that might be associated with the injection of HSV1716. The reduction in size of the tumour mass seen on imaging correlated well with his clinical improvement, however, a causal relationship between his clinical improvement and the procedure cannot be established. In other patients with detectable disease following tumour debulking, there was no tumour regression detected by imaging. In patients where there was no demonstrable tumour left following resection, macroscopic reduction in tumour could not be detected.

Following routine clinical practice, after surgical debulking of tumour, patients in the trial proceeded with further immunosuppressive treatments, either radiotherapy of chemotherapy. The data from this trial demonstrate unequivocally that injection of HSV1716 up to a dose of 10^5 pfu into the resection cavity rim following resection of high grade glioma in immunocompromised patients was safe and did not generate toxicity. There were no serious adverse events that were thought related to treatment with HSV1716. One patient only showed convincing evidence of response to treatment in that the clinical course following resection of his glioblastoma followed by cavity rim injection was highly atypical for this disease.

In retrospect, however, the injection of HSV1716 into the cavity rim following tumour resection was probably not optimal in terms of tumour treatment as the sparsely distributed permissive cells in this tissue would almost certainly be insufficient to sustain successive rounds of viral replication. In addition only ten

injection points were selected, into each of which a 0.1ml ($1x10^4$ pfu) aliquot of HSV1716 was delivered. Although areas of possible residual tumour were carefully sought and injected, the interface between normal tissue and tumour is not always distinguishable. In addition, the potential for failing to identify small islands of tumour cells is high. It would not be surprising therefore if HSV1716 in some instances failed to come into contact with replicating tumour cells and generate a productive lytic infection. Also, the nature of the resection cavity, with the inevitable inflammation response generated at the time of surgery, was probably not the ideal environment into which to inject HSV1716. Virus injection several days after surgery, which would have allowed time for the acute inflammatory response and the reactive oedema to settle, might have been preferable. This would, however, have involved two interventions with the associated increase in risk from anaesthesia, haemorrhage or infection.

Attempts were made to culture tumour samples from all of the patients *in vitro*, however only five samples grew sufficiently to perform multicycle growth experiments with HSV1716. In all five HSV1716 replicated efficiently, supporting the view that HSV1716 could potentially be efficacious, if delivered effectively to an optimum *in vivo* environment.

HSV1716 is the herpes simplex oncolytic virus currently in the most advanced clinical trials. Data from three published phase I clinical trials involving a total of 33 patients and doses up to 10^5 pfu has been gathered with no evidence of toxicity associated with HSV1716. In addition, as an extension to the first study, a further 12 patients were recruited who received an intratumoural injection of HSV1716 up to a higher total dose of 10^6 pfu. In this study there has also been no evidence of toxicity associated with HSV1716 (Papanastassiou V, personal communication).

In 2000, Markert *et al.* published data detailing an investigation of the selectively replication competent HSV-1 mutant G207 following intratumoural injection in 21 patients with high grade glioma. As G207 is a double mutant, lacking ICP34.5 and ribonucleotide reductase expression, it is considerably more attenuated than HSV1716 and consequently initiating doses of up to $3x10^9$ pfu were used. There

was no evidence of toxicity associated with the treatment but unfortunately the investigators were unable to demonstrate evidence of G207 replication in the tumours. One reason may lie in the trial design, in that for the first 18 patients, G207 in a small volume (0.1-0.3ml) was injected into a single tumour site. The investigators sought to minimise reflux up the needle tract by injecting the virus over two minutes. However, it is possible that using such small volumes, the proportion lost was high and the intended dose was not delivered.

Both HSV1716 and G207 are selectively replication competent virus and clinical studies with these agents generate novel problems of trial design. Conventional studies using escalating doses to achieve a toxic endpoint are not appropriate. Because the virus has the capacity to replicate, it is possible that after several rounds of replication, the virus titre will be orders of magnitude higher than the input. Therefore, although it is necessary to have an input dose sufficient to initiate viral replication, the nature of the tissue into which the virus is injected is possibly of greater importance, meaning that the issue of delivery is paramount.

HSV1716 may be at the forefront of oncolytic viral therapy in high grade gliomas, however, the investigators still face the challenge of designing a clinical trial to demonstrate efficacy. Before the treatment would be taken up in clinical practice, a clinical trial would unequivocally have to demonstrate increases in time to tumour progression and overall survival and possibly additional positive quality of life data. With the costs of running a phase II clinical trial high, the study design will be crucial.

In 2000 Rainov *et al.* presented data on a large randomised gene therapy trial in patients with newly diagnosed glioblastoma. Patients randomised to the treatment arm received an injection, of retrovirally transduced glioblastoma cells expressing HSV*tk*, into the resection cavity following tumour resection. The patients were treated later with Gancyclovir. Unfortunately the study failed to demonstrate any significant increase in the time to tumour progression or overall survival. It is probable that the investigators proceeded to a large phase III study with incomplete phase II data to support the protocol employed. This highlights the

necessity for efficacy studies to have clear objectives, be properly designed and be based on adequate preclinical and early clinical data.

Selection of the appropriate patient population will be important in future clinical trails of HSV1716 in glioblastoma. Investigators need to define clearly the clinical scenario in which they envisage HSV1716 will have clinical application. In the analysis of the three phase I clinical trials involving HSV1716 no one patient cohort has shown to have benefited more than any other. The *in vitro* data presented in this thesis support the view that HSV1716 will replicate more efficiently when injected into a replicating tumour mass than when used to mop up residual tumour cells in the cavity rim following tumour resection. It is arguably not ethical, in the absence of efficacy date, for HSV1716 to be used in trials in patients with *de novo* tumour where the current surgical and radiotherapy treatments have shown clear, if limited, efficacy. It is probable, therefore, that clinical trials to demonstrate efficacy are liable to be conducted in patients with tumour recurrence following first line adjuvant treatment. These patients, under current treatment guidelines, then receive chemotherapy. It has been shown that, despite the resultant immunosuppression, chemotherapy delivered following injection of HSV1716 is not associated with any additional toxicity (Harrow S *et al.*, 2004). Therefore, in theory the HSV1716 could be administered in conjunction with chemotherapy, although this has obvious repercussions for the analysis of data to demonstrate survival benefit. In addition, the recent demonstration of efficacy from combining ionising radiation with Temozolomide (Stupp R *et al.*, 2005) could complicate the issue of designing trials to assess the efficacy of HSV1716 in adjuvant settings.

It is anticipated that in future patients with newly diagnosed glioblastoma will undergo surgical tumour resection and then receive Temozolomide concurrently with IR. This is followed by a period of adjuvant Temozolomide. The data presented by Stupp *et al.* (2005) suggest that the additional survival seen in patients who received Temozolomide is largely due to the concurrent delivery of the drug with IR. The authors proposed that this may be because Temozolomide depletes levels of the enzyme 0^6-methylguanine-DNA methyltransferase (MGMT) thereby preventing repair of IR damaged DNA. It is unclear what the effect of

reduced MGMT levels would have on HSV1716 infection. It would therefore be interesting to perform some *in vitro* analysis to determine if there was any additional cell kill from combining HSV1716 and Temozolomide. It is possible that the DNA damage induced by HSV1716 infection could be enhanced by the reduction in MGMT levels resulting from Temozolomide treatment, to give enhanced cell kill. However it is recognised that the mechanism of MGMT is repair of methylation adducts which is not known to be directly caused by HSV infection. Alternatively as it appears that HSV1716 relies on the cellular DNA repair response to initiate its own replication, depletion in MGMT levels could be detrimental to HSV1716 replication.

