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**Radiobiological and magnetic resonance studies of combined radiation
and cisplatin therapy in the 9L rat brain tumour model**

by

David E. Wilkins, MSc(A)

**A thesis submitted to the
Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of**

Doctor of Philosophy

**Department of Physics
Carleton University
Ottawa-Carleton Institute of Physics
Ottawa, Ontario
February, 1993**

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ABSTRACT

The prognosis for adult patients with primary malignant brain tumours is poor. Radiation therapy is a standard adjuvant to surgery in the treatment of these patients, but is rarely curative. The extreme radioresistance of primary malignant brain tumours is due in part to their enhanced capacity for repair of potentially lethal radiation damage. The chemotherapeutic agent cisplatin has been shown to inhibit repair of radiation damage. Therefore combined cisplatin and radiation therapy could be a key to enhanced therapeutic gain in the treatment of primary malignant brain tumours.

In this project, the 9L rat brain tumour model was used to investigate combined radiation and cisplatin treatments. In vitro experiments showed the 9L cell line to be highly radioresistant and, like human malignant brain tumour cells, to have a high capacity for repair of potentially lethal radiation damage. These cells were found to be moderately resistant to the cytotoxic effects of cisplatin. In vitro exposure to cisplatin at clinically relevant concentrations caused inhibition of potentially lethal damage repair, with the amount of inhibition depending on cisplatin dose and treatment sequence.

Magnetic resonance imaging (MRI) was used to monitor the effects of combined radiation and cisplatin treatments of implanted intracranial 9L tumours in rats. A new technique for implanting experimental tumours was developed which resulted in more uniform tumour growth, and methods for radiation and cisplatin treatment of experimental intracranial tumours were developed and evaluated. Non-invasive measurements of tumour size using MRI were found to correlate well with measurements made from histological sections. Intraperitoneal administration of gadolinium contrast agent immediately before T1-weighted MRI was shown to be the most accurate and reliable method for MRI measurement of intracranial tumour size.

The capability of MRI to provide early indications of radiation injury to normal brain tissue was evaluated in the rat, and MRI changes were found to occur on average 130 days following partial brain irradiation. Combined radiation and cisplatin treatments of intracranial 9L tumours did not result in tumour regression observable by MRI, despite histopathological evidence of increased tumour necrosis compared with radiation or cisplatin treatments alone.

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LIST OF ABBREVIATIONS

AUC	area under concentration vs time curve, a measure of drug exposure
BBB	blood-brain barrier
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea, a chemotherapy drug
CDDP	cis-diaminedichloroplatinum(II), or cisplatin, a chemotherapy drug
CFA	colony-forming assay
cis	cisplatin
cis-DDP	cisplatin
CNR	contrast-to-noise ratio in MR images
CNS	central nervous system
Gd-DTPA	gadolinium diethylenetriamine pentaacetic acid, a MRI contrast reagent
Gy	Gray, unit of absorbed radiation dose
IB	integrated brightness, a measure of signal intensity in region of a MR image
i.c.	intracranial
i.p.	intraperitoneal
LDR	low dose rate irradiation
MR	magnetic resonance
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NMR	nuclear magnetic resonance
NRC	National Research Council of Canada
ORCC	Ottawa Regional Cancer Centre
PD	proton density
PLDR	potentially lethal damage repair
s.c.	subcutaneous
SEM	standard error of the mean
SLDR	sub-lethal damage repair
SF	surviving fraction
SNR	signal-to-noise ratio
T	Tesla, unit of magnetic field strength
T1	NMR spin-lattice relaxation time
T2	NMR spin-spin relaxation time
TE	echo time, the interval between echos in a spin-echo imaging experiment
TR	repetition time, the time between repetitions of a MR pulse sequence
X	cobalt-60 gamma radiation

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1.0 INTRODUCTION

1.1 Primary malignant brain cancer

The National Cancer Institute of Canada predicts that 2030 primary brain tumours will be diagnosed in Canada in 1992, and 70% of these will be fatal (NCIC 1992). Brain cancer is the twelfth (thirteenth) most common type of cancer incidence in males (females), but its poor prognosis results in it being ranked the tenth (ninth) most common cause of cancer mortality. Brain cancer accounts for 1.8% of cancer incidence in Canada, but 2.5% of cancer deaths. In children, brain cancer accounts for 17% of cancer incidence, and 24% of cancer mortality. This high pediatric component, together with a significant incidence among young and middle-aged adults, causes brain cancer to be ranked seventh of all cancers in person-years of life lost (NCIC 1992). No clear environmental or genetic factors have been linked with the majority of brain tumours (McKeran 1990).

The prognosis for patients with primary malignant brain tumours is poor. The five-year survival rate is 25%, compared with 50% for all cancers (NCIC 1992). Anaplastic astrocytoma and glioblastoma multiforme, which together account for more than two-thirds of all adult primary malignant brain tumours (Sherbet 1987, Seeger 1992), have five-year survival rates of 18-48% and 0-10% respectively (NCI PDQ 1992). Primary brain tumours have an impact beyond their relatively low rate of incidence because of peaks in incidence in the first and fifth decades of life, and because of their swift progression. Patients in the prime of life are often rapidly disabled by loss of intellectual function, paralysis, personality changes, headaches, epilepsy, dysphasia and loss of vision. Diagnosis remains difficult because of the non-specificity of symptoms; the delay between the time a patient becomes aware of an initial symptom of a brain tumour and the time of histological diagnosis averages 1.65 years (Thomas 1990). The five-year survival rate for primary brain tumour patients has not changed significantly over the past 20 years (NCIC 1992). Improvements in the methods of treatment of primary malignant brain tumours are clearly needed.

1.2 Current treatment approaches for primary brain tumours

1.2.1 Surgery

Extracranial metastases from primary brain tumours are extremely rare (Sherbet 1987), so the success of treatment depends on the response of the primary lesion. Surgery is a mainstay in the treatment of most malignant brain tumours. Surgery alone is not curative, but is directed at several important objectives (Bradford 1990): providing a sample for histological diagnosis; debulking of the tumour for symptom relief; tumour volume reduction to provide additional time for adjuvant therapy; and induction of active cycling in remaining cells to increase sensitivity to adjuvant radiotherapy and chemotherapy.

Surgical removal of intracranial neoplasms is fundamentally different from surgery of tumours in other sites. In the confined space inside the skull, approximately 100 grams of tumour is lethal due to increased intracranial pressure. Neurological symptoms are usually absent until the tumour is 30 - 60 g in size (Shapiro 1986). In this context, the distinction between benign and malignant intracranial lesions is less important than for visceral tumours -- benign brain tumours which are not completely removed are lethal. Cancer surgery normally involves removal of generous normal tissue margins surrounding the tumour, which in most sites causes minimal functional disruption. Neurosurgeons removing intracranial neoplasms must frequently leave tumour-infiltrated brain tissue, because its removal would cause unacceptable loss of neurologic function. Surgery typically removes only 20 - 90% of a malignant intracranial neoplasm (Shapiro 1986a); without adjuvant therapy, recurrence is inevitable and mortality is 50% within 3 - 6 months (Jelsma 1969, Walker 1978).

1.2.2 Radiotherapy

Radiotherapy is a standard adjuvant to surgery in the treatment of anaplastic astrocytoma and glioblastoma multiforme in adults (NCI PDQ 1992), usually delivered in 1.8 - 2 Gy daily fractions to a total dose of 60 Gy (Leibel 1991). The dose which can be delivered to brain tumours is limited by the tolerance of surrounding normal brain. Late delayed radiation injury to the brain is progressive, irreversible, often fatal, and represents the major dose-limiting complication of radiation therapy of malignant gliomas (Constone

1988, Pezner 1981). This constraint on total dose, coupled with the high intrinsic radiation resistance of gliomas (Gerweck 1977, Raaphorst 1989), limits the ability of radiotherapy to effect a cure.

Incompletely resected malignant gliomas treated with 60 Gy radiation usually recur within one year of surgery (Burger 1986). Anaplastic astrocytoma treated with surgery alone is associated with a 5-year survival rate of 0%; adjuvant radiotherapy increases this to approximately 20% (Leibel 1990). The 5-year survival rate of patients with glioblastoma multiforme remains 0% even with adjuvant radiotherapy, but the median survival time is increased by approximately 20 weeks with 60 Gy compared with surgery alone (Walker 1978, Kristiansen 1981). While radiotherapy provides several months of additional symptom-free survival, the prognosis for these patients remains poor.

1.2.3 Chemotherapy

The limited success of surgery and radiotherapy has led to the inclusion of adjuvant chemotherapy in the treatment of many primary malignant brain tumour patients. Multimodal therapy consisting of cytoreductive surgery, radiotherapy and chemotherapy has been actively investigated for the past 25 years. A large number of agents and regimens have been subjected to clinical trials, with no regimen emerging with dramatic effectiveness and acceptable toxicity (Dropcho 1990). The nitrosourea compounds (BCNU, CCNU, methyl-CCNU) have been the most commonly used agents for malignant gliomas, with a wide range of reported efficacy. One extensive study of the addition of BCNU to surgery and radiation therapy increased the median patient survival from 40 weeks to 50 weeks, and significantly increased the proportion of "long-term" (18 months) survivors (Green 1983). Other agents used in the treatment of primary brain tumours include procarbazine, 5-fluorouracil (5-FU), epipodophyllotoxin (VM-26), etoposide (VP-16), methotrexate, and cisplatin (CDDP). While present-day multimodal therapy can treat primary malignant brain tumours, permitting many patients to live longer with better quality of life, current therapy rarely produces a cure (Shapiro 1986a).

1.3 Combined radiation and cisplatin treatment of cancer

The high radioresistance of malignant gliomas and their local persistence after radiotherapy has been attributed to hypoxic cells (Nelson 1986), which are approximately three times more radioresistant than oxygenated cells (Hall 1988). Glioblastoma multiforme characteristically contains regions of necrosis which likely harbour hypoxic but viable cells (Burger 1986). However, a priority of cytoreductive surgery is the removal of these regions (Bradford 1990). Experiments with administration of hyperbaric oxygen prior to radiotherapy of malignant gliomas (Chang 1982), and with the hypoxic cell sensitizer misonidozole (Gutin 1980), have failed to show significant benefit (Nelson 1986, Leibel 1990). This suggests the radioresistance of malignant gliomas results from intrinsic cellular resistance (Gerweck 1977, Raaphorst 1989) more than hypoxic cell fractions.

Enhanced capacity for repair of potentially lethal radiation damage has been implicated in the extreme radioresistance of human malignant gliomas (Weichselbaum 1982, Weichselbaum 1986) and other radioresistant tumours (Weichselbaum 1988, Schwartz 1990). Agents which inhibit potentially lethal damage repair (PLDR) following irradiation could therefore improve the therapeutic response of radioresistant, repair-proficient tumours such as malignant gliomas. The chemotherapeutic agent cisplatin (cis-diaminedichloroplatinum (II)) has been shown to inhibit repair of radiation damage (Dewit 1987, Douple 1990), and thus could be a key to enhanced therapeutic gain in the treatment of malignant gliomas.

Cisplatin is one of the most important chemotherapy agents to enter clinical practice in the last 20 years (Dewit 1987, Borch 1987). Since the first report of its anti-tumour activity (Rosenberg 1969), and its licensing for clinical administration in 1979, it has been extensively used as a single agent or in combination with other drugs, with success in the control of testicular (McBrien 1986), epithelial ovarian (Avarete 1990), and squamous head and neck cancer (Douple 1990). The dose-limiting side effect of cisplatin treatment is nephrotoxicity; nausea and vomiting, ototoxicity, and peripheral neuropathy are also serious side effects (McBrien 1986). New analogues of cisplatin, such as carboplatin and paraplatin, appear to have similar antitumour activity but milder side-effects (Coughlin 1989, Skov 1991).

Cisplatin is a square planar molecule (MW = 300) which is believed to derive its antitumour activity from the formation of adducts and crosslinks with DNA (Pinto 1985, Eastman 1986). The cisplatin-DNA intrastrand crosslink between the N-7 position of adjacent guanine bases is the most abundant cisplatin-DNA product and is thought to be the

lesion responsible for mammalian cell death (Caradonna 1984, Eastman 1984, Douple 1990). Figure 1.3:1 schematically diagrams the cisplatin molecule and its DNA adducts.

The study of the radiosensitizing activity of cisplatin began with the first report of its interaction with radiation in mice (Zák 1971). Many *in vitro* studies have shown cisplatin radiosensitization through a change in the shoulder region of the radiation survival curve, which is often interpreted as being related to radiation repair processes (Alvarez 1978, Begg 1987, Douple 1990). Split-dose experiments have shown that cisplatin can inhibit the *in vitro* repair of sub-lethal radiation damage (SLDR) (Carde 1981, Schwachofer 1991). Several *in vitro* studies have shown modest inhibition of PLDR in mammalian cells at clinically relevant cisplatin doses (Carde 1981, Douple 1985, Dritschilo 1979, Momiki 1987, Wallner 1987). While a model analogous to the molecular theory of cell survival for radiation (Chadwick 1973) has been proposed for cisplatin radiosensitization (Chadwick 1976), the mechanism of inhibition of cellular radiation repair processes by cisplatin is complex and has not been clearly established (Coughlin 1989, Dewit 1987, Picker 1987, Schwachofer 1991).

In vivo studies of cisplatin-radiation interactions in experimental tumours have usually shown a radiosensitizing or supra-additive effect, although reports of no radiosensitization have also been published (DaSilva 1984, Höglmeier 1985, Stephens 1986). Some investigators have attributed cisplatin-radiation interactions to hypoxic cell sensitization (Douple 1979, Douple 1982); others have identified inhibition of SLDR as the mechanism of the interaction (Fu 1985, Kanazawa 1988). While *in vitro* studies have generally shown maximum interaction when cisplatin is administered at the time of irradiation (Dewit 1987, Schwachofer 1991), *in vivo* studies in experimental tumours have come to no consensus regarding the optimum treatment sequence (Coughlin 1989, Tanake 1987, Twentyman 1979).

Clinical trials of combined radiation and cisplatin treatment have generally reported improved response rates (Coughlin 1985, Coughlin 1989, Fountzilas 1991, Rosenthal 1991), but the outcome appears to be independent of any particular sequence of treatment, at least for the sequences studied to date (Coughlin 1989, Dewit 1987). Combined cisplatin and radiotherapy has been attempted in brain tumours but toxicity has limited the efficacy of the treatment (Eapen 1990, Feun 1983, Walker 1989). A large randomized trial of malignant glioma patients concluded that cisplatin did not enhance the effects of radiotherapy (EORTC 1991), but intermittent administration and relatively low doses of cisplatin were probably the reason for the lack of radiosensitization. High individual cisplatin doses concurrent with radiation fractions are likely to be most efficacious (Coughlin 1989). In order to guide the design of meaningful clinical trials, the use of

experimental tumour systems is necessary to elucidate the mechanisms of cisplatin radiosensitization and repair inhibition, the timescale over which these mechanisms operate, and optimum treatment sequence (Coughlin 1989, Dewit 1987).

1.4 MR imaging in the study and management of brain tumours

Since its introduction into the clinical setting in the early 1980's, MRI has become the method of choice for imaging the central nervous system. Superior soft tissue contrast and excellent resolution allow diagnosis, surgical planning, and treatment evaluation of patients with brain tumours. Following cerebral radiotherapy for brain tumours, many patients develop late radiation injury to brain tissue surrounding the tumour (Constine 1988). This injury represents the major dose-limiting complication for radiotherapy of brain tumours (Pezner 1981, Larsson 1992). Changes in MR images accompanying this injury have been observed clinically (Constine 1988, Valk 1990, Lindquist 1992) and in animal studies (Bernstein 1990, Lo 1992). MR imaging is potentially a useful technique in the study of the time course of development of late radiation injury, and in the management of patients who might develop this debilitating and often fatal complication of cerebral radiotherapy.

Traditional studies of the response of experimental tumours to treatment often use tumour volume as the measured parameter, estimated from caliper measurements of flank tumour diameter (von der Maase 1985, Gridley 1985). This technique is not applicable to animal models of brain cancer involving intracranial tumours; measurement after surgical resection, or survival of the animal are the usual endpoints. Resection of intracranial tumours in rats is technically difficult and subject to large errors (Barker 1973), while survival studies require every animal to suffer pain and discomfort. Both techniques provide only one measurement per animal. Magnetic resonance imaging of intracranial experimental tumours provides the opportunity to make non-invasive, serial measurements of tumour size in response to treatment, reducing the number of animals required for such research, and their suffering. MR imaging can therefore play an invaluable role in the study of new treatments for brain tumours.