The mode of delivery of HSV1716 is also important when considering the design of future clinical trials. In the previous trials, HSV1716 was injected either into a small number of sites (up to ten) within the tumour or into the cavity rim following tumour resection. It has been shown that following direct injection into the tumour there was viral replication within the tumour (Papanastassiou V *et al.*, 2002). However, if the delivery of HSV1716 could be improved to infect more tumour cells at a stage within the cell cycle conducive to the selective viral replication, subsequent virus replication might allow progeny virus to disseminate throughout the whole tumour. Improved delivery could be achieved by better targeting of delivery to actively replicating areas of the tumours, possibly by taking advantage of advances in functional imaging combined with automated stereotactic delivery systems or with convection enhanced drug delivery (CEDD).

CEDD uses a positive pressure infusion mechanism that can deliver a drug locally and bypass the blood brain barrier. CEDD distributes a therapeutic agent, down a pressure gradient through the brain interstitium, without causing structural or functional damage to the tissue. This approach is ideal for targeting areas of tumour that are unresectable due to inaccessibility or location in eloquent brain tissue. Groothius *et al.* (1999) demonstrated a 10,000 fold increase in the concentration of ^{14}C sucrose in rat brain compared with intravenous administration and the pattern of distribution was such that there was a central portion resulting from convection and a peripheral component in grey matter resulting from diffusion. Laske *et al.* (1997) reported encouraging efficacy results

following the treatment of 15 patients with CRM107, a conjugate of human transferrin and a modified diphtheria toxin delivered by CEDD. There was no local or systemic toxicity associated with the delivery of CRM107 by CEDD. In addition, *in vivo* data has been presented indicating that CEDD can be used to deliver gene therapy vectors (Cunningham J *et al.*, 2000). A feasibility study to investigate the applicability of this technology to the delivery of HSV1716 is therefore indicated. It would be useful to know if HSV1716 particles would successfully infiltrate tumours and if they would, like the small molecules in the Groothius *et al.* study, diffuse further to reach tumour cells which have migrated away from the main tumour mass.

Two large, multicentre, open label, randomised phase III clinical trials are currently underway using CEDD to deliver the drug under investigation. Both studies are investigating the efficacy of the agent in patients with recurrent high grade glioma with the primary objective of each trial being overall survival. In the first study patients are randomised to receive CRM107, renamed TransMID^{TM} or the preferred chemotherapy regime of the recruitment centre used to treat patients with recurrent or unresectable disease. CRM107, is being delivered in a total volume of 40ml directly into recurrent or progressive glioblastoma via two catheters. The total volume is delivered in a graduated manner up to a maximum rate of 0.4ml/hour over 4-7 days. Each patient will receive two infusions of TransMID^{TM} between 4 and 8 weeks apart. The other trial is investigating the convection enhanced delivery of IL13-PE38QQR, a recombinant, tumour targeted, chimeric cytotoxin composed of amino acids from human interleukin-13 (IL-13) and from Pseudomonas Exotoxin A. This study will compare post-resection, peritumoural infusion of IL13-PE38QQR to the use of Gliadel Wafer in patients with glioblastoma at time of first recurrence who are suitable for surgical resection. It is intended that there will be between 2 and 4 catheters inserted into the tumour. In this trial a flow rate of 3.12–6.25 μL/hr per catheter is proposed to deliver a total volume of 72ml over 4 days. The results of these trials will be interesting not only to appraise the drug under investigation, but also the novel delivery mechanism (Rampling R, personal communication).

Another dilemma for those involved in future trial design is the need to decide what would constitute an acceptable survival advantage. Persuading clinicians to adopt a radical new treatment such as oncolytic viral therapy for high grade glioma would require the demonstration of a clinically significant survival advantage (probably six months or 50% improvement in median survival). However, there is no point in designing such a trial if the end points are clinically unrealistic. When designing any future trial it will be necessary to select a treatment effect that is clinically acceptable, whilst considering the number of patients required to provide statistical significance in addition to the expected time for recruitment given the rarity of this disease.

Following the *in vitro* demonstration of additive (or even synergistic) glioma cell kill achieved by combining ionising radiation and HSV1716 infection, it is interesting to speculate on how this could be applied in practice. Although it is probable that extensive animal testing would be required to fully characterise the effect in terms of dosing scheduling etc, it is nevertheless worth considering possible scenarios in which the combination of ionising radiation and oncolytic HSV therapy might be combined in clinical practice.

Because of the long term effects and morbidity associated with radiotherapy of the brain, patients with HGG usually receive only one course of external beam radiation. Current clinical practice is to deliver this external beam radiotherapy to a 2cm margin around the cavity following maximal tumour resection. The radiotherapy is most effective when delivered soon after resection and ideally should commence as soon after surgery as is possible. As discussed previously, it is probable that HSV1716 therapy is liable to be less effective in the conditions existing immediately post surgery. However, as radiotherapy is the most effective treatment currently available for the treatment of HGG, it would be unethical to change the scheduling of the radiotherapy without very good evidence for the potential benefit of changing. Therefore, within the constraints of the current best practice, the only way to combine HSV1716 treatment with ionising radiation would be to administer the virus immediately before or during the radiotherapy course. In this scenario, either the patient would have to undergo two surgical procedures, only weeks apart or, if the radiotherapy was administered

immediately after surgery, the virus would have to be injected into the cavity rim immediately after resection, with all of the consequent disadvantages previously discussed.

Another option may be to follow the conventional treatment schedule with tumour resection followed by external beam radiotherapy to the tumour cavity. At the stage of tumour relapse the tumour could be injected with HSV1716 delivered by CEDD with a boost of ionising radiation delivered to the tumour via stereotactic radiotherapy. As has been discussed in the introduction stereotactic radiotherapy is reserved for the treatment of tumours that are less than 4cm in diameter. In this scenario however the radiation is not being delivered to offer tumour control but to enhance the replication of HSV1716 and therefore relies on irradiating the portion of tumour that is being maximally infused with HSV1716. Although the logistics of delivering stereotactic radiotherapy may be difficult, the localised nature of the treatment should not generate morbidity for the patient.

In some instances it is acceptable to re-irradiate high grade glioma using conformal external beam radiation techniques. The tolerance of glial tissue to re-irradiation allows a reduced dose, compared to the primary radical dose, to be delivered before serious damage of normal glial tissue is observed (Steel G., 2002). It is therefore conceivable to deliver HSV1716 by CEDD concurrent with re-irradiation of the whole tumour and not just a small part of the tumour as would be achieved by stereotactic radiotherapy. Earlier in this thesis experimental data was presented demonstrating that pre-irradiated tumour cells *in vitro* did not result in enhanced viral replication when compared with non-irradiated cells. Therefore, if the mechanism by which synergy was observed *in vitro* between HSV1716 and IR is due to an effect on the cells, such as an alteration in virus entry or egress, then irradiating the tumour as a whole may seem more logical. Pre-clinical animal work may be able to explore this.

If a synergistic relationship was to be conveyed *in vivo* then this might result in demonstrable reduction in tumour bulk or translate into prolonged progression free survival and an increase in overall survival.

One may argue that the optimum environment in which to deliver HSV1716 would be prior to any standard treatment. This would only be feasible provided tumour debulking surgery was not required to alleviate symptoms of increased intracranial pressure. Given that there is scant evidence to suggest a survival benefit from tumour debulking without adjuvant Temozolomide, it would be interesting to investigate the delivery of HSV1716 by CEDD into the tumour mass concurrently with radical radiotherapy prior to tumour debulking surgery. Confirmation of a high grade glioma could be obtained intraoperatively from a stereotactic biopsy after which HSV1716 delivery could be commenced via CEDD. It is possible that a radical course of radiotherapy could be scheduled to coincide with the delivery of HSV1716. Patients would then be monitored closely using imaging for evidence of tumour growth. At the time of tumour growth patients would be eligible for tumour debulking surgery if indicated and/or other treatment.

An international, multi-centre, phase III clinical trial to assess the efficacy of HSV1716 in patients with relapsed glioma will commence recruitment in March 2006. Since completion of the phase I clinical trial presented in this thesis the EMEA has awarded Crusade Laboratories orphan drug status for HSV1716 in the treatment of glioma. The EMEA has been able to provide both scientific advice and protocol assistance in order to optimise the design of the clinical trial in this next crucial step.

It is proposed that 382 patients with recurrent glioblastoma at first relapse will be randomised into one of two treatment arms: HSV1716 treatment alone or chemotherapy treatment alone. Patients in the HSV1716 treatment arm will receive HSV1716 either by stereotactic multipoint microinjection or by CEDD determined by local expertise. A total of $6x10^6$ pfu will be introduced into the tumour by either method. Patients will then be followed up every six weeks at which time cranial MRI will be acquired. If persistent active tumour is observed on MRI then further HSV1716 treatment will be administered, on up to a maximum of four occasions. Patients will be deemed to have failed HSV1716 therapy and will not receive further HSV1716 if there is tumour progression despite two or more virus administrations. Patients in the chemotherapy arm will

receive standard chemotherapy (Procarbazine, CCNU and Vincristine) at standard doses and schedule.

The primary objective of the forthcoming trial will be to determine whether HSV1716 given by intratumoural administration is effective, as measured by overall survival. In addition, secondary objectives such as quality of life and progression free survival will be assessed. It is proposed that recruitment will be complete by 2008.

As this discussion makes clear, the design of trials to test the efficacy of HSV1716 in human high grade glioma is extremely complex. Therefore, given the constraints of current best practice in the management of high grade glioma, it seems improbably that the *in vitro* demonstration of enhanced cell kill will be translated immediately into clinical application. However, if the efficacy of HSV1716 or another oncolytic viral therapy is proved, it is to be hoped that the possible advantages of combining radiotherapy and viral therapy will be readdressed. In addition, further experimentation to elucidate the mechanism behind the enhanced cell kill should be pursued as it might help improve our understanding of how the virus interacts with tumour cells.

17 Interaction of Ionising Radiation and HSV1716 *in vitro*

The aim of the work covered in this section was to investigate, *in vitro*, the effect on tumour cell kill of combining ionising radiation and infection with the ICP34.5 null mutant HSV1716. When this project was started, data from *in vivo* experiments combining ICP34.5 null HSV infection with ionising radiation demonstrating enhanced cell kill had been published (Advani SJ *et al.*, 1998; Bradley J *et al.*, 1999). The purpose of this project was to develop an *in vitro* experimental strategy to corroborate this effect on cell kill, by combining ionising radiation with HSV1716 infection of tissue culture cells. In addition, it was intended to quantify any such effect on cell kill due to combining the treatments, as being infra-additive, additive or synergistic.

In initial *in vitro* experiments, clonogenic and spheroid growth delay assays proved to be unsuitable for assessing combination therapies involving replication competent virus. In these systems the effect of the viral infection was "all or nothing"; as soon as the titre of input virus was sufficient to be detectable , the infection spread to kill all of the cells. A cytotoxicity assay demonstrated that there was a linear relationship between the number of live cells and the MTS colorimetric reading. Subsequent experiments used cell densities within this linear range.

In addition to BHK and 3T6 cells, which were well characterised with respect to lytic replication of HSV1716, a number of cell lines, primarily of human high-grade glioma lineage, were assessed for their permissivity to HSV1716 infection and their response to ionising radiation. Multicycle growth experiments investigating HSV1716 replication in the human high-grade glioma cell lines MOG and 373 demonstrated that there was less efficient replication of HSV1716 than the parental wild type HSV17^{+}. As HSV1716 infection failed to kill all of these cells, under selected conditions, there was the potential for additional cell kill due to ionising radiation to be achieved.

In vitro experiments were conducted investigating the reduction in cell viability when cell monolayers were exposed to various doses of ionising radiation. High initial cell densities resulted in cells becoming confluent too early in the course of the experiment. When the cultures reached confluence too early, any reduction in cell viability as a result of radiation damage could not be detected. In some cases a reduction in cell viability was evident in the absence of radiation; probably the result of reduced nutrients in the cell growth medium. Lower seeding densities, which enabled cell division to continue for 6-7 days allowing the detection of cell kill generated by ionising radiation, were determined. It was established that for all of the cell lines used in this study, a cell density of $2\text{-}3x10^3$ cells per (96 well plate) well allowed cell division to continue for 5-7 days. Separate experiments also proved that this density was suitable to support HSV1716 infections over the same time span. As HSV1716 only replicates in actively dividing cells, continued replication of the cells ensured that cell lysis due to viral replication was also optimal, mimicking the conditions in tumour cells where the cell cycle checkpoints are disrupted. It was shown that multiplicities of infection (MOI) greater that 0.1pfu/cell, would quickly cause complete cell kill and prevent further cell proliferation. A MOI of 0.1pfu/cell was adopted for subsequent experiments.

Interestingly, it was observed that at very low cell densities the ability of HSV1716 to replicate was markedly reduced. The observation that cell density has a profound effect on the ability of HSV1716 to kill tissue culture cells is also likely to have an impact on its ability to kill high grade glioma *in vivo* in regions where the tumour cells are sparsely distributed. The *in vitro* data indicate that, because of the observed cell-to-cell spread, a much higher MOI may be required to kill sparsely distributed cells than densely packed permissive cells. This would indicate that the best strategy for administering a replication competent virus is probably to direct it to areas with large numbers of actively dividing cells and not to mop up tumour cells following tumour resection.

Although some cytotoxicity was noted following irradiation of the various cell lines in culture, it was minimal. It appeared that the cell lines were relatively radio-resistant which corroborates the findings of other groups (Acevedo-Duncan M *et al.*, 2001). Published data (Advani SJ *et al.*, 1998; Bradley J *et al.*, 1999)

demonstrating additional cell kill when ionising radiation and oncolytic HSV were combined, used an ionising radiation dose rate (typically 1.91Gy/min) faster than the dose rate generated by the Alcyon II Teletherapy Co^{60} source unit (0.3Gy/min). Hall and Brenner, (1994) demonstrated that lower dose rates, particularly between 0.001Gy/min and 1Gy/min, generated reduced cell kill due to sub-lethal damage being repaired during the protracted delivery of the radiation. However, reducing the distance between the Alcyon II Teletherapy Co^{60} source and the tissue culture cells, which increased the dose rate by a factor of four to 1.2Gy/min, failed to generate any further reduction in cell viability.