1.5 Hypotheses and specific aims

The radiobiological and magnetic resonance investigations of cisplatin radiosensitization in the 9L rat brain tumour model undertaken in this project were directed at the following hypotheses:

1. In vitro exposure to cisplatin at clinically relevant concentrations causes radiosensitization by inhibition of repair of potentially lethal radiation damage, and this inhibition depends on the sequence of drug exposure and irradiation.
2. Magnetic resonance imaging provides accurate measurements of tumour size in the rat brain which agree with histological measurements. Serial MRI measurements of intracranial tumour size in individual animals can be used to monitor the effects of therapy.
3. Magnetic resonance imaging provides morphological information of predictive value in assessing the response of tumour and normal brain tissue to radiation and cisplatin therapy.
4. Concurrent in vivo cisplatin and radiation therapy of intracranial 9L rat brain tumours results in greater therapeutic response than either radiation or cisplatin alone.

The examination of these hypotheses was accomplished through an experimental program with the following specific aims:

- measurement of the in vitro radiation response of the 9L cell line, to establish that it represents a good model for the radiation response of human malignant gliomas;
- measurement of the in vitro response of 9L cells to cisplatin, to determine the relative resistance of these cells compared with other cell lines;

- measurement of the in vitro time course of potentially lethal damage repair following irradiation of 9L cells, and comparison with a human malignant glioma cell line;
- determination of the inhibition of potentially lethal damage repair in 9L cells following in vitro administration of cisplatin at 3, 4.5, and 6 $\mu\text{g/ml}$;
- investigation of the dependence of the sequence and timing of in vitro cisplatin administration relative to irradiation in the inhibition of potentially lethal damage repair;
- development and optimization of techniques for the implantation of intracranial 9L brain tumours in Fischer-344 rats, and characterization of the growth of these tumours using histopathological methods;
- development and optimization of techniques for in vivo magnetic resonance imaging of intracranial 9L rat brain tumours, and characterization of their MRI appearance and growth;
- comparison of MRI measurements of intracranial tumour size with measurements using histopathological methods;
- development and optimization of techniques for in vivo radiation therapy and cisplatin treatment of intracranial 9L rat brain tumours;
- investigation of MR imaging of radiation injury to normal rat brain tissue, to determine whether MRI can provide early indications of this dose-limiting complication of intracranial radiotherapy;
- serial MRI measurement of intracranial 9L brain tumour size in rats treated with radiation alone, cisplatin alone, and combined radiation and cisplatin, to determine whether in vitro cisplatin inhibition of potentially lethal damage repair translates into enhanced therapeutic gain in vivo.

This program of experimentation was aimed at several important questions which have not been adequately addressed in the literature:

- While inhibition of sub-lethal and potentially lethal damage repair by cisplatin has been reported in a number of in vitro studies, it is not universally supported in the literature (Coughlin 1989, Dewit 1987, Picker 1987). Cell cycle redistribution (Begg 1986, Tanabe 1987), hypoxic cell sensitization (Dewit 1987), and one-electron reduction of cisplatin leading to an active radiolytic product (Coughlin 1989, Richmond 1991), have also been suggested as possible mechanisms for the radiosensitizing effect of cisplatin.
- There are no published reports of cisplatin inhibition of PLDR in a repair-proficient cell line. Most investigators have demonstrated inhibition in cell lines with a recovery ratio < 5 (Carde 1981, Momiki 1987, Wallner 1987), or in V-79 cells, with a recovery ratio ~ 10 (Douple 1985, Dritschilo 1979). The 9L cell line, like human malignant glioma cell lines, exhibits a recovery ratio of approximately 20 at 18 Gy. This proficiency in PLDR is an important factor in the poor radiocurability of human malignant gliomas (Weichselbaum 1982, Weichselbaum 1986).
- No consensus has been reached on the optimum sequence of treatment for cisplatin radiosensitization. Studies on experimental tumours have produced conflicting results (Coughlin 1989, Tanake 1987, Twentyman 1979), while the outcome of clinical trials appears to be independent of any particular sequence (Coughlin 1989, Dewit 1987).
- There are no published reports of combined cisplatin and radiotherapy in an intracranial brain tumour model. Experimental tumours in extracranial sites are poor models for brain tumours because of the privileged vasculature of the brain, which affects oxygen and drug delivery to the tumour. In particular, studies are lacking in which in vitro experiments on combined cisplatin-radiation treatments have been carried into the in vivo situation using the same cell line.
- Resistance to chemotherapeutic drugs is a major cause of treatment failure. The multidrug resistance phenomenon is not likely involved in cisplatin resistance (Canon 1990, Carmichael 1991); increased removal of cisplatin-DNA adducts (Micetich 1983), decreased cellular drug uptake (Tashiro 1992), and increased capacity for repair of DNA damage (Canon 1990, Eastman 1988, Masuda 1988, Meijer 1990) are possible

mechanisms. Cross resistance between cisplatin and radiation has been observed in some cell lines (Behrens 1987, Louie 1985, Schwartz 1988, de Pooter 1991), but not in others (Wallner 1987). It has been postulated that radiosensitization by cisplatin occurs only in cells which are inherently sensitive to the cytotoxic effects of cisplatin (Coughlin 1989). Further experimental evidence is needed to clarify patterns of cross resistance, and to elucidate possible mechanisms of cisplatin radiosensitization.

Figure 1.3:1. The structure of cisplatin and its binding to DNA. The *cis* configuration of diaminedichloroplatinum(II) is active against tumours; the *trans* configuration has no antitumour activity. Four cisplatin-DNA adducts are shown: the monofunctional adduct; the DNA protein crosslink; the DNA interstrand crosslink; and the DNA intrastrand crosslink. The intrastrand crosslink between the N-7 positions of adjacent guanine bases account for more than 60% of cisplatin-DNA adducts (Eastman 1984), and is thought to be the primary lethal lesion in mammalian cells (Douple 1990).

2.0 MATERIALS AND METHODS

2.1 In Vitro Methods

2.1.1 The 9L brain tumour model system

The 9L cell line was originally established by investigators at the Massachusetts General Hospital in 1967. A total of 108 rats (60 Wistar, 48 Fischer) were given weekly injections of N-nitrosomethyl urea for 36 weeks. Primary brain tumours developed in 22 of these animals, and 11 were successfully propagated in cell culture (Benda 1971, Schmidek 1971). One such cultured tumour was frozen and sent to the Brain Tumor Research Center in San Francisco in 1968, where it was passaged as a flank tumour in a Fischer-344 rat and developed as a cell line (Barker 1986).

The tumours resulting from intracranial inoculation of 9L cells have been variously described as a malignant astrocytoma (Barker 1973), mixed glioblastoma multiforme and gliosarcoma, mixed glioma and fibrosarcoma, or sarcoma (Barker 1986). The consensus appears to be to classify the 9L as a gliosarcoma, although many authors refer to it simply as a rat brain tumour. It is a highly vascularized tumour with no evidence of cellular hypoxia, and does not metastasize (Leith 1975, Wallen 1980). 9L tumours are very radioresistant with a large capacity for repair of potentially lethal damage, a characteristic they share with human gliomas (Gerweck 1977, Raaphorst 1989), making the 9L brain tumour model an excellent system in which to evaluate the inhibition of repair by cisplatin.

2.1.2 General methods of cell culture

The methods of cell culture used in this project are standard methods in routine use in the tissue culture laboratory of the Ottawa Regional Cancer Centre and other laboratories. All cell manipulations were carried out in a laminar flow hood using standard sterile techniques. Cells were grown in a 50/50 mix of Dulbecco's Modified Eagle's Medium (Sigma catalog #D5648) and F12 Nutrient Mixture (Sigma catalog #N6760), supplemented with 0.1 mM MEM non-essential amino acids (Sigma catalog #M7145), fetal bovine serum (FBS 7.5% v/v, Gibco), and newborn calf serum (NCS 7.5% v/v, Gibco). No antibiotics were used for any cell culture. Medium was buffered with sodium bicarbonate (10 mM)

and HEPES buffer (20 mM), and pH adjusted to 7.4. Cells were maintained at 37° C in a CO₂ incubator, 97.5% air, 2.5% CO₂ at 100% humidity.

Exponentially growing cells were subcultured every 4 days by aspirating the medium and rinsing with isotonic citrate saline (KCl 134 mM, citric acid C₂H₈O₇Na₃2H₂O 17.6 mM) to remove cellular debris which might affect the accuracy of cell counting, and to remove trypsin-inhibiting substances remaining from the medium. Citrate saline was used because citric acid is a mild chelator of divalent cations; Ca⁺⁺ and Mg⁺⁺ present in the medium can inhibit monolayer dispersal and cause cell aggregation (Freshney 1983). Trypsin was added (0.5 ml, 0.2% w/v in citrate saline), rinsed over the cells, and the excess aspirated. Two minutes of incubation with trypsin was sufficient to obtain a single-cell suspension. Cells were resuspended in 5 ml complete medium and a sample was counted using an automatic cell counter (Particle Data Elzone 80, calibrated by hemocytometer). An aliquot containing 1x10⁵ cells was seeded into a T-25 tissue culture flask containing 4 ml complete medium. Cells were always subcultured while in exponential phase.

Upon receiving the initial stock of cells, a large number were grown and frozen to provide a bank of cell stocks of the same passage upon which to draw for experiments. Cells were frozen by trypsinizing exponential cultures into a single cell suspension, centrifuging to form a cell pellet, aspirating the medium and resuspending in freezing solution (complete medium containing 10% v/v dimethyl sulfoxide DMSO) at a density of approximately 10⁶ cells/ml. This cell suspension was placed in cryovials and frozen at -115 °C. Fresh cell stocks were thawed every 3 months; cells were never continuously subcultured for longer than three months. The bank of frozen cell stocks was renewed twice during the project.

A growth curve was defined for the 9L cells by seeding 5x10⁴ cells into 60 mm tissue culture plates containing 4 ml complete medium. Cells were incubated at 37°C and each day one plate was trypsinized and counted. At 80h after initial seeding, the remaining plates were divided into three groups: one was given a complete medium change, another was given a half medium change (2.0 ml medium aspirated, 2.0 ml fresh medium added), and the last group kept the original medium. Daily counts were performed until the cells were overconfluent.

The results of this growth curve experiment are shown in figure 2.1:1. The cell counts have been adjusted for the slightly larger growing surface in a T-25 tissue culture flask. The exponential portion of the curve has a doubling time of 16.0 +/- .75 hours. This is similar to the reported population doubling time for 9L cells of 18 hours (Barker 1986). The cell number reaches a plateau at about 144 hours after plating, and declines as the cell monolayer becomes overconfluent and cells lift off. There is very little difference in cell

number between the fed, partially fed, and unfed groups. The growth curve experiment was performed three times during the project, to ensure that the growth characteristics of the cells were not changing. Population doubling times of 16.0, 18.5 and 17.3 hours were measured.

All in vitro experiments were performed on plateau phase cultures of cells, to synchronize cells in G_0/G_1 phase and minimize cell cycle effects. Cells were seeded into T-25 flasks at 1×10^5 cells/flask. Half the medium (2.0 ml) was changed 3.5 days later, and experiments were performed 5.5 days after setting up the flasks. The half medium change was chosen because of a tendency of the 9L cell monolayer to lift away from the growing surface upon addition of fresh medium or rinsing with saline. This tendency was minimized by changing only half the medium, with little effect on plateau cell density. At plateau phase, each T-25 flask contained approximately 1×10^7 cells. The pH of the medium was initially 7.30; plateau phase cultures acidified their medium to a pH of approximately 6.8. Fluorescence activated cell sorting (FACS, Kreth 1974) analysis of plateau phase 9L cells showed that 89% of cells were in G_0/G_1 phase of the cell cycle, 11% were in G_2 or M, and 0.1% were in S phase.

In addition to the growth curve, chromosome karyotyping was performed on metaphase spreads to further characterize the cells, and ensure that the cell stocks were indeed representative of 9L cells based on the degree of aneuploidy. Exponentially growing cells were arrested in metaphase using colcemid, treated with hypotonic saline, fixed, dropped on a microscope slide, and stained with Giemsa (Worton 1979). The mean (s.d.) chromosome number was 66.2 (2.8), and the mode was 65. This agrees well with other investigators who have found the 9L cell line has a chromosome number that varies in the low 60's (Barker 1986).

2.1.3 Colony forming assay

The endpoint of all of the in vitro experiments was the colony forming assay, a measure of clonogenic cell survival originally described by Puck (Puck 1957, Freshney 1983, Hall 1988). This assay measures the proportion of cells which remain reproductively viable following an experimental treatment. It is an appropriate assay for treatments for cancer because in the clinical situation, the aim of treatment is not necessarily to kill tumour cells outright, but to render them incapable of producing viable offspring, thus halting tumour progression.

In all of the in vitro experiments of this project, plateau phase cells in T-25 tissue culture flasks were subjected to an experimental treatment consisting of cisplatin, radiation, or combinations of the two. At the time of assay for clonogenic cell survival, the medium was aspirated from the monolayer of cells, citrate saline added and aspirated to rinse the cells of debris and remaining medium, and trypsin added. Cells were incubated for 2 minutes with trypsin, and 5 ml complete medium was added with repeated pipetting to achieve a single cell suspension. A sample of this cell suspension was then counted using an automatic cell counter.

Using an estimate of cell survival for the particular treatment under investigation, the cell suspension was diluted appropriately and a known number of cells was added to a set of four 60 mm tissue culture plates each containing 4 ml fresh complete medium. The number of cells seeded into each plate was calculated, based on the estimated surviving fraction, to give approximately 50 surviving cells per plate. If the estimated surviving fraction was uncertain, two or three sets of plates were seeded, each with a different cell number. This ensured that at least one set would have approximately 50 surviving cells. Cells were never seeded at a density higher than 5×10^4 cells per plate; if the estimated surviving fraction was low enough to necessitate seeding at a density higher than 5×10^4 cells per plate, then 100mm tissue culture plates containing 10 ml medium were used. Control plates (untreated) were seeded for each experiment to determine the plating efficiency (the fraction of cells successful in attaching to the growing surface and cycling in the absence of treatment). The plating efficiency for 9L cells was in the range 60-80%.

After undisturbed incubation for 9-10 days to allow surviving cells to replicate, the plates were emptied of medium and crystal violet stain (0.2% w/v in 70% ethanol) was added for approximately 5 minutes. After gently rinsing away excess stain, colonies of diameter 2-3 mm, each containing several hundred cells were clearly visible. In the colony forming assay, each colony is assumed to have arisen from a single surviving cell which successfully attached to the growing surface and entered the cell cycle, surrounding itself with progeny.

Colonies were counted under a 10X stereoscopic microscope. Any colony consisting of less than about 50 cells was not counted as a survivor. The set of plates seeded with the appropriate number of cells to produce about 50 colonies per plate was counted; other seeding densities were discarded. Plates with significantly more than 50 colonies experience some growth inhibition and apparent reduced survival due to crowded conditions; plates with significantly less than 50 colonies gave poor statistics. The mean and standard deviation of each set of four plates was calculated. The surviving fraction was calculated according to the following formula:

$$\text{Surviving Fraction} = \frac{\text{mean number of colonies per plate}}{\text{number of cells seeded per plate}} \times \frac{1}{\text{Plating Efficiency}}$$

Each in vitro experiment was repeated at least 2 and usually 3 or 4 times. Results plotted are the mean surviving fraction, with error bars representing the standard error of the mean (SEM).

2.1.4 Irradiation of cells

Radiation treatments were administered to plateau phase cells in their T-25 tissue culture flasks using a clinical Theratron 780C cobalt-60 therapy unit operating at a dose rate of 1.0 - 1.3 Gy/min. Dosimetry was confirmed using TLD measurements. Cobalt-60 was used because the equipment was readily available, and the layer of medium over the cells and the upper layer of the flask provided the correct build-up layer to place the cells close to the depth of dose maximum for cobalt. Up to 8 flasks at a time were mounted in a specially constructed lucite jig which allowed the bottom of the flasks to be immersed in ice water. In addition to providing lateral build-up, the ice water cooled the cells to 0°C to arrest enzymatic radiation repair processes. This was important in experiments investigating the time course of repair of radiation damage, since enzymatic repair has a fast component which operates on a timescale of several minutes (Friedberg 1985). Since the time of irradiation was typically about 10 minutes (20-25 minutes with set-up), cooling was necessary to prevent repair during the time of irradiation.