Published *in vivo* data showing enhanced cell kill with a ICP34.5 null HSV mutant and ionising radiation had been produced by infecting tumour xenografts with the oncolytic virus six hours prior to irradiation (Bradley J *et al.*, 1999). Initially this schedule was mirrored in the *in vitro* experiments. Therefore, it was necessary to ascertain if ionising radiation diminishes the ability of HSV1716 to generate a lytic infection *in vitro*. Multicycle growth experiments using HSV1716 irradiated with 5Gy did not demonstrate any reduction in oncolytic activity compared to non-irradiated HSV1716.

After confirming that the dose of ionising radiation of 5Gy did not diminish the lytic replication capability of HSV1716, experiments combining the two modalities were devised. It was shown that in BHK cells, which are fully permissive to HSV1716 infection, any additional cell kill generated by ionising radiation would be impossible to detect as the virus killed all of the cells irrespective of the radiation. Therefore further combination experiments sought to investigate if ionising radiation would result in cell kill due to HSV1716 lytic replication in 3T6 cells which are normally non-permissive. Ionising radiation did result in some delay in exponential cell growth but did not appear to increase the permissivity of 3T6 cells to HSV1716 replication.

As multicycle growth experiments in human glioma cell lines had shown that HSV1716 replication was impaired in 373 and to a lesser extent in MOG cells, it was decided to continue combination experiments with these two cell lines. However, in initial experiments mirroring the scheduling used in the published *in*

vivo work, where ionising radiation was administered six hours after virus inoculation, there was minimal cell kill due to ionising radiation. Under these conditions both $HSV17^{+}$ and HSV1716 caused complete cell kill within 7 days.

During the course of experiments with alternative scheduling of radiation and virus treatments to elicit additional cell kill, Blank *et al.*, published data demonstrating enhanced cell kill, *in vitro*, when ICP34.5 null HSV was delivered one hour after irradiation of cells (Blank S *et al.*, 2002). In light of this data, the scheduling of the delivery of ionising radiation and HSV1716 was altered. Experiments delivering 5Gy radiation prior to infection with 0.1pfu/cell of HSV1716, reproducibly demonstrated additional cell kill detected by the MTS assay, by day 7 in both the 373 and MOG cell lines. A higher dose of 20Gy ionising radiation combined with HSV1716 was investigated under the same experimental conditions and additional cell kill in MOG and 373 cells was demonstrated by day 6.

Large fraction sizes such as those employed in the above experiments are used in stereotactic radiotherapy. However, these doses are larger than those used in the normal clinical radiotherapy management of patients with high-grade glioma. Experiments were therefore conducted to compare the effect on the level of cell kill when the ionising radiation was delivered in a single high dose or following a more clinically relevant regime of daily 2Gy fractions. Ten Gray of radiation were delivered either as a single dose or in 2Gy fractions over 5 days. In combination experiments, additional cell kill was achieved by combining the radiation 1 hour prior to HSV1716 infection but there was no difference in the reduction in cell viability when 10Gy was delivered as a single dose or following the fractionated regime.

There was evidence in the published data that enhanced cell kill in combined radiation and HSV therapy was due to enhanced viral replication (Advani SJ *et al.*, 1998). Following the demonstration of additional cell kill when monolayers were infected with HSV1716 one hour after irradiation, an investigation was undertaken to determine if there was an increased viral yield from the irradiated cells. There was no evidence of increased virus yield assessed by multicycle

growth experiments in pre-irradiated compared to control non-irradiated MOG or 373 cells.

In the studies with HSV1716 it was noted that if the cells used to seed the 96 well plates were dissociated from fully confluent cultures, the cell kill generated by radiation and virus was reduced and no additive relationship between ionising radiation and HSV1716 could be detected. It has been postulated that ICP34.5 null HSV mutants rely on active host cell replication to provide the machinery required to maintain viral replication. Also when cells are irradiated, if they are confluent, contacted inhibited and therefore growth arrested, they are more liable to be in a radio-resistant phase of the cell cycle such as G_0. Therefore when a cell culture is actively replicating a significant proportion of the cells is in a phase of the cell cycle in which they are more sensitive to the effects of ionising radiation and viral replication.

Following the characterisation of experimental conditions under which additional cell kill could be demonstrated reproducibly, experiments were performed to investigate whether or not the additional cell kill was synergistic. As the dose response curves of the two modalities do not follow first order kinetics, it is not possible to demonstrate synergy by showing that the observed reduction in cell viability when the two modalities are combined, is greater than the sum of the two modalities used independently. Therefore, isobologram analysis was used to define the relationship between HSV1716 and ionising radiation in cell kill.

The isobologram of the 373 data demonstrated a region of additive cell kill between the Mode I and Mode IIb lines. Plots below the Mode IIb line are supportive of a synergistic relationship between HSV1716 and ionising radiation. Results from three MTS assay experiments, administering HSV716 one hour after ionising radiation, each at doses known to generate les than 50% cell kill independently, when plotted onto the isobologram fell on or below the Mode IIb line. These results indicate that there is a synergistic relationship between HSV1716 and ionising radiation when 373 cells are irradiated one hour prior to infection with 0.1pfu/cell HSV1716.

The isobologram of MOG cell data also demonstrates an envelope of additivity, which lies between the Mode IIa and Mode IIb line. Two MTS experiments combining HSV1716 one hour after ionising radiation were plotted onto the isobologram. Each modality was used at a dose which was known to generate less than 30% cell kill independently. Both plots lie within the region between the Mode IIa and Mode IIb lines and hence the relationship between HSV1716 and ionising radiation under these conditions is additive.

In 2002, when experimental conditions to demonstrate additive cell kill with HSV1716 and radiation were still being developed, Blank *et al.* published a paper including the first data demonstrating additive cell kill *in vitro*. They described experiments to assess the efficacy of four different ICP34.5 mutants of HSV-1 when combined with low-dose radiation. The mutants, 4009, 7020, 3616 and G207 were assessed in the human cervical squamous carcinoma cell lines Me180 and C33a. Their conclusions, were that there had been a greater than additive effect when two of the viruses were independently combined with ionising radiation and an additive relationship with a third. This warrants further discussion as it is the only comparable experimental report in the field.

The conditions that Blank *et al.* (2002) used to detect cell kill due to viral replication and ionising radiation were similar to the conditions used for HSV1716 in MOG and 373 cells. For example, $2x10^3$ cells were seeded per well of a 96 well plate. However, by day 3 in their system, the control cells were confluent and further analysis of cell kill discontinued. In the comparable studies, MOG and 373 cells did not reach confluence until day 6-7. It appears therefore that the human cervical cancer cell lines replicated at a significantly higher rate. This high replication rate may account for the high levels of cell kill they reported for the oncolytic virus alone.

Blank *et al.* (2002) used doses of radiation (1.5Gy or 3Gy) which, in pilot studies, demonstrated less that five-fold cell kill. Because their cell lines were extremely permissive to oncolysis, the dose of virus they used was an order of magnitude less than that used with HSV1716 in the glioma cell lines; a MOI of 0.01pfu/cell of R3616 killing 50% of Me180 cells by day 3. In fact, the C33a cell line proved

to be too permissive to infection to be of use in combination experiments; each of the four mutants at a MOI of 0.001pfu/cell demonstrating greater than 75% cell death by day three (although, interestingly, this did not translate to *in vivo* experiments where the injection of G207 or R3616 demonstrated only modest growth delay of tumours in xenograft models).

It was also reported by Blank *et al.* (2002) that radiation had been demonstrated to cause a 3-fold decrease in the replication of HSV1716. This conclusion was drawn from data by Lambright *et al.* (2002) who investigated the use of EJ-6-2Bam-6a cells as carriers of HSV1716, injuected into the peritoneal cavity to infect and lyse intraperitoneal disease. However, the growth experiment from which this conclusion was derived was extremely flawed. What was described as a single cycle growth curve, showed the yields of virus over forty eight hours from irradiated and unirradiated EJ-6-2-Bam-6a cells infected with 0.01pfu/cell of HSV1716. Not only did this represent multi-cycle virus replication rather than the single cycle replication claimed, but there was more than a 2 log difference in the titres of the 0 hour samples. In addition, the extrapolation is invalid as Lambright *et al.* treated their cells with 30Gy in order to sterilise them, making their data in no way comparable with the experiments presented in this thesis and in the paper by Blank *et al.*(2002).

Blank *et al.*(2002) assessed the cell kill in Me180 cells irradiated with 1.5Gy or 3Gy one hour prior to inoculation with the HSV mutants G207, R3616 or R7020. The multiplicities of infection ranged from 0.0001pfu/cell to 0.1pfu/cell. At 48 hours the cell kill generated by virus, radiation or the combination of the two modalities was assessed. The cell kill generated by each of the three viruses alone in Me180 cells was similar. Interestingly there did not appear to be any significant increase in cell killing with increasing MOI. This may be explained by the fact that, at 48 hours many cells which have already been infected and are destined to die are still alive. It should be noted that in the comparable experiments with HSV1716 infected MOG and 373 glioma cells, at 48 hours the MTS readings would remain high although the monolayers at the time showed a high proportion of rounded, swollen cells indicative of advanced viral infection. This observation supported the decision to allow the experiments to run for 6-7

days to ensure a true reflection of the toxicity generated by both viral infection and ionising radiation. The Me180 cell line appeared to be radiosensitive with a 33% reduction in cell viability by 48 hours with 1.5Gy and a 50% reduction with 3Gy.

When the oncolytic viruses R3616 and G207 were combined with ionising radiation the reduction in cell viability was greater than the sum of the cell viability reduction generated individually by either alone. With R3616 this observation was only detected when combined with 3Gy and not 1.5Gy. The cell kill generated when R7020 and ionising radiation were combined was no better than additive. The authors state that, as the cell kill generated by G207 and R3616 and ionising radiation was greater than the sum of the cell kill exerted by either of the modalities alone, the relationship is "more than additive". However, as these two models of cell kill do not follow first order kinetics and as analysis of cell kill at 48 hours was arguably too early to measure the true potential of either viral lysis or radiation, the conclusion of a supra-additive or synergistic relationship does not appear to be substantiated by their data.

The only other published *in vitro* study on the interaction between ionising radiation and oncolytic HSV, involved the mutant hrR3 (Spear M *et al.*, 2000). hrR3 is not deficient in ICP34.5 but contains an insertional mutation of *E.coli* lacZ cDNA into the ICP6 locus disrupting the expression of viral ribonucleotide reductase. Using an MTT assay, Spear *et al.* (2000) assessed the cell kill generated in tumour cells, *in vitro*, by the oncolytic virus hrR3 in combination with ionising radiation. Their data indicated that hrR3 is much more attenuated than HSV1716 and they therefore required a much higher MOI in their experiments; 5pfu/cell compared to the HSV1716 MOI of 0.1 pfu/cell. Cell monolayers were irradiated two hours prior to virus inoculation and the MTT assay was performed over the course of 72 hours to demonstrate any enhanced cell kill. As the duration of the experiment was only three days, it is surprising that the cell kill generated by ionising radiation alone was detectable by MTT assay. One possible explanation for this is that the dose rate in this experiment was 2.77Gy/min, considerably higher than the 0.3Gy/min delivered by the Cobalt source used in the experiments with HSV1716. The authors concluded that the

cell kill generated when ionising radiation and hrR3 were combined was greater than either modality on its own and that the relationship was synergistic. Once again, as they failed to take into account the non-linear relationships between cell death and radiation or lytic replication, their data are inconclusive.

Other research investigating cell kill when ICP34.5 null HSV is combined with ionising radiation has been conducted *in vivo*. In fact the premise behind this research project, that ionising radiation may augment the cell kill of HSV1716, was based on the observations of Advani *et al.* (1998) using R3616. They demonstrated a reduction in xenograft growth following injection of the ICP34.5 null HSV mutant, R3616, alone. However, they observed significant additional growth delay and even tumour eradication when R3616 was combined with ionising radiation. There was no evidence of tumour eradication using virus alone, despite the injection of high doses (up to $2x10^7$pfu). It must be noted that the ionising radiation doses used, 45Gy in two fractions over two days, is far in excess of clinically acceptable doses. It is possible that the ionising radiation at this dose resulted in virtual tumour sterilisation reducing the clonogenic potential of the tumour to a level where the virus was merely required to 'mop up' the small proportion of viable cells remaining. As doses of 20Gy are not clinically applicable, it remains to be proven that clinically relevant doses of radiation could be useful. The authors stated that preliminary experiments demonstrated that smaller radiation doses would also be effective. Unfortunately this data was not published. They did not quantify the effect on cell kill of combining R3616 and ionising radiation. They did, however, state that the observed delay in growth rates of the tumours treated with virus and ionising radiation was significantly greater than that of viral infection or irradiation alone.

The same group published data the following year, investigating the replication of the HSV mutant, R7020 (Advani SJ *et al.*, 1999). R7020 is a multi-mutated HSV vaccine candidate with the HSV-2 genes encoding glycoproteins G, J, D and I inserted and one copy of the long repeat region deleted from HSV-1 strain F DNA. The deletion removes one copy each of the genes $\alpha 0$, $\alpha 4$, $\gamma_1 34.5$,ORF P and ORF O and hence R7020 only encodes one copy of ICP34.5. Radio and

chemo-resistant cell line xenografts inoculated in this study were approximately four times the size of the xenografts investigated previously using the more attenuated ICP34.5 null mutant R3616. The docs of virus was slightly lower, $2x10^6$ pfu, and the radiation dose was reduced to 32Gy in 4Gy fractions over the course of two weeks. Once again they demonstrated that the growth delay when ionising radiation and virus are combined is significantly more than with either modality on its own. In their discussion the authors state that the effect on cell kill between R3616 and ionising radiation is synergistic. How this conclusion had been derived is not stated.