The lucite jig containing the flasks of cells was placed on the patient couch centred under the treatment head. The distance from the source to the surface of the medium was 80 cm. The field size was set at the maximum (35 cm x 35 cm) to provide dose uniformity over all the flasks. The treatment time was typically about 10 minutes for a dose of 18 Gy.

After irradiation, the flasks were taken upstairs to the tissue culture lab, placed briefly in a water bath to warm the medium to approximately 37°C, and manipulated according to the protocol of the particular experiment being performed. For experiments in which the time after irradiation was an independent variable, the $t = 0$ time was considered to be the time of emergence from the water bath, i.e. the time of return to 37°C. Control (unirradiated) flasks were placed on ice for the same time as irradiated flasks.

2.1.5 Cisplatin treatment of cells

Cisplatin was added directly to the existing medium covering plateau phase 9L cells in T-25 tissue culture flasks. Cisplatin was obtained from the pharmacy of the Ottawa Regional Cancer Centre (Cisplatin Injection, David Bull (Canada) Inc., Vaudreuil, Quebec). This solution consisted of cisplatin at a concentration of 1 mg/ml (3.33 mM) in isotonic saline (NaCl 9 mg/ml) and pH adjusted to 7.3. A micro-pipette was used to measure 24 μ l of the Cisplatin Injection solution, which was added to the 4.0 ml of medium in a T-25 flask, to give a concentration in medium of 6 μ g/ml (20 μ M). This was the concentration used in most in vitro experiments. Other concentrations were achieved by using greater or less than 24 μ l of the Cisplatin Injection solution.

For experiments requiring lower concentrations of cisplatin, or experiments where the amount of medium covering the cells was small (e.g. low dose rate experiments performed in small vials with only 0.5 ml medium), the stock cisplatin solution was diluted 1:10 in citrate saline immediately before adding a measured amount to the cells. This 1:10 diluted solution was discarded immediately after use, and fresh diluted solution made up as required. The reason for this is that the concentration of chloride ions affects the proportion of aquated cisplatin species versus native species. In water, cisplatin loses its chloride ions and they are replaced by hydroxyl groups. The half-time for this reaction is approximately 2.5 hours. This aquated species of cisplatin is the compound which reacts with DNA (Daley-Yates 1986, Goel 1992). The chloride ion concentration of citrate saline is slightly lower than that of Cisplatin Injection (134 mM versus 154 mM). By using only freshly diluted cisplatin, the proportion of aquated and native species was controlled.

At the end of the period of exposure of cells to cisplatin, the medium containing cisplatin was aspirated and the cells were rinsed thoroughly with citrate saline. The saline was aspirated and cells were either trypsinized for immediate plating, or depleted medium was added for delayed plating experiments. Depleted medium was collected from other plateau phase cultures previously and refrigerated until needed. By adding depleted medium, cells were maintained in the non-cycling plateau phase by conditions of nutrient depletion.

Some early cisplatin experiments were performed using HPLC grade cisplatin supplied as a yellow powder. The powder was dissolved in citrate saline just before adding to the medium on cells. Its use was discontinued because of a limited supply and its poor solubility. An experiment was performed comparing the toxicity of the HPLC-grade cisplatin preparation with that of the Cisplatin Injection preparation (see figure 2.1:2). No significant differences were observed over the range of exposures 0 - 16 μ g/ml for 1 hour.

2.1.6 Preparation of 9L cells for tumour inoculation in rats

In the early stages of this project, 9L tumours were inoculated intracranially in Fischer rats according to the standard method described in the literature (Barker 1973, Barker 1986). For this method, exponentially growing cells were trypsinized, centrifuged, and resuspended in serum-free medium at a density of 4×10^6 cells/ml. This cell suspension was placed on ice to slow cell metabolism and prevent cell clumping, and transported to the animal facility at M-40, NRC where the tumour inoculation surgery was performed (see section 2.2.3).

A new tumour inoculation technique was developed and used for approximately 85% of the animals inoculated with intracranial tumours in this project. This technique is described in detail in section 2.2.3. Cells were prepared for tumour inoculation by harvesting exponentially growing cells from T-80 tissue culture flasks. Cells were trypsinized for 2 minutes, resuspended in complete medium, and a sample of this suspension was counted with an electronic cell counter. The cell suspension was centrifuged, the supernatant discarded, and the cell pellet resuspended in enough freezing solution (75% medium, 15% serum, 10% DMSO) to give a cell density of 2.5×10^7 cells/ml. Approximately 80 μ l of this cell suspension was placed into individual cryovials, and frozen to -115°C . In this way, a large number of individual cryovials could be prepared from a single passage of cells, and kept on hand in the freezer to be used as needed. The vials of cells were transported in liquid nitrogen to NRC and thawed immediately prior to inoculation into rats.

2.1.7 Passaging 9L cells as a flank tumour

Approximately halfway through this project, the success rate of intracranial tumour inoculation dropped precipitously. The rate of success of tumour inoculation had been close to 100%; it dropped to approximately 30%, and the growth rate was slower than normal. This coincided with a switch in animal supply, from Fischer 344 rats bred in-house at the NRC animal facility, to rats purchased from an outside supplier (Charles River). It is likely that a difference in the immunological characteristics of the animals was responsible for the drop in tumorigenicity, since the 9L tumour model has been shown to be immunogenic (Blume 1974, Barker 1986).

To rectify this problem, approximately 2×10^6 cells were injected subcutaneously into the flank of each of 6 rats (Charles River). Flank tumours grew in 2 of these animals. Three weeks after injection, the tumours (~1cm diameter) were resected as follows (Freshney 1983): The animal was anesthetized with sodium pentobarbital 80 mg/kg (Somnitol 1.2 ml/kg) and the flank was shaved. The flank area around the tumour was swabbed with alcohol (ethanol 70% v/v) and the animal was placed in a sterilized tray in a laminar flow hood. An incision was made into the tumour with a sterile scalpel and the contents of the tumour were removed with a fresh sterile scalpel. The tumour chunks were placed in a sterile glass petri dish containing citrate saline, and sterile scalpel blades were used to mince the tumour into fine pieces. The citrate saline was aspirated, and the tumour fragments were covered with trypsin and incubated at 37°C for 20 minutes. The trypsin solution was mixed by pipetting, and placed into a centrifuge tube, taking care to leave larger chunks behind. After centrifuging, the supernatant was discarded and the pellet was resuspended in complete medium. This cell suspension was placed into primary culture T-25 tissue culture flasks (to inhibit fibroblast growth) containing 4 ml fresh medium and incubated for four days. The cells were then trypsinized and transferred to standard T-80 tissue culture flasks.

Passaging the cells through an animal as a flank tumour restored the tumourogenicity of the cells. Subsequent intracranial inoculations using this new passage had a success rate approaching 100%. A large number of cells of this new passage were grown and frozen at -115°C to provide a bank of cells for further inoculations into animals. In addition, subsequent in vitro experiments were performed using cells from this new passage. There was no significant change in growth parameters or radiation response with the new passage.

Figure 2.1:1. A representative *in vitro* growth curve for 9L cells. The exponential portion of the curve shows a population doubling time of 16.0 hours. Three replicates of this experiment yielded exponential doubling times of 16.0, 18.5, and 17.3 hours. Curves for three cell groups are shown: unfed, half medium change at $t = 80$ h, and full medium change at $t = 80$ h. For clarity, error bars are shown for the first five points only, and represent the standard deviation in the counts for individual flasks. There is no significant difference between the fed, half-fed, and unfed cell groups (t -test, $p < .05$).

Figure 2.1:2. Cisplatin dose-response curve for 9L cells comparing HPLC-grade cisplatin with clinical cisplatin (Platinol), one hour exposure time at various concentrations. Points are the mean of two replicate experiments, 4 plates per point, error bars are standard error of the mean. There is no significant difference in cytotoxicity between the two cisplatin preparations (t-test, $p < .05$).

2.2 In Vivo Methods

2.2.1 General handling of animals

All protocols involving manipulation of animals were submitted for approval by the Animal Care Committee of the Institute of Biological Sciences, National Research Council of Canada. Regular updates were submitted by the investigator and reviews of the project were performed by the ACC. All experimental protocols involving animals were designed and carried out in accordance with the guidelines of the Canadian Council of Animal Care (CCAC 1984). Animals were housed in a facility regularly inspected and approved by representatives of the Canadian Council of Animal Care. Animals were given food and water ad libitum and monitored regularly for signs of distress or discomfort. Animals were euthanized by anesthetic (sodium pentobarbital) overdose.

2.2.2 Source of animals

The rats used for this project were male Fischer-344 rats. The 9L rat brain tumour will not grow intracranially in females (Wheeler 1981). Rats were initially obtained from a Fischer-344 breeding program in the animal care facility of the National Research Council. Partway through the project, the source of supply was switched to Charles River Breeding Laboratories, Wilmington, Massachusetts. We believe this change in supplier caused a drop in tumourogenicity of the 9L cells. This was rectified by passaging the cell line as a flank tumour, as noted in section 2.1.7. Rats weighed from 180 - 220 g when received. They were given 5 - 7 days to adjust to their new surroundings before being subjected to experimental procedures.

2.2.3 Inoculation of intracranial 9L tumours

Intracranial 9L tumours were initially inoculated according to a standard method outlined in the literature (Barker 1973, Barker 1986, Wheeler 1981). A number of significant modifications to this standard technique were made, and these are discussed in

detail in section 3.2.2. A brief description of the major changes to the standard technique is provided here.

Cells were prepared for tumour inoculation by freezing individual aliquots, as described in section 2.1.6. The standard technique calls for preparing a cell suspension in balanced salt solution, which is kept on ice until inoculated into the brain. This method works well when the time between cell preparation and inoculation is small. In this project, however, cells were cultured and prepared for inoculation at one laboratory, and transported on ice to another laboratory where the inoculation surgery was performed. As a result, the last rat inoculated on a given day received cells which had been on ice for up to 6 hours. In vitro experiments showed a significant drop in clonogenic survival (to less than 20%) for cells which had been on ice for 4 hours. To overcome this problem, cells were prepared in advance as described in section 2.1.6, and kept in liquid nitrogen until just before inoculation. The cells were then thawed quickly and inoculated directly into the brain of the rat. In this way, each rat received cells which had been handled identically.

The volume of inoculated cell suspension was reduced compared with the standard technique, which calls for inoculation of 10 μ l of cell suspension through a hole in a stainless steel or plastic injection screw threaded into the skull. Since the presence of such a screw in the skull could affect the results of magnetic resonance experiments and radiation dosimetry measurements, the cells were introduced directly through a hole in the skull, without using injection screws. To reduce the possibility of cell suspension flowing up through the hole and out of the brain during injection, the injection volume was reduced to 2 μ l. The number of cells injected (5×10^4) was kept the same by increasing the density of the cell suspension to 2.5×10^7 cells/ml.

The standard positions in the brain for inoculation of 9L tumours described in the literature were not ideal for this project. Preliminary phosphorus-31 NMR spectroscopy experiments using surface coil localization showed that the standard inoculation sites were too close to the insertion points in the skull of the large muscles of the neck and jaws, leading to problems due to high levels of phosphocreatine in muscle compared with brain and tumour. Hence the inoculation site was moved to a position further from these muscles, where the skull and scalp were thinner. Dissection of a rat head and preparation of a phantom from the skull assisted in the choice of an injection site. (See figure 2.2:1)

The following is a detailed set of instructions for inoculating 9L intracranial tumours according to the method used in this project. Approximately 85% of the intracranial tumours used in this project were inoculated according to this technique. This procedure successfully produced intracranial tumours in 96% of the animals inoculated.

9L RAT GLIOMA INOCULATION PROCEDURE

1. Use male Fischer 344 rats, 200-300 g. Anesthetize with intraperitoneal injection of sodium pentobarbital, 65 mg per kg body weight (e.g. Somnitol, 1.0 ml per kg).
2. Shave head with clippers or razor blade.
3. Mount rat in stereotaxic head frame (Paxinos 1982), taking care to insert the ear bars correctly. Hook incisor teeth over the incisor bar and screw down the nose bar. An electric heating pad placed under rat will help to maintain normal body temperature during surgery. Eye drops such as IsoptoTears will keep eyes moist during the procedure.
4. Swab scalp with alcohol. Make a 1 cm cranial-caudal incision starting about 5 mm caudal to the eyes. The incision should be 1-2 mm to the right of the midline.
5. Scrape away the periosteum with the scalpel until the skull is exposed. Mop blood with a swab or Q-tip until the bone is dry.
6. Mount the micrometer apparatus on the frame. With a blunt needle in the holder, locate Bregma and note the vertical coordinate. Then locate Lambda and note the vertical coordinate. Adjust the height of the incisor bar until the flat skull position is achieved, i.e. vertical coordinates of Bregma and Lambda are equal. (This step does not need to be repeated for every rat, provided the vertical position of the incisor bar is kept fixed, and provided rats of similar weights are used.)
7. Locate Bregma with the needle. Raise the needle tip slightly and move caudal (back) from Bregma 0.3 mm (30 of the smallest divisions on the graduated knob.) Move to the right of the midline 3.0 mm.
8. Mark this position on the skull using a pick or 26G needle.
9. Remove the micrometer apparatus from the frame.
10. Use a hand drill to drill through the skull at the marked position. A 0.79 mm (1/32") drill bit mounted in an Exacto knife handle works well. Cover the drill bit to within 1 mm of the tip with intramedic tubing, to act as a stop to prevent the bit from plunging down through the skull and puncturing the dura.
11. Remove a vial of 9L cells from liquid nitrogen and allow to thaw (about 80 μ l of cell suspension, 2.5×10^7 cells per ml suspended in freezing solution of complete medium plus 10% DMSO). Using a 1 or 3 ml syringe, flush the inoculation tube with several ml of sterile saline. Draw back slightly on the syringe to create a small air bubble in the tube. Draw cell suspension into inoculation tube. Draw and expel several times to mix cells.
12. Replace 3 ml syringe with a 10 μ l Hamilton microsyringe filled with sterile saline, taking care to ensure that the Luer-lock connection between the syringe and the inoculation tube has no air bubbles. Mount the micrometer apparatus on the stereotaxic frame, and mount the inoculation tube on the micrometer apparatus.
13. Touch the tip of the inoculation tube to Bregma, and note the vertical coordinate.

14. Position the tip of the inoculation tube above the hole in the skull, and carefully descend into the hole, watching to ensure that the tube is descending smoothly without binding. Descend to a position 3.5 mm below the vertical coordinate of Bregma, then return upwards by 0.5 mm, so that the final position of the tip of the tube is 3.0 mm below Bregma (and 3 mm right of midline, 0.3 mm caudal to Bregma). The purpose of descending an extra 0.5 mm then returning is to clear a small space in the brain to receive the cells.
15. Slowly inject 2.0 ul of cell suspension using the Hamilton microsyringe. Watch for movement of the bubble in the inoculation tube to ensure that there is no blockage of flow.
16. Wait about 30 seconds for the pressure to equalize as the injected fluid is absorbed, then withdraw the inoculation tube and remove the micrometer apparatus from the frame.
17. Plug the hole in the skull with a dab of sterile bone wax, applied using the end of a pair of tweezers or other blunt object. Make sure the hole is thoroughly sealed.
18. Dip a Q-tip in hydrogen peroxide and swab the wound thoroughly to kill any cells which may have spilled on top of the skull. Flush the wound with sterile saline to wash away the peroxide, then mop dry with a swab or Q-tip.
19. Close the wound with 3 or 4 stitches. Using absorbable sutures helps to minimize the damage to the wound which may be caused if the rat picks at the stitches.
20. Wipe away any blood from around the wound with a swab moistened with sterile saline. Carefully remove the ear bars and incisor bar, and place the rat in a warm and quiet area to recover. Return to cage only when rat is fully conscious and able to maintain its body temperature.

IMPORTANT POINTS:

The 9L cells should be treated as a potential BIOHAZARD. Wear good quality gloves during the procedure. After the procedure, fill the cell vial with alcohol to kill any remaining cells, and dispose of the vial in the regular garbage. Any spilled cells should be doused with alcohol. Cells should be handled with care. Flush the inoculation tube with alcohol after the procedure to kill the leftover cells, then flush with copious amounts of saline to get rid of the alcohol before treating the next rat.

Sterilize instruments by wiping with alcohol or soaking in Zepherin to minimize the rat's risk of infection. Pay special attention to cleaning and sterilizing the drill bit and the inoculation tube, since these instruments contact the brain. Keep the work area clean.