Further *in vivo* data, published prior to the commencement of the *in vitro* research with HSV1716, was presented by Bradley J *et al.* (1999). This group demonstrated delayed growth in hind-limb human U-87 malignant glioma xenografts when high doses ($1x10^7$ pfu) of ICP 34.5 null mutant, R3616, were combined with ionising radiation. None of the xenografts appeared to decrease in size, implying that the tumour growth matched or outstripped the rate of tumour destruction by R3616. Mice with intracranial xenografts treated with $1x10^7$ pfu of R3616 and ionising radiation, appeared to show improved survival compared to either modality on its own. Using statistical methods described by Machado and Bailey. (1985), based on a proportional hazards model, the authors claimed that the relationship was synergistic. None of the intracranial tumours were assessed to see if there had, in fact, been a decrease in the tumour burden, accounting for the increase in survival.

Since the commencement of the *in vitro* study, further data has been published investigating the *in vivo* interaction of various HSV mutants and ionising radiation in a range of different tumour cell lines. As the sensitivity of cells to oncolytic virus is a problem for *in vitro* analysis, further investigation of the interaction between HSV1716 and ionising radiation could involve the use of animal models and therefore it is pertinent to review this research.

Chung SM *et al.* (2002) investigated the interaction between the multi-mutated HSV-1 variant, R7020, and ionising radiation in hepatoma xenografts. In Huh7

tumour cell line xenografts there was minimal tumour regression. The authors suggested that this was, as had been described before, due to the Huh7 tumour growth outstripping R7020 viral lysis. In another hepatoma cell line, Hep3B cells, the cell kill caused by R7020 was enhanced by ionising radiation. They explained the reduced ability of R7020 to delay the growth of Huh7 xenografts as a consequence of a decrease in the ability of virus to gain entry into the cells. They believed that progress in understanding the cellular receptors responsible for HSV-1 entry into cells, should allow improved targeting to tumour cells. To date, HSV1716 has not been known to fail to gain entry into any tumour cell type and lack of replication, for instance in 3T6 cells, has not resulted from a failure to enter cells but rather from an inability to replicate and egress from the cells (Brown SM *et al.*, 1994b).

Jorgensen T *et al.* (2001) investigated the interaction of the ICP34.5 null mutant, G207, in LNCaP prostate xenografts. Although they found that delivering either G207 or radiation delayed tumour growth, they were unable to show any enhancement by combining the treatments. In these studies, G207 was not administered as intratumoural injection as this resulted in such extensive tumour lysis that a potential interaction with ionising radiation could not be detected. Instead the virus was administered intravenously, which it was argued would generate a less robust treatment response. During the *in vitro* investigations of response to radiation and HSV1716, attempts were made to reduce the efficiency of the viral infection, by using a cell line not fully permissive to HSV1716. This proved to be difficult because, although cell kill was enhanced when HSV1716 was combined with ionising radiation, HSV1716 independently was extremely effective in lysing the cells *in vitro* and if left for longer than six days would have probably completely eradicated the cell monolayer, independent of radiation.

Although evidence has been presented in this thesis and elsewhere for enhanced cell kill when replication competent HSV and ionising radiation are combined, the mechanism remains to be elucidated. As discussed previously, Blank *et al.* (2002) demonstrated *in vitro* that there was increased viral replication in Me180 cells treated with low dose radiation one hour prior to virus inoculation. The 48 hour

viral yield from the irradiated cells was 4.45 fold greater than from the non-irradiated cells.

The majority of the research to confirm the mechanism behind the observed relationship has, however, been performed *in vivo*. Viral yields from tumour xenografts treated with radiation and a variety of HSV mutants have been compared with yields from xenografts treated with virus alone. Advani SJ *et al.* (1999) reported that the yield of R3616 and R7020 obtained from irradiated xenografts was 2-5 times higher than that recovered from non-irradiated xenografts. They suggested that the observed increase in viral yields actually underestimated the amount of virus produced following irradiation, as *in situ* hybridisation studies of irradiated xenografts showed that the distribution of infected cells exhibiting viral DNA was greater and more distal to the point of injection than in non-irradiated xenografts. However, the elevated viral yield obtained from irradiated xenografts appears to be short lived. Two days after the peak in viral yield, the titre of virus recovered from irradiated and non-irradiated cells was identical. Bradley J *et al.* (1999) demonstrated enhanced viral proliferation by immunohistochemical staining of R3616 in irradiated intracranial tumours compared to controls. In their study the radiation was delivered as a fractionated schedule and they reported a 2-5 fold enhancement in viral proliferation. Stanziale S *et al.* (2002) showed that R7020 viral yields from Hep3B xenografts were 2.5 fold higher in the irradiated cells six hours after inoculation. This was not demonstrated in the less permissive Huh7 irradiated xenografts.

If the enhanced cell kill is due to an increase in viral replication and viral yield, by what mechanism does ionising radiation enhance the replication of the virus? Stanziale S *et al.* (2002) showed that R3616, deleted in both copies of ICP34.5, demonstrated greater independent cell kill than G207, which also has both copies of ICP34.5 deleted but in addition has an insertion of the *E.coli lac*Z gene disrupting expression of the viral ribonucleotide reductase (RR) gene ICP6. Radiation increased the cell kill of both oncolytic viruses but the increase in tumouricidal effect was greater in the cells treated with G207. Ionising radiation

has been shown to increase the levels of cellular RR and it is proposed that the enhanced levels of this protein within the irradiated tumour cells could enhance viral proliferation and subsequent oncolysis. Hydroxyurea, an inhibitor of RR, was demonstrated by Stanziale *et al.* to abrogate the enhanced replication of G207 in irradiated HCT-8 cells. Kuo and Kinsella. (1998) have confirmed an increase in cellular RR following irradiation and Blank *et al.* (2002) also propose that an increase in RR following irradiation complements the G207 deletion in ICP6.

Interestingly, Spear *et al.* (2002) failed to demonstrate an increase in hrR3 viral titre from irradiated pancreatic tumour cells compared to non irradiated cells, despite hrR3 being deficient in RR. They argued that possibly ionising radiation augments complementation of the small subunit of RR and not the large subunit, in which hrR3 is deficient.

Multicycle growth experiments to investigate the replication of HSV1716 in irradiated MOG and 373 cells failed to demonstrate an increase in viral yield compared to non-irradiated cells. The synergistic relationship between ionising radiation and HSV1716 in 373 cells cannot therefore be explained by enhanced viral replication. Chung *et al.* (2002) say that the mechanism by which ionising radiation enhanced the replication of R7020 remains unclear, but consider the relationship to be cell type dependent. Blank *et al.* (2002) also could not explain the mechanism by which ionising radiation enhanced the cell kill of the oncolytic viruses R3616 and R7020. As neither R3616 nor R7020 is deleted in RR, an elevation in cellular RR due to ionising radiation should not be of importance to these mutants. It is therefore probably that other cellular proteins are involved.

Stanziale *et al.* (2002) investigated, by Northern blot, the levels of GADD34 in HCT-8 cells following irradiation. They demonstrated that 2.5Gy increased the levels of GADD34 by 48 hours and that the level reduced slightly by 72 hours. Following 5Gy irradiation, the level of GADD34 at 48 hours was twice the level detected following 2.5Gy and this was seen to rise further by 72 hours. The GADD34 gene, encoding one of a group of growth arrest and DNA damage proteins, has been shown to share homology with the carboxyl domain of the

ICP34.5 gene RL1 (McGeoch DJ and Barnett BC, 1991). Therefore they proposed that the upregulated GADD34 may substitute for the lack of viral ICP34.5 and create an environment within the cell conducive to ICP34.5 null HSV.