2.2.4 Growth characteristics of the intracranial 9L tumours

The tumours were approximately 2.5 mm in diameter on day 14 following tumour inoculation, which is in agreement with reported tumour weight of 9 mg at 14 days for the 9L/SF intracranial tumour (Wheeler 1981). Further growth of the tumour resulted in symptoms of increased intracranial pressure: ataxia of the hindquarters, somnolence, cachexia, and occasionally periorbital bleeding. These symptoms appeared a mean (s.d.) of 19.5 (+/-4.4) days after tumour inoculation, followed by death approximately one day later. This agrees with median times to death reported in the literature (Wheeler 1981, Barker 1986) ranging from 16-18 days (Rochester) to 23-25 days (San Francisco). Host-tumour interactions are likely responsible for the variation in median day of death (Wheeler 1981).

Measurement of tumour size by MRI was the designed endpoint of the in vivo experiments. The survival time of the animals was not an endpoint, as this would require that every animal suffer a protracted death. Animals were always sacrificed by anesthetic overdose (sodium pentobarbital, Somnitol) or perfusion fixation (section 2.2.6) at the first sign of increased intracranial pressure or distress. Only a few animals were allowed to die from their tumours, to determine the growth characteristics. The time to appearance of symptoms of increased intracranial pressure was recorded as an endpoint when it occurred, even though the intention was always to image and sacrifice animals before appearance of these symptoms. These data were used for the survival analysis of section 3.2.9. Only those animals whose tumours grew unusually quickly, or animals which did not have MR imaging performed on the designated day due to equipment malfunction or unavailability, experienced the symptoms of increased intracranial pressure.

2.2.5 Flank tumour inoculation

Flank tumour inoculation was accomplished by lightly anesthetizing a rat with sodium pentobarbital or isoflurane and shaving its flank. A vial containing frozen 9L cells (see section 2.1.6) was thawed and 1.0 ml of sterile isotonic saline was added to the vial. A volume of 0.5 ml containing 10^6 cells was inoculated subcutaneously into the flank of the rat. In two weeks, a tumour approximately 1 cm in diameter would result.

2.2.6 Perfusion fixation and preparation of sections for histology

Most of the animals bearing intracranial tumours were sacrificed by perfusion fixation and their brains were removed for histopathological analysis. This was done to help characterize the tumours and their growth, to observe at a cellular level the effects of treatments on the tumours and surrounding normal tissue, and to correlate morphological features and size measurements from MRI with histopathological observations. Whenever possible, perfusion fixation was performed immediately following MRI, to provide maximum opportunity for correlation between MRI and histopathology.

Standard procedures of perfusion fixation were used (Humason 1972). Formalin was used because it does not dissolve lipids, making it a good fixative for brain. The important feature of formalin fixation is formation of cross-links between protein end groups. These reactions are pH dependent, hence the formalin solution must be buffered (Pearse 1968).

Perfusion fixation was performed by administering a heavy, near-lethal dose of sodium pentobarbital intraperitoneally (150 mg/kg, Somnitol 2.2 ml/kg). At the onset of heavy anesthesia, the rat was placed ventral side up in a pan and pinned to a dissection pad. A midline ventral incision was made in the abdomen and continued cranially on both sides of the sternum to expose the thoracic contents. The descending aorta was clamped to prevent perfusion of the lower body (to conserve formalin).

Table 2.2:1. Recipe for buffered formalin used in perfusion fixation.

Ingredient	Amount for 1 litre	for 4 litres
37% formaldehyde	100 ml	400 ml
distilled water	900 ml	3600 ml
Sodium phosphate monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)	4.0 g	16.0 g
Sodium phosphate dibasic (Na_2HPO_4)	6.50 g	26.0 g

The left ventricle was catheterized at the apex of the heart using a 14-gauge i.v. catheter. The right atrium was nicked to drain the venous return into the thoracic cavity. A 50 ml syringe filled with isotonic saline was connected to the cardiac catheter. The saline was infused into the heart to flush the blood from the circulatory system. A 50 ml syringe filled with buffered formalin was connected to the cardiac catheter and formalin was infused into the heart and throughout the circulatory system. This was repeated with a second 50 ml syringe of buffered formalin. The cardiac catheter was then connected to a hanging drip bottle containing approximately 400ml buffered formalin, and this was allowed to slowly perfuse the rat (~ 0.5 hours). The rat's head was removed by guillotine, the skull peeled away, and the fixed brain was removed intact and placed in a small glass bottle of buffered formalin for a minimum of two weeks for complete fixation.

Fixed rat brains were prepared for microscopic examination in one of two ways. Some brains were sectioned using a cryotome at NRC, producing a minimum section thickness of 40 microns; no staining was done. Detailed histopathological analysis was not possible from these sections, but accurate measurements of tumour size could be easily obtained. Most of the fixed brains were sectioned by the technicians in the neuropathology labs of the University of Manitoba Medical School, Winnipeg, Manitoba. Fixed brains prepared in Winnipeg were embedded in paraffin, sectioned with a slice thickness of approximately 2 microns, dehydrated in alcohol to remove the paraffin, and stained with eosin (tetrabromfluorescein) and hematoxylin ($C_{16}H_{14}O_6$). Two representative sections through the approximate centre of the tumour were prepared.

2.2.7 Method of irradiating intracranial tumours in rats

2.2.7.1 Field size for irradiation

Irradiation methods for intracranial tumours in rats which involve irradiating the whole head or a large portion of the head can lead to unnecessary suffering of the animal due to the radiosensitivity of the salivary glands, which lose function several days after a dose of 15 Gy (Vissink 1991), resulting in excess mortality from starvation (Wheeler 1980). To avoid this, a highly collimated beam with a full width at half maximum of 5.1 mm was used to irradiate the tumour and a small margin of surrounding brain, while sparing the majority of the brain and the salivary glands.

A collimator was built consisting of a block of lead 12 cm x 12 cm x 9 cm thick with a 5 mm vertical hole under which the tumour was positioned. The 9 cm thickness was based on a calculation of the amount of lead collimation necessary to reduce the whole body dose to the rat to less than 0.15 Gy for a tumour dose of 80 Gy. A whole body dose of 0.15 Gy is sufficient to cause a measurable reduction in numbers of circulating lymphocytes, and thus could conceivably influence experimental results. This collimator allowed irradiation of the tumour, while avoiding complications due to damage to the salivary glands and possible systemic effects.

2.2.7.2 Radiation dose for treating intracranial 9L tumours

The radiation dose used for treating intracranial 9L tumours was chosen to provide a reasonable expectation of a response to treatment which would be reflected in a change in tumour size or morphology visible on MRI. Pilot experiments were performed in which rats with intracranial 9L tumours were irradiated at 30, 45, 60, 75, and 90 Gy (2 rats each dose). MRI changes were visible 3 days after irradiation in the animals receiving 90 Gy, and MRI changes were apparent 5 days after irradiation in the animals receiving 75 Gy. No changes were apparent in the animals receiving 30, 45, or 60 Gy. A dose of 80 Gy was chosen as a standard radiation treatment for this project.

A comparison of the radiation responses of the 9L cell line and human malignant gliomas cell lines supports this choice of treatment dose. In the high dose region, 9L cells are approximately 1.8 times more resistant than human malignant glioma cell lines (see section 3.1.1.3). Since clinical doses for radiation therapy of human malignant gliomas are typically about 55 Gy (Pezner 1981, Constine 1988), a reasonable treatment dose for intracranial 9L tumours would be approximately 100 Gy. However, since the radiation dose to the 9L tumours was delivered in a single fraction and these tumours have no significant hypoxic cell population (Wheeler 1981), a lower dose of 80 Gy was considered appropriate.

In clinical radiotherapy, the total dose is delivered in multiple fractions, typically 15 - 20 daily fractions of 2 - 3 Gy each. The rationale for fractionation is based on allowing normal tissues surrounding the tumour to repair sublethal damage between dose fractions, and to repopulate if the total treatment time is sufficiently long. Clinically, fractionation increases damage to tumours due to reoxygenation of hypoxic (and thus radioresistant)

regions of the tumour, and redistribution of cells into more radiosensitive phases of the cell cycle (Hall 1988). Fractionation is particularly important in clinical radiotherapy of brain tumours, since the brain is less tolerant of large dose fractions and dose schedules with few fractions per week than other tissues (Pezner 1981). However, brain is considered a late-responding tissue for radiation injury; the time for onset of clinical manifestations of brain radiation injury ranges from 3 to 40 months (Pezner 1981, Constine 1988). Since the animals in this project were usually monitored for no more than a week after radiotherapy, fractionation to avoid late radiation injury of the brain was unimportant. Although fractionated radiotherapy is more clinically relevant, the experimental difficulties of administering multiple fractions to rats with intracranial 9L tumours were considerable; in this project, the simple case of a single, high dose radiation fraction was chosen.

2.2.7.3 Rat irradiation technique

All rats were irradiated using a clinical Theratron 780C cobalt-60 therapy unit located at the Ottawa Regional Cancer Centre. The lead block collimator was placed on a lucite tray with a 10 mm hole in the centre mounted in the accessories holder of the treatment unit. Anesthetized, shaved rats were mounted in a stereotaxic head frame placed on the patient couch. Using stereotaxic coordinates recorded during inoculation surgery, the micrometer attachment of the stereotaxic frame was used to locate the position of the hole in the skull through which the tumour cells had been inoculated. A mark was made on the skin over this hole with a marker pen. Pre-irradiation MR images were used to confirm that the tumour was centred under the inoculation hole. The field light on the treatment unit was used to project the field centre crosshairs through the hole in the lead collimator onto the rat's head, and the mark over the inoculation hole was centred under the crosshairs. The patient couch was raised to a source-to-skin distance of 60 cm, and the positioning reconfirmed. A field size of 10 cm x 10 cm was used.

Figure 2.2:2 shows a photograph of the irradiation set-up. The rat was covered from the neck down with a surgical drape to help maintain body temperature during the irradiation. Time of irradiation was 25-30 minutes for a tumour dose of 80 Gy.

2.2.7.4 Anesthesia of rats for irradiation

Anesthesia for restraint during radiation therapy was initially accomplished using a light dose of sodium pentobarbital (50 mg/kg i.p., Somnitol 0.8 ml/kg). NMR studies in the literature prompted concern about possible radioprotective effects of brain hypoxia induced by barbituates. While some studies found no difference in P-31 NMR spectra of rat brain between conscious rats and those anesthetized with sodium pentobarbital (Deutz 1986), others found P-31 NMR spectral changes showing reduced energy status following anesthesia with sodium pentobarbital at 150 mg/kg (Wehrle 1987, Fenton 1989), which is consistent with reduced blood pressure flow in tumours following sodium pentobarbital administration (Johnson 1976, Cullen 1985). In addition, sodium pentobarbital has been reported to cause an increase in radioresistance in subcutaneous mouse tumours, attributable to a decrease in blood flow (Rockwell 1980).

These concerns prompted a change to a combination of acepromazine (4 mg/kg) and ketamine (40 mg/kg) for anesthesia during radiation therapy. Acepromazine is a dissociative agent used as an antipsychotic drug in humans which does not produce surgical anesthesia when used alone. Ketamine is a cyclohexylamine analogue which produces unconsciousness and somatic analgesia, but unlike barbituate agents, it produces no reduction in cardiopulmonary function (Green 1981). This combination of acepromazine and ketamine induced a relaxed and docile state in rats during which they were easily mounted in the stereotaxic frame for radiotherapy.

Concerns were later expressed by the NRC Animal Care Committee, about restraint of animals in a state of less than complete surgical anesthesia. It was decided that restrained animals should be fully unconscious, marked by a loss of righting reflex and lack of response to tactile stimulation. For this reason, the method of anesthesia for radiotherapy was changed back to sodium pentobarbital (50 mg/kg i.p., Somnitol 0.8 ml/kg). Of 35 animals treated with radiation alone, 15 were anesthetized with acepromazine/ketamine, 20 with sodium pentobarbital. There was no evidence of a difference in radiation response with anesthetic agent.

2.2.7.5 Dosimetry for radiotherapy of intracranial tumours in rats

Measurements were performed to determine the distribution of radiation dose for the rat irradiation technique described in section 2.2.7.3. A scanning X-ray diode system was used to obtain the spatial distribution of relative dose, and film dosimetry was used to compare the measured dose to the calibrated configuration for the cobalt-60 system. Thermoluminescent dosimetry was used as a final check of the dose delivered.

2.2.7.5.1 Scanning X-ray diode dosimetry

A Therados computer-controlled scanning X-ray diode system was used to measure the relative depth dose distribution along the collimator axis, as well as cross-plots of relative dose distribution at various depths. This system consists of a large water tank with a thin mylar window in one side. Computer controlled stepping motors move a gantry in three dimensions inside the water tank. An X-ray diode with a sensitive volume of 0.15 mm³ mounted on the gantry measured relative dose.

Since the mylar window is mounted on the side of the water tank, the rat irradiation configuration described in section 2.2.7.3 was duplicated in a horizontal geometry. The gantry of the Co-60 unit was rotated to 90°, and the lead block collimator was mounted 1.5 cm from the mylar window. The distance from the source to the mylar window (SSD) was 60 cm, and the field size was 10 x 10 cm. The X-ray diode detector integrated measurements of current over 1 second, and the scanning steps were 0.2 mm.

The results of these scans are shown in figure 2.2:3. The upper plot shows the depth dose distribution, with a depth of dose maximum at 4.3 mm. This was approximately coincident with the centre of the intracranial tumours, assuming the distance from the top of the scalp to the top of the brain was about 2 mm (Paxinos 1982) and the vertical dimension of the tumour was about 4 mm. The lower plot shows the series of measurements of dose distribution across the collimator axis, for depths from 0 to 20 mm at 2 mm intervals. The minor asymmetry of the peak was due to a slight misalignment of the collimator axis with the origin of the scanning X-ray diode system, and does not influence the results. The width of the beam at 50% relative dose was 5.1 mm, and was constant with depth, except in the build-up region where the beam width was 3.7 mm at a depth of 0.6 mm. Since the tumour diameter in the horizontal plane was typically 2-3 mm at the time of treatment, this beam size allows for a positioning error of at least 1 mm in either direction.

2.2.7.5.2 Film dosimetry

The large gradient of dose across the beam axis due to the high collimation of the irradiation geometry precluded the use of ionization chambers, with their relatively large sensitive volume. The reproducibility of film and its high spatial resolution (Johns & Cunningham 1983) make it a good choice for measuring the dose under the collimator and comparing it with calibrated ionization chamber measurements.

The X-ray film was cut into strips and the edges sealed light-tight with electric tape. The film strips were placed in a polystyrene backscatter phantom with 5mm of polystyrene above to place the films at the Co-60 $d_{\max}$. The phantom was placed in a 10 x 10 cm field at a SSD of 80 cm. For this configuration, previous calibrated ionization chamber measurements for this machine had established a doserate of 89.8 cGy/min (the machine output factor). The films were irradiated to 8 doses from 5 to 60 cGy, and this was repeated 3 times for reproducibility. The films were processed and measured with a densitometer to produce a film calibration curve.

To measure the dose distribution with depth for the rat irradiation set-up, film strips were oriented vertically in a polystyrene backscatter phantom and irradiated for 0.3 min and 0.5 min in a 10 x 10 cm field at SSD 60 cm under the lead block collimator. The films were processed and the density measured as a function of depth along the film. These density measurements were converted to dose using the film calibration curve, and corrected to dose in tissue by dividing by 1.036 (Johns & Cunningham 1983). The resulting depth dose distribution is shown in figure 2.2:4, for 0.5 minute irradiation time. By taking the mean of the dose at $d_{\max}$, a dose rate of 138 cGy/min was determined for the rat irradiation technique.

2.2.7.5.3 Thermoluminescent dosimetry

A final check of dosimetry was performed using lithium fluoride thermoluminescent dosimeters (TLD). Four calibration TLDs were placed one at a time in a polystyrene backscatter phantom with 5 mm buildup material. At SSD=80 cm and a field size of 10 x 10 cm, a known dose of 200 cGy was delivered (from the machine output factor, previously established by calibrated ionization chamber measurements). The TLDs were read out by standard methods, and a calibration constant of 31.24 cGy/ μ C was established. The same four TLDs were then placed individually in the phantom under the lead block collimator at

SSD=60 cm and irradiated for the same time. The TLDs were read out and the readings multiplied by the calibration constant. The resulting mean doserate was 131.5 ± 3.0 cGy/min for the rat irradiation set-up. This compares favourably with the generally more accurate film dosimetry method, which gave a result of 138 cGy/min. Along the collimator axis, inserting the lead block collimator reduced the dose to 84.5% of the dose which would have been obtained without the collimator.

2.2.7.6 Effect of irradiation on rats

The irradiation procedure had no apparent adverse effect on rats. A dose of 80 Gy to a 5 mm column vertically through the brain resulted in no detectable systemic effects. Rats without tumours irradiated to 80 Gy showed a ~5 mm circular region of desquamation marked by hair loss which appeared approximately 3 weeks following irradiation, at both the entrance and exit points of the beam. These desquamated regions were not ulcerated or inflamed. The hair returned to these areas at 6-8 weeks following irradiation. No weight loss, difficulty in eating, or behavioural changes attributable to the radiation treatment were observed.

2.2.8 Cisplatin treatment of rats

The cisplatin used for injection in rats was Platinol (Bristol Laboratories Oncology Products, Bristol-Myers Company, Evansville, Indiana). This drug was supplied as a dry powder in an injection vial, which was reconstituted with 50 ml of sterile water immediately prior to use. Upon reconstitution, each ml of solution contained 1 mg cisplatin, 9 mg NaCl, and 10 mg mannitol. The mannitol acts as an osmotic agent to ameliorate nephrotoxicity. Rats were injected within 2 hours of reconstituting the drug; any remaining reconstituted drug was discarded.

Cisplatin is administered clinically by intravenous infusion, which in the rat would involve surgery under anesthesia to expose a vein for cannulation (usually the femoral vein). Given the burden of anesthesia these animals were already bearing (tumour inoculation, MR imaging, radiotherapy), it was considered preferrable to administer the

cisplatin intraperitoneally, since this route does not require anesthesia. Intraperitoneal administration was found to be suitable, considerably more convenient and easier on the experimental animals than intravenous administration.

The dose of cisplatin used for intraperitoneal injection in rats was 6 mg cisplatin per kg body weight. This was increased to 8 mg/kg based on the results of the experiments of section 3.2.7. See section 3.2.7 for a complete discussion of in vivo cisplatin doses.

Figure 2.2:1. Upper: Diagram of a rat skull showing the location of the stereotaxic reference points Bregma and Lambda. The location of the hole drilled in the skull during tumour inoculation surgery is marked by an x. (Diagram from Paxinos 1982.) Lower: Diagram of transverse slice through rat brain at location of tumour inoculation corresponding to the diagram above, showing the point of tumour cell inoculation in the caudate putamen region. (Diagram from Paxinos 1982.)

Figure 2.2:2. The rat irradiation technique. The rat was anesthetized and mounted in a stereotaxic frame and placed on the patient couch of a Theratron 780C cobalt-60 radiation therapy unit. The lead block collimator was mounted in the accessories tray under the treatment head.

Figure 2.2:3. Measurements of the Co-60 radiation beam below the lead block collimator, made with a scanning X-ray diode system in a water tank. The upper graph shows the depth dose curve along the beam axis. The depth of dose maximum is 4.3 mm. The lower graph shows the cross-beam dose curves at various distances from the collimator. The beam width at 50% relative dose is 5.1 mm.

Figure 2.2:4. Film depth dose curve. The Co-60 depth dose curve along the beam axis below the lead block collimator in a polystyrene phantom. The mean doserate (in water) at the depth of maximum dose was 138 cGy/min.

2.3 Magnetic resonance imaging methods

2.3.1 Magnetic resonance system

Magnetic resonance experiments were performed on a Bruker Spectrospin Biospec MR imaging/spectroscopy system, which has a 4.7 Tesla horizontal bore superconducting magnet with a bore diameter of 30 cm, and operates at a resonant frequency for protons of 200.17 MHz.

2.3.2 Gradient systems

The magnetic field gradient systems supplied with the Bruker Biospec were inadequate for imaging the rat brain, which is approximately 20 mm x 14 mm x 9 mm in size. A custom gradient set was designed and built by the imaging science group at the Institute for Nuclear Physics in Krakow, Poland.

The first custom gradient set consisted of a Helmholtz pair to generate the z-gradient, and Golay-type saddle-coil systems to generate the x and y gradients (Bottomley 1981). The conductors were imbedded in epoxy in a composite former which fit snugly inside the existing Bruker gradients, providing a clear bore diameter of 12 cm. This gradient set could generate gradients of approximately 250 mT/m when driven by Techtron 7570 power supply amplifiers. Rat brain images with a slice thickness of 150 μm and in-plane pixel size of 75 μm could be generated using this system (see figure 3.2.3:1). These gradients suffered from slow gradient rise times and transient magnetic field inhomogeneities after gradient switching due to induction of eddy currents (Boesch 1991). Lengthy pre-emphasis adjustment (shaping of gradient pulses to compensate for inductive coupling and to minimize eddy currents) was required whenever the gradient set was installed. (Other ongoing experimental programs requiring the full 30cm bore of the magnet necessitated frequent removal and re-installation of the gradients.)

An active shield (Mansfield 1986) was designed and built in Krakow to minimize these eddy currents (Jasinski 1992), allowing faster gradient rise times and shorter delays before signal acquisition, and reducing the need for pre-emphasis adjustment upon gradient installation. When operated with the active shields, this gradient system generated maximum gradient fields of 34 mT/m; with the shields disconnected, the maximum gradient strength was 204 mT/m (Jasinski 1992).

The evolution of gradient technology resulted in improvements in image quality and reductions in experiment time as the project progressed. These changes in equipment and imaging methods accounted for some variations in resolution, tumour contrast, image signal-to-noise and imaging experimental protocol throughout the project.

2.3.3 Imaging methods

Magnetic resonance imaging was done using a spin-echo sequence based on the spin-warp method (Edelstein 1980). Figure 2.3:1 shows the pulse sequence used for most of the imaging experiments.

Whenever the gradient coils were reinstalled, the spatial accuracy of images was checked by imaging a water phantom of known dimensions in all three orientations. Scaling of the gradients was adjusted as required to ensure that distance and area measurements taken from images were accurate. Accuracy of linear dimensions measured from MR images was estimated to be ± 0.2 mm.

The early images in this project were acquired with a pulse sequence repetition time TR of 1200 ms. This was increased to 3000 ms when more detailed experiments showed improved contrast between tumour and surrounding normal brain at longer TR (see section 3.2.3.1). At a TR of 3000 ms, spin echo images of the rat brain are predominately T2 or spin density weighted. However, at 4.7 Tesla the rat brain has a T1 relaxation time in the range 1500 - 2000 ms (Bottomley 1987), resulting in some T1 dependence in images even when acquired with a TR of 3000 ms.

The inter-echo time TE was fixed at 34 ms in the original imaging software. The installation of shielded gradients allowed TE to be reduced to 22 ms, resulting in slightly improved signal-to-noise ratio and improved acquisition of signal from short T2 regions of the sample. However, tumour-brain contrast was not improved at 22 ms, and with the installation of a new version of the imaging software, subsequent imaging was performed with a TE of 30 ms. Images were acquired with multiple echoes (6 or 8 echoes) to allow exponential fitting of the signal and calculation of apparent T2 (see section 2.3.9).

Tumour-brain contrast could be dramatically enhanced by using the injectable MRI contrast reagent gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA), which was obtained as Magnevist® (Berlex, Montreal) approximately halfway through the project. When Gd-DTPA became available, it was used in all subsequent imaging, since it made the definition of the tumour size easier and more precise compared with long TR multiecho

imaging. Gd-DTPA was administered intraperitoneally at a dose of 0.5 mmol (1ml) per kg body weight. (See section 3.2.3.2 for results of experiments on the kinetics of Gd-DTPA.)

Gadolinium contrast reagent causes selective enhancement on MRI by reducing the spin-lattice (T_1) relaxation time of extracellular water in tissues where it accumulates (Sobol 1991). Gadolinium-DTPA does not cross the blood-brain barrier unless it is compromised, as is the case in intracerebral tumours, where Gd-DTPA accumulates in the extracellular water compartment (Norman 1989). T_1 -weighted imaging was started within 10 minutes of Gd-DTPA injection. A spin-echo sequence with a repetition time of 300 ms was used, causing saturation of signal from long T_1 tissues which could not fully relax in 300 ms. Tissues which accumulated Gd-DTPA (tumour), with their resulting short T_1 , could fully relax in this time, and would contribute their full, unsaturated NMR signal. Only the first echo was acquired in these T_1 -weighted images.

All images were acquired on a 256 x 256 image matrix. The field of view used was the minimum allowed (for maximum resolution) by the configuration of gradients, shields, and gradient amplifiers in place at the time. This ranged from 2.0 cm (for an in-plane pixel size of 78 μm) to 4.0 cm (156 μm). Slice thickness was 0.8 - 1.0 mm. Signal averaging was done to improve the signal-to-noise ratio of the images. Typically 4 averages were accumulated for long TR images ($TR=3\text{s}$), for an imaging time of 51 minutes. Duration of anesthesia and temperature stability of the rat were limiting factors on imaging time. Gd-DTPA T_1 weighted images ($TR=300\text{ ms}$) were averaged over 16 accumulations, for an imaging time of approximately 20 minutes.

2.3.4 Imaging probe

The probe used for imaging rat brains is shown in figure 2.3:2. It was designed and built in collaboration with Paul Morris Instruments (Gloucester, Ont.). The probe consisted of acrylic tube 76 mm i.d., with a saddle coil made of adhesive copper foil applied to the outside of the tube used for excitation. A separate 14 mm surface coil resting on the rat's head was used for detection of NMR signals. Separate tuning and matching capacitors were present for both the excitation and detection coil circuits. Decoupling of the excitation and detection circuits was achieved by incorporating a quarter-wave cable terminated by crossed diodes into the excitation coil tuning circuit. These diodes turned on only when RF excitation pulses were being applied to the saddle coil from the RF transmitter, causing the

quarter-wave cable to be switched into the tuning circuit and the coil tuned to the transmitter frequency. With the transmitter pulses off, the diodes turned off, causing the quarter-wave cable to be switched out of the tuning circuit and the coil detuned. Crossed diodes were also incorporated into the tuning circuit of the surface coil used for detection. In this way, RF pulses from the transmitter did not induce currents in the detection coil, and NMR signals from the sample induced signals only in the detection coil (Jasinski 1992).

The large saddle coil used for transmission provided uniform excitation of NMR spins throughout the volume of the rat head, while the separate detection coil provided the improved sensitivity inherent in surface coils. A disadvantage of this system was a gradient of intensity present in the images due to the reduction in detection sensitivity with distance from the coil. Signal was generally good from intracranial tumours, since these were implanted near the top of the brain close to the detection coil. However, structures deep in the brain were often difficult to visualize. For this reason, a new probe was constructed (by Paul Morris Instruments) during the course of this project. This new probe incorporated a single 35 mm saddle coil for both excitation and detection. The saddle coil was made of adhesive copper foil applied to a 35 mm acrylic cylinder into which the rat's head was snugly inserted. This new probe resulted in images with uniform signal intensity from all regions of the brain, with little reduction in sensitivity.

The rat's head was held in place by a specially constructed plastic incisor bar which was fully adjustable and allowed the head to be fixed in the centre of the coil. The rat lay on a plastic i.v. bag filled with sand, which helped to damp vibrations from switching gradients. The bag of sand contained coils of plastic tubing connected to a circulating water bath, to maintain the correct body temperature during anesthesia. A rectal thermistor connected to a digital display outside the Faraday cage provided continuous monitoring of the rat's body temperature during imaging.

Anesthesia for imaging was achieved using the inhalation anesthetics isoflurane (1.75% v/v in 70% air, 30% O₂) or halothane (1.25% v/v in 70% air, 30% O₂). Anesthetic gas mixture was delivered to the probe where it emerged from a tube directly adjacent to the rat's nose. A vacuum hose exhausting to the outside cleared overflow anesthetic gas from the magnet bore.

Rats were positioned in the probe so that the approximate position of the tumour was centred in the RF excitation coil. The height of the incisor bar was adjusted to place the rat in the flat-skull position. The surface coil was centred over the tumour; the position of the tumour was judged from the scar from the tumour inoculation surgery. The tumour was positioned in the approximate isocentre of the magnet by sliding the probe into the magnet to a predetermined distance from the end of the probe to the end of the magnet bore. This position could be quickly checked using a patient positioning routine in the imaging software, and adjusted as required.

The image orientations of the Bruker Biospec are labelled as if a human patient were lying supine in the horizontal bore of the magnet. Hence a transverse image (T) is in a vertical plane perpendicular to the magnet axis (the x-y plane). A sagittal image (S) is in a vertical plane containing the magnet axis (the y-z plane). A coronal image (C) is in a horizontal plane containing the magnet axis (the x-z plane). Image orientations contain a second letter specifying the direction of the read gradient, e.g. TX is a transverse image with the read gradient in the X direction.

Intracranial tumours were located for imaging by first performing a CZ scout image with a slice thickness of 10 mm (TR=500 ms, 1 average, 128x128 image matrix, imaging time 70 s). From this image, it was usually possible to identify the bone wax plug in the inoculation hole drilled in the skull during tumour implantation. The CZ scout image was used to position a single TX slice centred on the hole in the skull. If the hole was not identifiable, the TX slice was positioned 9.5 mm caudal to the centre of the eyeballs. Previous measurements with the stereotaxic frame had established that this was a reliable indicator of the tumour position. A quick TX image was taken to verify positioning, then the full image acquisition was performed as described in section 2.3.3.

The reproducibility of positioning the MRI slice through the centre of the tumour was assessed by acquiring quick images from the same tumour-bearing rat removed and repositioned four times. Tumour size measurements from these images varied by less than 10%. However, uncertainties in positioning the slice through the centre of the tumour contributed to some error in comparing tumour size measurements between animals, and in correlating tumour size by MRI with tumour size from histological sections.

2.3.6 Image analysis

Classical studies of tumour growth and regression in response to treatment use tumour volume as the measured variable, usually estimated from caliper measurements of three orthogonal diameters of a flank or subcutaneous tumour (von der Maase 1985, Gridley 1985). Intracranial tumours are inaccessible to this technique, and even surgical resection of intracranial tumours after sacrifice, in addition to being technically difficult and providing only one measurement per animal, is subject to large errors (Barker 1973). MRI provides a method of non-invasively obtaining multiple measurements of tumour size from each animal. However, direct measurement of tumour volume proved elusive.

One way to sample intracranial tumour volume by MRI is to scan the region of the tumour with multiple thin, contiguous slices. Tumour volume can then be estimated by either: measuring tumour area from each slice, multiplying by the slice thickness, and summing over all slices; or, use image processing software to perform 3-dimensional volume reconstruction based on the set of slices, and integrate to find tumour volume. However, technical considerations prevented the acquisition of a contiguous set of slices.

The Bruker Biospec system was originally configured with slice selective RF pulses consisting of truncated Gaussian pulses amplified by a vacuum tube RF amplifier operating in the class C mode. The imperfect slice profile generated by this configuration led to "bleeding" of signal between adjacent slices, and significant degradation of image signal-to-noise ratio if contiguous slices were acquired. Given the small size of the tumours (~2-4 mm diameter), placement of a single 1 mm thick slice through the centre of the tumour precluded the acquisition of other slices in the tumour. This limitation was addressed towards the end of the project, when a linear RF amplifier (ENI 5100L-NMR RF power amplifier) was installed, as well as new imaging software which incorporated improved selective pulses. However, it was decided not to change the protocol for imaging intracranial tumours at that stage of the project.

An alternate approach to estimating tumour volume is to position a single slice through the centre of the tumour, in each of three orthogonal directions. This provides two measurements each of three orthogonal tumour diameters, allowing an estimation of tumour volume based on an assumption of ellipsoidal tumour geometry. However, acquisition of three orthogonal slices proved impractical in this project. Since it is impossible to acquire orthogonal slices simultaneously, total time in the magnet for three orthogonal non-contrast enhanced image acquisitions was 3 - 4 hours. This was considered excessive for animals already stressed by the presence of a brain tumour and previous multiple anesthesia, and would have led to increased mortality during imaging. Furthermore, the first custom

gradient set was considerably more difficult to trim (adjustment of gradient pulse amplitudes to maximize echo amplitude) and pre-emphasize (pre-shaping of gradient pulses to minimize eddy current ringing) in the coronal and sagittal image orientations. Frequently, time constraints allowed optimization of only the transverse (TX) image orientation. The installation of integral active shields in the gradient set significantly reduced the time required for optimization of the imaging sequence in all orientations. Again, it was decided not to change the imaging protocol developed during the project.