It is of interest that, following 5Gy or radiation, the levels of GADD34 were highest at 72 hours. The fact that many research groups terminated their *in vitro* analyses at 72 hours, means that they could have missed any effect from the upregulation of proteins such as GADD34 and failed to take advantage of the enhanced intracellular environment created by ionising radiation. Isobologram analysis with HSV1716 and ionising radiation was performed following analysis at six days post irradiation, which may account for the detection of synergy.

It is likely that ionising radiation alters the expression of a number of different genes, as yet unidentified. It is conceivable that these changes could enhance viral entry, replication, assembly or egress of progeny, explaining the synergistic cell kill demonstrated with HSV1716 and ionising radiation. Irradiation of human colon carcinoma cells *in vitro* has been shown to significantly increase the uptake of adenovirus and improve gene transfer (Zhang M *et al.*, 2003). Since it has been shown that ionising radiation can produce cell membrane changes, Zhang *et al.* (2003) proposed that an increase in the number of tumour cell membrane receptors may account for the increased adenoviral uptake.

Ionising radiation has been demonstrated to increase the levels of cellular proteins that may facilitate the cell kill from oncolytic HSV mutants. *In vitro* analysis has demonstrated that the alterations to the levels of these proteins are transient (Stanziale S *et al.*, 2002). This phenomenon was also demonstrated in xenografts. Two days following the peak in viral yield difference, the viral titre was noted to be the same in the irradiated and non-irradiated xenografts. One explanation is that the cellular factors induced by the ionising radiation decrease over time and therefore cease to maintain high levels of viral replication. This raises the point that temporally fractionating the ionising radiation may cause the repeated induction of genes resulting in prolonged and accentuated gene expression. There

was, however, no difference in the yield of HSV1716 following the irradiation of *in vitro* cultures with a single 10Gy dose compared to the same dose fractionated equally over five days.

The cell kill generated when HSV1716 was combined with ionising radiation, was shown to be additive in one glioma cell line and synergistic in another. Whether an additive or synergistic response between these two modalities can be demonstrated *in vivo*, remains to be seen. Further research might involve the delivery of HSV1716 and ionising radiation to animal xenografts to determine if the observed *in vitro* relationships are translated to the *in vivo* situation. The majority of the *in vivo* research has involved the use of immunocompromised murine models; either athymic of SCID mice. Lambright *et al.* (2000) demonstrated that in immunocompetent mice the survival following injection of HSV1716 into intraperitoneal tumours in HSV immunised mice was identical to that of HSV naïve mice. It would be of interest to investigate, in immunocompetent mice, the relationship between HSV1716 and ionising radiation in xenografts. In addition, the radiation could be delivered in clinically relevant fraction sizes to help inform the direction of any clinical research.

Given that there is robust clinical data demonstrating that the injection of HSV1716 into the CNS of humans is safe, it may be possible to bypass *in vivo* animal model research. Instead a phase II clinical trial protocol could be conceived to test the safety and efficacy of combination therapy, using date from *in vitro* experiments to ensure optimal scheduling of the treatments. The demonstration that irradiation with 5Gy did not reduce the titre of HSV1716, indicates that there should be no reduction in efficacy if radiation and virus therapies are combined. Any additional advantage as a result of irradiating tumours prior to, or immediately following, virus injection might be measured as reduction in tumour bulk and/or prolonged survival. The ethics and the practical problems of designing such a trial are considerable, not least in light of the probable changes in accepted practice for chemotherapy in glioblastoma.

The data in this section show that an *in vitro* assay, using tumour cell lines to study the combination of HSV1716 infection and ionising radiation, was

successfully developed. The effect on cell kill of combining the treatments was assessed using isobologram analysis, which demonstrated an additive relationship in the MOG cell line and a synergistic relationship in the 373 cell line by six days. The results did not elucidate the mechanism(s) behind these relationships but, as irradiation of the cells did not result in an increase in viral yield, they differed from the findings of others demonstrating an increase in viral replication. These *in vitro* results and those of the preceding phase I clinical trial, support the development of a clinical trial using HSV1716 in combination with spatially and temporally specified doses of ionising radiation.

18 Generation and Characterisation of Stably Transfected Cell Lines Expressing ICP34.5 and ICP34.5-GFP

This section describes a project to investigate the effect on cell cycle regulation of expression of ICP34.5 in the absence of viral infection. The proposal was to engineer cells to express ICP34.5 and to investigate if this plasmid expressed protein would interact with PCNA to allow initiation of DNA replication. In non-dividing cells PCNA is predominantly located in the cytoplasm. PCNA in the nucleus is regulated by interacting proteins to allow only DNA repair, and not replication processes. The working hypothesis was that ICP34.5 interacts with replication inactive PCNA switching it to a replication active form. It appears that as part of the infection process, HSV needs the cellular replication machinery to be active before it can initiate its own replication. In non-dividing cells, as there is normally very little PCNA in the nucleus, HSV manages to recruit PCNA to the nucleus by inducing DNA damage. The PCNA recruited to repair the DNA damage is regulated to stop any DNA replication and the virus therefore requires the expression of ICP34.5 to switch the PCNA function to initiate replication.

If time had allowed, the intention for this project was to investigate the effect of ionising radiation on 3T6 cells expressing ICP34.5 or ICP34.5-GFP. This would have permitted the study of PCNA, recruited to the sites of DNA damage similar to those caused by HSV infection, but without all of the concomitant effects of viral infection. It was hoped to demonstrate if the ICP34.5 expressed in the transfected cells interacted with the PCNA recruited to the sites of DNA damage, and, if so, what effects the interaction produced.

In this study 3T6 cells were successfully transfected to produce stable expression of ICP34.5 or ICP34.5-GFP. Review of the literature failed to show any data using ICP34.5 transfected cell lines. Mao *et al*, (2003) stated that the best approach for studying the effects of different ICP34.5 protein variants on the tissue culture behaviour of HSV-1 would be to develop stable cell lines that express the ICP34.5 protein. However, they were unable to establish stably

transfected cell lines and proposed that the expression of ICP34.5 exerted a toxic or growth limiting effect on the cells.

During the generation and maintenance of the stably transfected 3T6 cells there was no observed growth limiting or toxic effect attributable to the expression of ICP34.5 or ICP34.5-GFP. However, in BHK cells transfected under the same conditions with pc4-34.5 or pc4-34.5-GFP there was only transient expression of ICP34.5 and ICP34.5-GFP detected by Western blotting (data not shown). Subsequent Western blot analysis failed to confirm ICP34.5 or ICP34.5-GFP expression although the cells continued to exhibit Zeocin resistance. As the Zeocin resistance and the ICP34.5 expressing genes were in the same plasmid, the selective pressure required to favour the survival only those Zeocin resistant cells which failed to express ICP34.5, indicates a strong survival disadvantage for constitutive expression of ICP34.5 in BHK cells. It is possible therefore that in BHK cells expression of ICP34.5 caused toxicity similar to that described by Mao *et al.* (2003).

The demonstration that the expressed ICP34.5 could complement the defect in HSV1716 shows that the expressed protein is functionally active. The ability to stably express functionally active ICP34.5 in cells could aid future research in elucidating the mechanism of action of ICP34.5 in the replication cycle of HSV.

The cell cycle is tightly controlled by a complex molecular mechanism, governed by a series of structurally related serine/threonine protein kinases, which consist of catalytic subunits, known as cyclin-dependent kinases (Cdks), and regulatory subunits, cyclins. Several types of cyclins are made, and their expression and the activation of the different cyclin-Cdk complexes at different points of the cell cycle are required for cell cycling to occur. Different stages of the cell cycle have internal checkpoints and progression is regulated by activating or inhibiting phosphorylation of the Cdk subunits (Akashi M *et al.*, 1999).

An important mechanism in the maintenance of genomic integrity in response to DNA damage relies on the ability of a cell to induce cell cycle arrest. Delays in progression through the cell cycle allow time for the repair of damaged DNA, thereby preventing the replication and propagation of genetic mutations. When cells are exposed to DNA damaging agents, negative regulation of the cell cycle occurs; Cdk activities are inhibited and the cell cycle is arrested in either G_1 or G_2 phase, thus preventing the cells from entering the next stage of the cell cycle before their DNA is repaired. The main cellular factors activated by DNA damage which interfere with the cell cycle controls are: p53, delaying the transition through the G_1-S boundary; p21WAF/Cip1, preventing the entrance into S phase and; proliferating cell nuclear antigen (PCNA) and the replication protein A (RPA), blocking DNA replication (Loher HD, 1996).

DNA repair machinery has evolved to maintain genomic integrity after various kinds of DNA damage. Nucleotide excision repair eliminates pyrimidine dimers caused by UV light. Base excision repair targets base modifications caused by DNA hydrolysis and alkylation. Mismatch repair corrects errors of DNA replication. DNA strand breaks are remedied by DNA recombination using unbroken alleles as templates (Liu Y and Kulesz-Martin M, 2001).

Single-strand breaks (SSBs) in the backbone of one strand of a DNA duplex can be caused by exposure to many environmental factors including ionising radiation. If not repaired, SSBs can be converted into potentially lethal double-strand breaks during chromosomal replication. In general, two routes for SSB repair, distinguished by their DNA polymerase and DNA ligase usage, occur in mammalian cells. Polβ-XRCC1-Lig3 is thought to conduct both short-patch and long-patch repair, with Polδ/ε-PCNA-Lig1, operating primarily during S phase, conducting long-patch (2-7nt gap filling) repair. To avoid DNA replication being re-initiated before the SSB in the template strand is removed, replication-coupled SSB repair and replication-restart are tightly coordinated through a switching mechanism involving PCNA function (Caldecott K, 2001).

PCNA, a processivity factor for DNA polymerase δ and ε, is involved in DNA replication as well as being an integral component of the diverse DNA repair pathways; nucleotide excision repair, base excision repair and mismatch repair (Karmakar P *et al.*, 2001). In S phase, PCNA forms a toroidal trimer with replication factor-C and DNA and enables the loading of DNA polymerase δ and ε onto the complex. Two forms of PCNA exist in cells: (i) a detergent-insoluble trimeric form stably associated with the replicating forks during S phase and (ii) a soluble form in quiescent cells in G_1 and G_2 phases. Treatment of quiescent cells with DNA damaging agents such as ionizing radiation or HSV infection, triggers the redistribution of PCNA from a soluble to an insoluble chromatin-bound complex analogous to that found in S phase cells. A rapid assembly of chromatin-bound PCNA is observed soon after IR, which disappears from the majority of foci by 6 hours after treatment. It is thought that when PCNA is recruited to sites of DNA damage it is regulated by interacting proteins to allow only repair functions. When the repair is complete the PCNA function may switch back to DNA replication (Balajee A *et al.*, 2001).

It has been demonstrated that ICP34.5 complexes with PCNA (Brown SM *et al.*, 1997). The authors proposed that ICP34.5 interacts with PCNA to activate the cellular replication machinery to allow initiation of viral replication. It has also been demonstrated through immunofluorescence studies that ICP34.5 co-localises with PCNA in the cell nucleus at early stages of HSV infection and that later ICP34.5 accumulates in the cytoplasm (Harland J *et al.*, 2003). It was of interest to investigate if the ICP34.5 expressed in the transfected cells would influence cellular DNA replication. However, as expected the 3T6 cells expressing either ICP34.5 or ICP34.5-GFP were growth arrested by serum depletion and did not exhibit PCNA bound to the DNA.

Future experiments should investigate the distribution of PCNA in 3T6 cells following IR damage to DNA. In confluent 3T6 cells it would be expected that PCNA would be recruited to the sites of DNA damage whilst IR induced DNA repair was underway. To test the hypothesis that the presence of ICP34.5 might prevent PCNA from switching to a repair only mode, it should be possible to

investigate the effect of IR induced DNA damage in ICP34.5 transfected cells. This will give insight into the possible mechanisms of interaction between ionising radiation and viral damage.

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20 Appendices

1.Mini Mental State

1 Date
2 Address or set of numbers as a test of memory immediate
3 Place
4 Home address
5 Date of birth
6 Date of the First World War
7 Name of present monarch
8 Counting back from 100 by subtracting 7
9 What has been happening in the news
10 Repeat of address or set of numbers given at the beginning

2. Karnofsky Score

1 100 – Normal; no complaints; no evidence of disease.
2 90 - Able to carry on normal activity, minor signs or symptoms of disease.
3 80 - Normal activity with effort; some signs or symptoms of disease.
4 70 – Cares for self, unable to carry on normal activity or to do active work.
5 60 – requires occasional assistance, but is able to care for most of own needs.
6 50 – Requires considerable assistance and frequent medical care.
7 40 – Disabled, requires special care and assistance.
8 30 – Severely disabled, hospitalisation indicated although death not imminent.
9 20 – Very sick; hospitalisation necessary, active, supportive treatment necessary.
10 10 – Moribund, fatal process progressing rapidly.
11 0 – Dead

(Karnofsky D *et al.*, 1949)

3. Barthel Score

1	Bowels	0	incontinent
		2	occasional accident
		3	continent
4	Bladder	0	incontinent or catheterised and unable to manage
		5	occasional accident (maximum 1x 24 hours)
		6	continent (for over 7 days)
7	Grooming	0	needs help
		8	independent, face / hair / teeth / shaving
9	Toilet use	0	dependent
		10	needs some help but can do something
		11	independent (on and off, dressing, wiping)
12	Feeding	0	unable
		13	needs help cutting, spreading butter etc
		14	independent
15	Transfer	0	unable
		16	major help (1-2 people, physical)
		17	minor help (verbal or physical)
		18	independent
19	Mobility	0	immobile
		20	wheel chair independent including corners etc
		21	walks with help of one person (verbal or physical)
		22	independent (but may use any aid, e.g. stick)
23	Dressing	0	dependent
		24	needs help, but can do about half unaided
		25	independent
26	Stairs	0	unable
		27	needs help (verbal, physical, carrying aid)
		28	independent up and down
29	Bathing	0	dependent
		1	independent