As a result of the above constraints, it was necessary to estimate changes in tumour size from a single transverse image through the centre of the tumour. The area of the tumour on transverse images was used as a surrogate measure for tumour volume. Any quantitative estimation of tumour volume based on a single transverse slice would have involved assumptions about tumour geometry which were unlikely to be universally valid. Measurements of tumour area contained more information about variations in the geometry of the individual tumours than measurements of tumour diameter. While the relationship between tumour area on transverse images and tumour volume depends on the geometry of each individual tumour, the assumption that changes in volume are mirrored by changes in area on TX images is undoubtedly valid for the majority of tumours.

The area of the tumour on TX images was measured using the image display and processing software available with the Bruker Biospec. The tumour boundary was defined by freehand tracing of a region of interest boundary around the tumour using a trackerball. The software provided a count of the number of pixels within that region of interest. Multiplying by the area of each pixel gave the tumour area in square millimetres.

For correlations with tumour size measurements from histological sections, tumour diameter was the measured parameter. This is because there is no accurate way to measure tumour area through the microscope. Tumour diameter was measured from histological sections simply by centering the microscope cross-hairs on the tumour boundary, reading the position of the stage on the vernier scales, then moving the stage to the opposite tumour boundary and reading the distance from the stage vernier scales. Locating the microscope beside the MRI console ensured that tumour diameters measured through the microscope were positioned in the same location in the tumour as diameters measured from MR images.

2.3.7 Measurement of apparent T2 relaxation times and signal intensity

Measurements of apparent T2 relaxation time from multiple echo image series were performed using a "T2 fit" routine in the Bruker image processing software. The aim of these measurements was not to try to estimate tissue T2, but to attempt to quantify inter-animal variations in tumour/brain contrast in long TR multi-echo imaging. A 16 pixel region of interest in the image was selected for calculation of apparent T2, and the software fitted the average signal intensity in this region to a single exponential decay as a function of echo time (6 or 8 echoes). The routine calculated and displayed the inverse of the decay constant ("T2") and the correlation coefficient. A parameter related to the proton density (the y-intercept of the exponential fit) was also calculated and displayed. An example of a T2 fit performed using this routine is shown in figure 2.3:3.

In general, these fitted T2s were avoided as a quantitative parameter. The reason is that the value of T2 obtained is not dependent purely on properties of the tissue and its pathology, but also depends on factors related to the equipment and the methods of measurement (Majumdar 1986, Majumdar 1987, Crawley 1987). RF field inhomogeneities and imperfect selective pulses resulting in variable flip angle across slices also causes errors in apparent T2 measurements due to stimulated echo artifacts and imprecise refocussing of transverse magnetization (Wong 1987, Majumdar 1986). Time-dependent (e.g. from gradient-induced eddy currents) and static magnetic field inhomogeneities (Majumdar 1986a) also reduced the measured value of T2. Since the induction of eddy currents by the gradients changed with pre-emphasis adjustment each time the gradients were removed and replaced, and varied with changes in gradient-shield configurations, this effect was not constant through the project. More importantly, the fitted value of T2 depended on gradient trimming (the adjustment of gradient pulse amplitude to optimize spin refocussing, maximizing spin echo signal and minimizing stimulated echo signal). This lengthy trimming procedure was performed each time the gradients were removed and replaced, resulting in variations in the apparent T2 decay of tissues from imaging session to session. Variations in fitted values of T2 due to poor gradient trimming were considerably larger than any intrinsic tissue T2 variations due to pathology or treatment. These instrument-dependent variations reduced the value of the fitted T2 as a quantitative parameter for comparing tissue and its pathology between individual rats and treatment groups. The fitted T2 was only used in MRI characterization of intracranial 9L tumours (section 3.2.4), since all of the images for this experiment were obtained in a single imaging session, with no changes in gradient trimming or pre-emphasis. Instrument-dependent variations in fitted T2

due to gradient pre-emphasis and trimming were largely eliminated with installation of the matched gradient-shield set towards the end of the project.

Measurements of ratios of signal intensity were used for quantifying images. The "integrated brightness" (IB) was calculated using the Bruker image processing software. This parameter is the average signal per pixel in a specified region of interest of size and shape defined by the operator. The IB was measured for the region of pathology and a "normal" region and expressed as a ratio. In this way, images were quantified independent of animal-to-animal variations in instrument sensitivity or scaling.

Figure 2.3:1. Schematic diagram of the spin-echo pulse sequence used for most of the imaging experiments. This sequence is a variation of the spin-warp sequence first described by Edelstein et.al. (1980).

Figure 2.3:2. Photograph of imaging probe used for MR imaging. This probe incorporated a 78 mm saddle coil used for excitation, and a 14 mm surface coil placed on the rat's head for detection. The rat's head was held in place with the plastic incisor bar.

Figure 2.3:3. An example of a T2 fit using the Bruker image processing software. A region of the image was selected for T2 calculation, and an exponential curve was fitted to the average signal intensity in this region as a function of echo time. A threshold value was set to terminate the fit if the later echo images had signal to noise ratios approaching unity in the region of interest. A parameter related to the proton density was calculated by extrapolating the curve back to $t = 0$.

3.0 THE EXPERIMENTS

3.1 In Vitro Experiments

3.1.1 Radiation response of 9L cells

3.1.1.1 Purpose of radiation response experiment

The purpose of this experiment was to generate a cell survival curve of clonogenic survival versus radiation dose for 9L cells in vitro. Comparison of this curve with 9L survival curves in the literature allowed identification and characterization of the cell line. The relevance of the 9L cell line as a model for the radiation response of human malignant glioma was determined by comparison of the 9L cell survival curve with those of human malignant glioma cell lines.

3.1.1.2 Method

The 9L cell survival curve was generated according to standard radiobiological methods (Hall 1988, Puck 1957). Plateau phase 9L cells in T-25 flasks were irradiated on ice as described in section 2.1.4. Cobalt-60 doses of 0, 2, 3, 4, 6, 8, 10, 12, 16, and 18 Gy were used. Control flasks (for plating efficiency) received sham radiation treatment. Cells were then trypsinized immediately without warming (in order to minimize repair of radiation damage), then counted, diluted and plated for the colony forming assay as described in section 2.1.3. The experiment was repeated on 4 separate occasions, and a plot was generated of the mean surviving fraction $\pm$ standard error of the mean, as a function of radiation dose.

The resulting 9L cell survival curve is shown in figure 3.1.1:1. Application of the multi-target single hit model (Hall 1988) to this curve results in the following parameters: the quasi-threshold dose D_q is 5.5 Gy, the extrapolation number (y-intercept) n is 16, and the reciprocal of the slope in the exponential cell-kill region, D_0 , is 1.94 Gy. The large shoulder on this survival curve indicates that the 9L cell line has a large capacity to accumulate and repair radiation damage. The extrapolation number n can be interpreted as the average number of hits on critical cellular targets required to produce cell death (Hall 1988). Most mammalian cells in tissue culture have extrapolation numbers in the range of 2 - 4 (Puck 1964). The value of D_0 for most mammalian cells in vitro lies in the range 1 - 2 Gy (Hall 1988). The 9L cell line is clearly highly radioresistant.

The observation by cytogeneticists that many chromosome aberrations visible after irradiation are clearly the result of two separate events led to the linear-quadratic, or alpha-beta model (Chadwick 1973, Chadwick 1978, Leenhouts 1983). In this model, there are two components to radiation cell killing, one proportional to the dose, the other to the square of the dose:

$$S = e^{-\alpha D - \beta D^2}$$

where S is the surviving fraction of cells after delivery of a radiation dose D , and α and β are constants which depend on the cell population and the irradiation conditions. The ratio α/β is the dose at which the linear and quadratic components of cell killing are equal. The α component is believed to represent the intrinsic sensitivity of the cell to lethal radiation events. The β component is believed to reflect the repair of radiation damage (Steel 1989). The relative radiosensitivity of tumour cells can be quantified by the ratio of α/β , this ratio being more useful than absolute values of α or β (Withers 1983). Radioresistant cells have large α/β ratios; radiosensitive cells have smaller α/β ratios.

An estimate of α/β can be made by plotting $(-\ln S)/D$ versus D ; the ratio of the intercept to the slope of the resulting straight line is α/β . A least squares fit of such a plot using the data of figure 3.1.1:1 gives a straight line with a correlation coefficient $r^2 = 0.971$, and a ratio of $\alpha/\beta = 3.4$ Gy. This value is indicative of a radioresistant cell line.

Table 3.1.1:1 presents a summary of the survival curve parameters D_0 and D_q extracted graphically from 9L cell survival curves from various sources in the literature. There is considerable variation in survival curves measured by different investigators,

Table 3.1.1:1. Summary of some published 9L cell survival curves

Source	Cell cycle phase	D ₀ (Gy)	D _q (Gy)
Ross-Riveros 1979	exponential	1.66	2.6
Deen 1979	exponential	2.02	5.5
Keng 1980	early G1	2.40	2.6
Seidentfeld 1980	not specified	2.14	7.0
Weizsaecker 1981	not specified	2.52	4.3
Wheeler 1981 9L/SF	not specified	2.48	4.3
Wheeler 1981 9L/Ro	not specified	1.80	5.4
Mean (sd) of above studies		2.15 (0.34)	4.5 (1.6)
Figure 3.1.1:1	plateau	1.94	5.5

likely due to various sub-populations of 9L cells, and different conditions of irradiation. However, all 9L survival curves show a large shoulder and radioresistance, and the survival curve parameters measured from figure 3.1.1:1 fall within the range of values reported in the literature.

Figure 3.1.1:2 shows cell survival curves for various cells lines measured in the laboratories of the Ottawa Regional Cancer Centre under identical conditions of irradiation. While the 9L is the most radioresistant of the cell lines shown, it shares the characteristic of a large shoulder with the human malignant glioma cell line 87-MG. This supports the use of the 9L cell line as a good model for the radiation response of human malignant gliomas. The large shoulder on the human malignant glioma survival curve is consistent with the clinical observation that gliomas are resistant to radiotherapy (Gerweck 1977, Raaphorst 1989), and that this radioresistance is correlated with a large capacity for repair of radiation damage (Weichselbaum 1982, Weichselbaum 1983).

Table 3.1.1:2 summarizes the radiation survival curve parameters for the 4 cell lines shown in figure 3.1.1:2. (A linear regression of $-\ln S/D$ vs. D was used to calculate α/β ratio; HT-144 data could not be fit because the data is nearly exponential, i.e. no β component). The α/β ratio for SK-MEL 3 is smaller than α/β for 87-MG, despite the large shoulder on the SK-MEL 3 survival curve. This reflects the steeper final slope ($D_0 = 0.96$ vs. 1.12 for 87-MG) and continuing downward curve, suggesting a dominant β term for SK-MEL 3. The 9L is the most resistant of the 4 cell lines shown.

Table 3.1.1:2 Survival curve parameters for 4 cell lines from ORCC laboratory

Cell line	α/β (Gy)	D_0 (Gy)	D_q (Gy)
HT-144	--	0.50	0.3
87-MG	3.1	1.12	2.0
SK-MEL 3	2.3	0.96	6.5
9L	3.4	1.94	5.5

Figure 3.1.1:1. Cell survival curve for 9L cells irradiated in vitro. Plateau phase cells were irradiated on ice using cobalt-60 and plated immediately. Each point represents the mean surviving fraction of 4 replicate experiments; the error bars represent the standard error of the mean for each point.

Figure 3.1.1:2 Radiation survival curve for 4 different cell lines. The curves were obtained in the same laboratory under similar conditions. The 87-MG is a human malignant glioma cell line (data courtesy of D. Heller, ORCC). The HT-144 and SK-MEL 3 are radiosensitive and radioresistant human malignant melanoma cell lines, respectively (data courtesy of A. Bussey, ORCC). Each point represents the mean surviving fraction of 3 or 4 replicate experiments; error bars are SEM.

3.1.2 Cisplatin response of 9L cells

3.1.2.1 Purpose of cisplatin dose response experiment

The purpose of this experiment was to determine a dose response curve for 9L cells exposed to cisplatin *in vitro*. This was to help in establishing an appropriate range of cisplatin doses and exposure times to be used in further *in vitro* experiments combining radiation and cisplatin, as well as providing guidance for *in vivo* cisplatin protocols. In addition, this experiment was intended to test the reciprocity of cisplatin concentration and exposure time *in vitro*, and to provide information on the timescale over which cisplatin in tissue culture medium remains toxic to 9L cells.

3.1.2.2 Method

Plateau phase cells in T-25 tissue culture flasks were exposed to cisplatin for one hour as described in section 2.1.5. Cisplatin concentrations of 0, 2, 3, 4, 6, 8, 10, 16, and 20 $\mu\text{g/ml}$ were used. Additional plateau phase cultures were exposed to cisplatin at 6 $\mu\text{g/ml}$ for various times: 0.33, 0.5, 0.66, 1.0, 1.33, 1.66, 2.25, 2.66, and 3.33 hours. At the end of the exposure period, the medium containing cisplatin was aspirated, the cells were rinsed and processed for the colony forming assay as described in section 2.1.3. Control flasks (plating efficiency) received sham cisplatin treatments.

A further experiment was performed to determine the extent to which the toxicity of cisplatin was diminished with incubation on cells and in cell-free tissue culture medium. In this experiment, cisplatin at 6 $\mu\text{g/ml}$ was added to the medium of plateau phase cell cultures, and also to cell-free depleted medium, and incubated for 0, 6, 11, 23, and 27 hours. This cisplatin-containing medium was then placed on other plateau-phase cultures for one hour, after which these cells were rinsed and processed for the colony forming assay.

Figure 3.1.2:1 shows the cisplatin dose-response curve for in vitro exposure of plateau-phase 9L cells. Exposure to 6 $\mu\text{g/ml}$ cisplatin for one hour reduces the survival of 9L cells to approximately 35%. A reduction in survival to 10% requires 8.8 $\mu\text{g/ml}$ for 1 hour.

The cisplatin dose-response curve has a noticeable shoulder region at low cisplatin dose. This can be interpreted in two possible ways (Wheeler 1978): 1) In analogy with radiation survival curves, 9L cells might be able to accumulate and/or repair sublethal cisplatin damage; 2) the shoulder might predominantly represent the time required to hydrolyze native cisplatin to its toxic form, i.e. replacing chloride ions with hydroxyl groups (Daley-Yates 1986, Goel 1992). There is evidence for repair of cisplatin DNA lesions; a caffeine-sensitive repair process in Chinese hamster ovary cells has been shown by Van den Berg and Roberts, who abolished the shoulder on the cisplatin dose-response curve by post-treatment incubation in a non-toxic concentration of caffeine (Van den Berg 1975). In addition, Roberts and Fraval have shown that different sensitivities of cell lines to cisplatin are correlated with different capacities to remove DNA-platinum lesions (Roberts 1980).

In analogy with the molecular theory of cell survival (linear-quadratic model) for radiation (Chadwick 1973), the shoulder of the cisplatin dose-response curve can be interpreted as being due to the combination of two independently produced DNA lesions into a single, lethal lesion. For cisplatin, the predominant lesion is likely the intrastrand crosslink between adjacent guanines (Eastman 1986). The probability of two such lesions occurring on opposite strands in close proximity is proportional to the square of the cisplatin concentration; such an event is analogous to the combination of two neighbouring radiation-induced single-strand breaks to form a lethal double-strand break, which is related to the square of the radiation dose. A lesion which depends linearly on the cisplatin dose is the interstrand crosslink, which probably accounts for only about 1% of total DNA platination (Eastman 1986). This model of cisplatin action leads to a linear-quadratic dose-response curve with a pronounced shoulder (Chadwick 1976).

Table 3.1.2:1 summarizes the relative sensitivities of some cell lines to cisplatin reported in the literature. The 9L cell line can be categorized as being resistant to cisplatin, although there are other cell lines which are more resistant.

Table 3.1.2:1 Relative sensitivity of various cell lines to cisplatin

Cell line	Cisplatin concentration to reduce survival to 10% ($\mu\text{g/ml}$ for 1 hour)	Source
RIF-1 fibroblast	0.9	Barcellos-Hoff 1987
TR 175 ovarian ca.	3	Shellard 1991
A2780 ovarian ca.	4	ORCC data
A2780 ovarian ca.	8	Terheggen 1987
U373-MG human glioma	8	ORCC data
9L rat glioma	8.8	Wilkins, fig 3.1.2:1
CHO hamster	12	Terheggen 1987
SK-OV-3 ovarian ca.	12	Shellard 1991
A1847 ovarian ca.	15	Terheggen 1987
A2780 ^{CP} cis res ov ca	19	ORCC data

3.1.2.4 In vitro cisplatin "exposure dose"

From figure 3.1.2:1, it is clear that there is good reciprocity between cisplatin concentration and exposure time up to approximately $10 \mu\text{g/ml} \times \text{h}$. This shows that the definition of cisplatin "exposure dose" (Wheeler 1978) as the product of cisplatin concentration and exposure time is valid in vitro for exposure times of less than about 2 hours. For exposure times longer than 2 hours, the curve for $6 \mu\text{g/ml}$ for various times diverges from the curve for 1 hour exposure to various concentrations. This is because of binding of the cisplatin to serum proteins, which inactivates the cisplatin and reduces the toxicity towards cells. HPLC studies of in vivo cisplatin metabolites by other investigators have found that 6 hours after i.v. administration of cisplatin in rats, 50% of total Pt is protein bound and inactive (Mistry 1989); 3 hours after i.p. administration in rats, 95% of total Pt is protein bound and inactive (Daley-Yaies 1986). Human cells in vitro were found to be impermeable to protein-bound cisplatin; cisplatin in human serum has a half-life of 2.1 h before becoming protein-bound (Melvick 1992).

The diminished toxicity of cisplatin in vitro due to serum protein binding for exposure times longer than about 2 hours proves that there is no advantage to using long cisplatin exposure times for in vitro experiments. A standard one hour exposure time for all further in vitro cisplatin and radiation plus cisplatin experiments was adopted.

3.1.2.5 Diminished cisplatin toxicity with incubation

As described in section 3.1.2.2, a further experiment was performed to elucidate the rate of inactivation of cisplatin by serum protein binding. The results of this experiment are shown in figure 3.1.2:2. Cisplatin incubated at 37°C in the medium covering plateau phase 9L cells, or in depleted medium without cells present, became less toxic against other cultures of plateau phase 9L cells. Figure 3.1.2:2 indicates that it made little difference whether or not cells were present during this incubation period. This supports the interpretation that the inactivation of cisplatin is due to binding to serum proteins in the medium, as opposed to removal of cisplatin from the medium by cellular binding or metabolism. The cisplatin was reduced to half of its original toxicity after approximately 7 hours of incubation in depleted medium or on cells. This agrees with the *in vivo* HPLC studies on rat plasma ultrafiltrate showing half of cisplatin inactivated by protein binding 6 hours after *i.v.* administration (Mistry 1989). The results of this experiment support the *in vitro* protocol of one hour cisplatin exposure times, as figure 3.1.2:2 shows little loss of cisplatin toxicity with one hour incubation on cells or in depleted medium. This loss of cisplatin toxicity due to protein binding is significant in the design *in vitro* cisplatin protocols involving extended exposure times, for example induction of cisplatin resistance, or combining cisplatin with low dose rate irradiation.

Figure 3.1.2:1. In vitro 9L cisplatin dose response curve. The closed symbols represent the surviving fraction of cells following exposure to 6 $\mu\text{g/ml}$ cisplatin for various times. The open symbols represent the surviving fraction following exposure to various concentrations of cisplatin for 1 hour. The data is plotted as "cisplatin dose" along the abscissa, where cisplatin dose is the product of cisplatin concentration and exposure time. Points are the mean surviving fraction of 3 replicate experiments; error bars are SEM. The curves diverge at high cisplatin dose due to inactivation of cisplatin with serum protein binding at long incubation times.

Figure 3.1.2:2. Cisplatin toxicity after prior incubation in plateau phase cultures of 9L cells, or in depleted medium without cells. Cisplatin ($6 \mu\text{g/ml}$) was added to the medium over plateau phase cells, or to depleted medium without cells, and incubated for various times. This medium was then used to expose fresh plateau phase cells to cisplatin. The reduction in cisplatin toxicity with incubation time is due to binding to serum proteins, which inactivates the cisplatin. Points are the mean surviving fraction of 3 replicate experiments; error bars are SEM.

3.1.3 Potentially lethal damage repair (PLDR) experiments

3.1.3.1 Purpose of experiments

The way in which cells are treated following irradiation can affect the surviving fraction after a given dose because of the expression or repair of potentially lethal damage (PLD). This damage is potentially lethal because it causes cell death only if it remains unrepaired. PLD is damage which under one set of post-irradiation conditions is lethal, but under other conditions is not lethal (Bedford 1991). Allowing cells to cycle generally fixes PLD and causes its expression, reducing the proportion of cells surviving the radiation dose. However, if cells are held in a non-cycling state following irradiation, then the proportion of surviving cells increases, implying that repair of damage has occurred. In the experiments described in this section, PLD repair was evaluated according to the method of Little et.al., who studied PLDR in density-inhibited plateau phase cell cultures (Little 1973), which are considered to be a better in vitro model for tumour cells in vivo than exponential cell cultures (Hall 1988). The magnitude and kinetics of recovery from potentially lethal radiation damage correlate well with rejoining of DNA strand breaks, leading to the interpretation of PLD recovery as resulting from repair of this DNA lesion (Chadwick 1978, McWilliams 1983, Curtis 1986, Wheeler 1991).

The experiments described in this section measured the capacity of 9L cells to recover from radiation damage when the cells were held in plateau phase for a post-irradiation incubation period. The kinetics of this recovery were investigated, and recovery at two different doses (12 and 18 Gy) was assessed. The effect of cisplatin on recovery from potentially lethal radiation damage was evaluated at three different cisplatin doses.

3.1.3.2 Method

All PLDR experiments were conducted on half-fed plateau phase 9L cell cultures (see section 2.1.2). Cells were irradiated in T-25 flasks on ice (see section 2.1.4) to either 12 Gy or 18 Gy. These doses were chosen to give an initial cell survival of approximately 1% and 0.1% respectively. After irradiation, cells were warmed by immersing the flasks for 1 minute in a 40°C water bath, and placed in a CO₂ incubator at 37°C for the post-irradiation incubation period in density-inhibited plateau phase. The starting point of the post-

irradiation incubation period was considered to be the instant of removal from the water bath. Flasks were taken from the incubator and processed for the colony forming assay (see section 2.1.3) at various times from 0 to 24 hours after irradiation.

For experiments on the effect of cisplatin on PLDR, cisplatin was added after irradiation to the existing medium in the flasks, immediately before placing cells in the incubator. Cisplatin doses of 3, 4.5, or 6 $\mu\text{g/ml}$ for 1 hour were used (10, 15, 20 μM). After 1 hour, the medium containing cisplatin was aspirated, the cells were thoroughly rinsed with citrate saline, and new depleted medium was added. (Depleted medium was previously harvested from identical plateau phase cell cultures and kept refrigerated until needed). The cells were then returned to the incubator for the rest of the post-irradiation incubation period.

All experiments were repeated a minimum of two and usually three times, and results plotted as the mean $\pm$ standard error of the mean. Control flasks exposed to cisplatin alone were used to determine cisplatin toxicity for each experiment. All flasks received either real or sham radiation and cisplatin treatments. The surviving fraction of cells receiving radiation plus cisplatin was divided by the surviving fraction of the cells receiving cisplatin alone, in order to correct for the toxicity of cisplatin to allow direct comparison with the PLDR curves for cells receiving radiation only. The recovery ratio was determined from PLDR curves by taking the maximum surviving fraction reached during the post-irradiation incubation period (usually at $t = 12$ h) and dividing by the initial surviving fraction at $t = 0$ h.

3.1.3.3 Results and discussion: 9L PLDR without cisplatin

The results of the PLDR experiments without cisplatin at 12 and 18 Gy are shown in figure 3.1.3:1. The recovery ratio at 12 Gy is 4.6; the recovery ratio at 18 Gy is 18.1. This agrees well with recovery ratios for 9L cells reported in the literature (Wheeler 1981). Recovery occurs quickly after irradiation, with half the recovery occurring in less than two hours. Other investigators have found that DNA repair in 9L cells occurs with a fast and slow component; the fast component has a half-time of approximately 6 minutes, whereas the slow component has a dose-dependent half-time ranging from approximately 42 minutes at 15 Gy to approximately 87 minutes at 50 Gy (Wheeler 1991).

The importance of PLDR on the outcome of radiotherapy has been emphasized by experiments showing that repair occurs in vivo in experimental tumours. Using intracranial

9L tumours, Wheeler and Wallen showed that PLDR, comparable in magnitude to that found *in vitro*, occurs *in vivo*, and that cell survival measured after the completion of recovery from radiation-induced PLD was the determinant of response as measured by increased lifespan of tumour-bearing animals (Wheeler 1980a). Enhanced PLDR has been implicated in the extreme radioresistance of human malignant gliomas (Weichselbaum 1982, Weichselbaum 1986) and other radioresistant tumours (Weichselbaum 1988, Schwartz 1990), although not all authors agree with this interpretation (Deschavanne 1990).

Figure 3.1.3:2 shows the PLDR curves for both 9L cells and the human malignant glioma cell line U-87-MG, acquired under identical experimental conditions. The radiation doses used (18 Gy for 9L, 9 Gy for U-87-MG) were chosen to give approximately the same surviving fraction at the zero time point. The magnitude and kinetics of the PLDR are almost identical for the two cell lines. This reinforces the usefulness of the 9L cell line as a model for the radiation response characteristics of human malignant gliomas.

3.1.3.4 Results and discussion: 9L PLDR with cisplatin

Figures 3.1.3: 3, 4 and 5 show the results of PLDR experiments in which cisplatin was present in the medium for the first hour after irradiation, at concentrations of 3, 4.5, and 6 $\mu\text{g/ml}$ respectively. The survival of cell cultures treated with radiation plus cisplatin has been corrected for the toxicity of cisplatin alone. Cells exposed to cisplatin alone show no recovery in cell survival with incubation up to 21h following cisplatin exposure (see fig 3.1.6:3 on page 85).

The data clearly show a cisplatin dose-dependent inhibition of PLDR. While several studies have shown modest inhibition of PLDR in mammalian cells *in vitro* at similar cisplatin doses (Douple 1985, Dritschilo 1979, Momiki 1987, Wallner 1987), the inhibition has not been as definite as that shown here. More studies have shown inhibition of sub-lethal damage repair (SLDR) (Carde 1981, Dewit 1985, Schwachofer 1991) or a change in the shoulder of the radiation cell survival curve (Alvarez 1978, Begg 1987, Douple 1990). However, while inhibition of repair of radiation damage by cisplatin has been observed in many SLDR and PLDR studies, it is not universally supported in the literature (Coughlin 1989, Dewit 1987, Picker 1987).

The mechanisms of radiosensitization by cisplatin have not been clearly established (Coughlin 1989). A radiochemical, free-radical mediated radiation potentiation mechanism

has been identified involving one-electron reduction of cisplatin leading to an active radiolytic product (Coughlin 1989). While this mechanism is important in radiosensitization of bacterial cells at high radiation doses, and might play a role in hypoxic mammalian cell radiosensitization (Dewit 1987), it is not likely a significant factor in oxic mammalian cells irradiated at clinically relevant doses (Douple 1979, Richmond 1991), where repair inhibition is the more important mechanism of radiosensitization (Coughlin 1989).

Cisplatin radiosensitization may occur in part through cell cycle redistribution (Begg 1986, Tanabe 1987). Cisplatin treatment can arrest cycling cells in G₂ (Begg 1986), which is a radiosensitive phase of the cell cycle. Since the present experiments were performed on plateau phase cell cultures, this is not likely to be a significant mechanism of cisplatin radiosensitization.

The mechanism of inhibition of cellular radiation repair processes by cisplatin is complex and has not been clearly elucidated (Dewit 1987, Coughlin 1989, Picker 1987, Schwachofer 1991), but a model for the interaction of radiation and cisplatin has been proposed (Chadwick 1976). In analogy with the molecular theory of cell survival for radiation (Chadwick 1973), cisplatin could cause a lethal event by forming either a DNA interstrand crosslink, or two intrastrand crosslinks on each of two complementary DNA strands. A radiation-induced single strand break opposite a cisplatin-induced intrastrand crosslink could result in a supra-additive or synergistic interaction, where one DNA lesion would interfere with the repair of the other, leading to a disruption of the two complementary strands. In analogy with radiation-induced double-strand breaks, this would be a lethal lesion for the cell (Chadwick 1978), with reduced repair of these interaction lesions (Begg 1990). While this model appears to be reasonable, it remains speculative (Dewit 1987, Coughlin 1989).

Figure 3.1.3:1. Potentially lethal damage recovery in plateau phase 9L cells (without cisplatin). Cells were irradiated to 12 or 18 Gy and incubated at 37°C for various times before plating for the colony forming assay. Points are the mean surviving fraction of 3 replicate experiments, error bars are SEM.

Figure 3.1.3:2 Comparison of potentially lethal damage recovery in 9L cells and U-87-MG human malignant glioma cells, assessed using identical experimental methods. The radiation doses used were chosen for iso-survival at the origin of the time axis. Points are the mean surviving fraction of 3 replicate experiments, error bars have been left omitted for clarity. (U-87-MG data courtesy of D. Heller, Ottawa Regional Cancer Centre).

Figure 3.1.3:3. Potentially lethal damage recovery in plateau phase 9L cells irradiated to 18 Gy and treated with 3 $\mu\text{g/ml}$ cisplatin for 1 hour immediately following irradiation. Points are the mean surviving fraction of 2 replicate experiments, error bars are SEM. The surviving fraction of cells treated with cisplatin has been corrected for the toxicity of cisplatin alone, based on the survival of control flasks treated with cisplatin.

Figure 3.1.3:4. Potentially lethal damage recovery in plateau phase 9L cells irradiated to 18 Gy and treated with 4.5 $\mu\text{g/ml}$ cisplatin for 1 hour immediately following irradiation. Points are the mean surviving fraction of 2 replicate experiments, and have been corrected for the toxicity of cisplatin alone. Error bars are SEM.

Figure 3.1.3:5. Potentially lethal damage recovery in 9L cells irradiated to 18 Gy and treated with 6 $\mu\text{g/ml}$ for 1 hour immediately following irradiation. Points are the mean surviving fraction of 3 replicate experiments, corrected for the toxicity of cisplatin alone. Error bars are SEM.

3.1.4 In vitro radiation and cisplatin: delayed plating experiments

3.1.4.1 Purpose of experiments

The experiments of section 3.1.3 were performed at a single radiation dose (18 Gy). The delayed plating radiation survival experiments described in this section were performed to elucidate the radiation dose response of repair inhibition by cisplatin. These experiments were intended to confirm the results of section 3.1.3, and verify that inhibition of PLDR by cisplatin occurs over a range of radiation doses. In these experiments, the post-irradiation recovery time was kept constant at 8 hours, while the radiation dose was varied. From figure 3.1.3:1, it can be seen that almost full PLD recovery is completed within 8 hours of irradiation.

3.1.4.2 Method

Plateau phase 9L cell cultures were irradiated on ice as previously described, at the various doses from 0 to 18 Gy, three flasks at each dose. The first group of flasks was plated for the colony forming assay immediately, in order to generate the acute cell survival curve as in section 3.1.1 (immediate plating, IP). The second group of flasks was incubated at 37°C for 8 hours prior to plating, in order to allow full PLD recovery to occur (delayed plating, DP). This group received a sham cisplatin treatment (saline, followed by rinse and depleted medium change.) The third group of flasks was treated with 6 µg/ml cisplatin for 1 hour immediately after irradiation and incubated at 37 °C. After 1 hour, the cisplatin-containing medium was aspirated, the cells rinsed with warm isotonic saline, and fresh depleted medium was applied before returning the flasks to the incubator for the remainder of the 8 hour post-irradiation incubation period (delayed plating with cisplatin, DP+cis). Control flasks treated with cisplatin alone were included to allow the surviving fraction of the DP+cis flasks to be corrected for the toxicity of cisplatin alone.

3.1.4.3 Results and discussion

The results of this experiment are shown in figure 3.1.4:1. The lower curve is the immediate plating radiation only group (IP), which is directly comparable to the survival curve in figure 3.1.1:1. The upper curve is the radiation only delayed plating group (DP), in which almost full PLDR has occurred. At 12 and 18 Gy, the recovery ratios are 5.8 and 13.5 respectively. This is in approximate agreement with the recovery ratios from figure 3.1.3:1, which were 4.6 and 18.1 for 12 and 18 Gy, respectively. The middle curve is the radiation plus cisplatin delayed plating curve, showing significant inhibition of PLDR by cisplatin (t-test, $p < .05$). Two points, at 12 and 18 Gy, are shown for cells treated with cisplatin for 1 hour after irradiation then plated immediately. These points coincide (after correction for the cytotoxicity of cisplatin alone) with the radiation only immediate plating curve. This confirms that the radiosensitizing effect of cisplatin occurs through inhibition of post-irradiation recovery.

Eight hour recovery ratios were calculated from the data of figure 3.1.3:1. These recovery ratios are plotted as a function of dose in figure 3.1.4:2. At 18 Gy, the 8 hour recovery ratio for radiation plus cisplatin is 4.0 ± 1.5 . This agrees within experimental error with the recovery ratio determined in the PLDR experiments of figure 3.1.3:5, which was 2.3. Significant inhibition of PLDR by cisplatin clearly occurs in both versions of the experiment.

For radiation doses less than 8 Gy, the recovery ratio for cells treated with cisplatin is less than 1, indicating more than complete inhibition of PLDR. This phenomena is known as "enhanced chemotoxicity", and has been observed in other cell lines (Douple 1985). This total inhibition of PLDR at lower doses is potentially significant in the clinical situation (Skov 1991), because in standard fractionated radiotherapy the daily fractions are typically 2 - 3 Gy .

Figure 3.1.4.1. Delayed plating radiation survival curve for plateau phase 9L cells with and without cisplatin. The lower curve is the immediate plating acute cell survival curve without cisplatin, and is directly comparable to figure 3.1.1:1. In the upper curve, cells were allowed a 8 h post-irradiation incubation period to allow PLDR to occur. In the middle curve (delayed plating + cisplatin) irradiated cells were treated with cisplatin at 6 $\mu\text{g/ml}$ for the first hour of the 8 h post-irradiation recovery period. Two points (12 and 18 Gy) are plotted for cells treated with cisplatin for 1 h following irradiation then plated immediately. The survival of cisplatin-treated cells has been corrected for the toxicity of cisplatin alone. Points are the mean surviving fraction of 3 replicate experiments, error bars are standard error of the mean.

Figure 3.1.4:2. Eight hour recovery ratio as a function of radiation dose for plateau phase 9L cells with and without cisplatin. The data from figure 3.1.4:1 was used to generate recovery ratios for radiation only, and radiation plus cisplatin at 6 $\mu\text{g/ml}$ for 1 h immediately following irradiation.

3.1.5 Low dose rate irradiation combined with cisplatin

3.1.5.1 Purpose of experiments

Radiotherapy is usually delivered in daily fractions of 2 - 3 Gy, which allows for repair of potentially lethal and sublethal radiation damage. This permits normal tissues surrounding the tumour to repair radiation damage, enhancing the therapeutic ratio. The choice of an optimum dose and dose-delivery schedule which minimizes the probability of normal tissue injury and cancer recurrence depends on many factors, including the intrinsic radiosensitivity, growth kinetics and volume of the tumour and surrounding normal tissue. Intrinsic radiosensitivity depends primarily on the proportion of hypoxic cells and the capacity for repair (Weichselbaum 1983). Low dose rate (LDR) irradiation (0.5 - 2.0 cGy/min), which is used extensively in brachytherapy (implanted radioactive sources) and immunotherapy, maximizes the possibility for repair due to the dose rate effect (Hall 1988).

Brachytherapy is used only in approximately 5% of radiation-treated cancers, but in this group it is considered the treatment of choice (Hall 1988). The advantages of brachytherapy include: a sharp decrease in dose with distance, improving localization of dose in the tumour and allowing a higher total dose to be delivered to the tumour without concomitant damage to surrounding normal tissue; and low dose rate, continuous irradiation which maximizes opportunity for repair. However, in tumours with a large intrinsic capacity for repair, the advantage of brachytherapy may be maximized with administration of a radiosensitizing repair inhibitor such as cisplatin. A disadvantage of brachytherapy is the difficulty in delivering a uniform and adequate dose over the entire tumour volume. This situation could be ameliorated with the administration of a radiosensitizer such as cisplatin.

In this section, experiments are described in which cisplatin treatment was combined with low dose rate irradiation in plateau phase 9L cells. The aim was to determine whether there was increased survival under low dose rate conditions compared with acute irradiation, and to investigate the effect of several in vitro cisplatin treatment regimes on cell survival under low dose rate conditions. The experiments were preliminary in nature and do not represent a thorough investigation of combined in vitro cisplatin - low dose rate treatments.

Cells were grown to confluency in small glass vials with a growing surface of 1.4 cm². Irradiation was performed in a temperature controlled low dose rate cell irradiator consisting of eight 100 mCi Radium-226 sources arranged in a circular array inside a lead shielded chamber, mounted inside a standard cell incubator. Up to 12 cell vials at once were mounted in a lucite jig and positioned on an isodose contour resulting in less than 1% variation in dose across the cell growth surface. Cells were irradiated at 37°C to a dose of either 12 or 18 Gy. The doserate was 0.882 cGy/min, resulting in a total irradiation time of 22.68 h for 12 Gy or 34.01 h for 18 Gy. Cells were processed for the colony forming assay immediately following irradiation.

Cisplatin was added to the cells during LDR irradiation according to three different schemes, as described below.

- Scheme #1: 6 µg/ml for the first hour of LDR irradiation, aspirate, replace with fresh depleted medium (1h)
- Scheme#2: 6 µg/ml for the first hour of LDR, aspirate, fresh depleted medium with new cisplatin 6 µg/ml for second hour, aspirate, replace with fresh depleted medium (1h - 1h)
- Scheme #3: 6 µg/ml for the first hour of LDR, aspirate, replace with fresh depleted medium, new cisplatin 6µg/ml for the 13th hour of LDR, aspirate, replace with fresh depleted medium (1h - 12 h - 1 h).

3.1.5.3 Results and discussion

Figure 3.1.5:1 shows the results of the low dose rate experiments for 12 and 18 Gy, without cisplatin and with cisplatin in the three treatment schemes. Low dose rate irradiation results in considerably enhanced survival compared with acute (Co-60) irradiation, due to increased repair during LDR. Even when compared with the 8 hour delayed plating survival in figure 3.1.4:1, the LDR survival is a factor of 3 higher at 12 Gy, and a factor of 6 higher at 18 Gy. This indicates the greater opportunity for repair of radiation damage under low dose rate conditions.

The reduced survival after administration of cisplatin is due to inhibition of this repair, since the plotted survival has been corrected for the toxicity of cisplatin alone. Cisplatin administration schemes 2 and 3 show greater inhibition than scheme 1, indicating that a 1 hour exposure at the start of LDR irradiation does not have a lasting inhibitory effect throughout the course of irradiation. Inhibition would likely be maximized by continuous exposure to cisplatin during LDR irradiation (Fu 1985), but the results of section 3.1.2.6 show that it is necessary to refresh the cisplatin over the course of irradiation, since the drug loses its effectiveness due to binding to serum proteins in the medium.

Under acute irradiation conditions at 18 Gy, exposure to cisplatin reduced survival to 30% of the level without cisplatin (8 hour delayed plating, fig. 3.1.4:1). Under LDR irradiation conditions, exposure to cisplatin reduced survival to as little as 10% of the level without cisplatin (scheme #2, fig. 3.1.5:1). These results indicate that radiosensitization by cisplatin is greater when irradiation conditions are conducive to repair (LDR), compared with acute irradiation.

Figure 3.1.5:1. Low dose rate irradiation of plateau phase 9L cells combined with cisplatin. Surviving fraction has been corrected for the toxicity of cisplatin alone. The 12 Gy dose was delivered over 22.69 h; the 18 Gy dose was delivered over 34.01 h. Cisplatin was administered according to three different treatment schemes, as described in section 3.1.5.2. Surviving fraction is the mean of 3 replicate cell cultures, error bars are SEM.

3.1.6 Sequencing of radiation and cisplatin treatments

3.1.6.1 Purpose of experiments

The effects of time sequence and dose schedule of cisplatin and radiation administration has been investigated in a number of in vitro, in vivo and clinical studies, and no clear conclusion has emerged. Clinical trials have revealed that the combination of cisplatin with radiation is apparently independent of particular sequence, at least for the sequences studied to date (Coughlin 1989, Dewit 1987). While in vivo studies of combined cisplatin and radiation therapy in a variety of experimental tumour systems generally indicate that maximum radiosensitizing effect is observed when cisplatin is given a short time before irradiation (Dewit 1987, Kanazawa 1988), one study found maximum effect when cisplatin was administered 24 h prior to irradiation (Tanabe 1987). A study of three in vivo tumour systems found no optimum radiation and cisplatin treatment sequence which gave consistent results in the different tumour types (Twentyman 1979). Another in vivo study found cisplatin did not act as a radiosensitizing agent (von der Maase 1985). In vitro studies have generally shown maximum radiosensitizing effect when cisplatin is present at the time of irradiation (Schwachofer 1991, Dewit 1987).

There is little information in the literature on the effect of treatment sequence on cisplatin inhibition of potentially lethal damage repair following irradiation. The experiments described in this section investigated the effect of treatment sequence on PLDR in plateau phase 9L cells.

3.1.6.2 Method

Plateau phase 9L cell cultures were exposed to cisplatin at 6 $\mu\text{g/ml}$ for 1 hour at 12, 6, or 1 hours before irradiation, or immediately following irradiation with 18 Gy Co-60. After the 1 hour cisplatin exposure, cells were rinsed, the medium was replaced with depleted medium, and the cells were incubated until irradiation. Six flasks were used for each time point: radiation only, immediate plating and 8 h delayed plating; radiation plus cisplatin, immediate plating and 8 h delayed plating; cisplatin only; and a control flask for plating efficiency. All flasks received either real or sham radiation and cisplatin treatments. After

irradiation, cells were plated either immediately, or incubated for 8 h prior to plating for the colony forming assay.

3.1.6.3 Results and discussion

Figure 3.1.6:1 shows the results of these experiments. Maximum PLDR inhibition occurs when cisplatin exposure occurred immediately after irradiation; there was no significant inhibition of PLDR when the drug was administered 6 or 12 hours prior to irradiation (t-test, $p < .05$).

In figure 3.1.6:2, a parameter "PLDR inhibition" has been calculated and plotted from the data of figure 3.1.6:1. This parameter is defined as

$$\text{PLDR inhibition} = \frac{(\text{recovery ratio radiation}) - (\text{recovery ratio radiation} + \text{cisplatin})}{(\text{recovery ratio radiation})}$$

PLDR inhibition was maximum ($0.92 \pm .15$) when cisplatin was administered for the first hour following irradiation, compared with $0.61 \pm .10$ when administered for the hour immediately prior to irradiation. These results suggest that the cellular lesions responsible for cisplatin inhibition of PLDR are removed quickly, and that maximum repair inhibition is achieved only when cisplatin is present in the medium during the period of potentially lethal damage repair.

In contrast, the toxicity of cisplatin alone is independent of the time of administration. Figure 3.1.6:3 shows the results of experiments in which cisplatin was administered ($6 \mu\text{g/ml}$ for 1 h) at various times before plating for the colony forming assay. The survival of cells exposed to cisplatin and incubated for up to 20 hours in plateau phase is not substantially different from the survival of cells plated immediately after exposure to cisplatin. These data show that any cellular repair mechanisms for removing lethal cisplatin lesions operate on a long timescale, possibly as long as 24 - 60 hours (Dewit 1987, Barot 1985, Drewinko 1975). The markedly different timescale of removal of cellular lesions responsible for inhibition of PLDR by cisplatin suggests that the lesions which cause cell death by cisplatin may be fundamentally different from the lesions which inhibit repair of potentially lethal radiation damage.

Figure 3.1.6:1. Effect of sequence of radiation and cisplatin treatment on PLDR. Plateau phase 9L cells were treated with cisplatin ($6 \mu\text{g/ml}$ for 1 hour) at 12, 6, or 1 hour before irradiation, or immediately after irradiation (18 Gy Co-60). The 8 h recovery ratio is plotted versus time of cisplatin treatment before irradiation. Vertical error bars represent the standard error of the mean ($n=2$), horizontal error bars represent the 1 hour duration of the cisplatin treatment.

Figure 3.1.6:2. PLDR inhibition in plateau phase 9L cells as a function of sequence of cisplatin and radiation treatments. PLDR inhibition was calculated from the data of figure 3.1.6:1, with PLDR inhibition defined as

$$\text{PLDR inhibition} = \frac{(\text{recovery ratio X only}) - (\text{recovery ratio X + cis})}{(\text{recovery ratio X only})}$$

2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

Figure 3.1.6:3. Cisplatin toxicity as a function of time between exposure and plating. Plateau phase 9L cells were exposed to cisplatin (6 $\mu\text{g}/\text{ml}$ for 1 h) then incubated for various times at 37°C in depleted medium before plating for the colony forming assay. Vertical error bars represent standard error of the mean (n = 3 experiments). Horizontal error bars represent the 1 hour duration of the cisplatin exposure.

3.2 In Vivo Experiments

3.2.1 Intracranial inoculation of human glioma cells

3.2.1.1 Purpose

The 9L rat glioma cell line is considered to be a good model for the human malignant astrocytoma which does not metastasize and is radioresistant with a large capacity for radiation repair (Barker 1987). However, the clinical relevance of an experimental program is increased with the use of human cell lines. The slow growth rate of human tumour cells compared with rat tumour cells allows a better adaptation of vascularization to the host environment (van Vaals 1991). For this reason, experiments were performed to investigate the possibility of using the human glioblastoma cell line U-87-MG and the human malignant astrocytoma cell line U-373-MG to grow intracranial tumours in Fischer rats. Since the brain comprises an immunologically privileged site, interspecies implants can sometimes be successful, as shown by reports of 9L tumours grown intracranially in cats with no immunosuppression (Lazareff 1988), and human malignant astrocytoma cells inoculated into rats (van Vaals 1991).

3.2.1.2 Method

The U-87-MG and U-373-MG cell lines were developed by J. Pontén and B. Westermarck at the University of Uppsala, Sweden, 1966-69 (Bigner 1981). Cells were cultured according to standard techniques described previously (section 2.1, also Raaphorst 1989). Cell suspension (2.5 µl) containing 6×10^4 cells was inoculated into the brains of 6 Fischer rats (3 each U-87-MG and U-373-MG) according to the technique described in section 2.2.3. Magnetic resonance imaging was performed at 3 and 6.5 weeks following tumour inoculation.

The MR images acquired at 3 and 6.5 weeks following tumour inoculation showed no indication of growth of intracranial tumours. Animals were sacrificed at 8 weeks following tumour inoculation, and were asymptomatic at that time. Since the in vitro generation time of U-87-MG and U-373-MG cells is approximately twice that of 9L cells (34 and 32 hours respectively, compared with 17 hours for 9L cells), and 9L cells produce a tumour easily visible by MRI after 2 - 2.5 weeks, it was expected that tumours from the human glioma cell lines would be visible after 4 - 5 weeks. This was not the case.

Figure 3.2.1:1 shows a MR image taken 3 weeks following tumour inoculation. The track of the inoculation needle is clearly visible in the brain, but this track is considerably wider than the needle itself. The extra width of the track and its jagged nature is believed to be evidence of an immunological attack mounted against the injected cells, resulting in scarring and edema in adjacent brain tissue. A similar enlarged and jagged track was observed in five of the six animals inoculated with human glioma cells.

In a report in the literature of successful inoculation of human astrocytoma cells into rat brains, cells were passaged as a flank tumour through nude mice prior to inoculation into the brains of rats (van Vaals 1991). Possibly this procedure, or immunosuppression through whole body irradiation or drugs such as cyclosporin, is necessary in order to ensure growth of a tumour. It is clear from these experiments that simply injecting human malignant glioma cells into the brains of Fischer rats does not result in the growth of intracranial tumours.

Figure 3.2.1:1. Magnetic resonance image of a rat brain 3 weeks after inoculation with U-373-MG human astrocytoma cells. The track of the inoculation needle is visible in the brain.

3.2.2 New inoculation technique for intracranial 9L tumours

3.2.2.1 Purpose

As described in section 2.2.3, changes were made to the standard technique of intracranial 9L tumour inoculation, in order to improve uniformity of viable cell number injected and increase efficiency of inoculation. In this section, data are presented in which the new inoculation technique is compared with the standard technique. In order to conserve experimental animals, these data were obtained from experiments designed to provide MRI and histopathological characterization of the growth of intracranial 9L tumours, and from untreated control tumours.

3.2.2.2 Method

A complete description of the new inoculation technique is presented in section 2.2.3, as well as a discussion of the differences between the new technique and the standard technique. To assess the effect of maintaining 9L cells on ice, an experiment was performed in which cell suspensions of 10^7 cells in balanced salt solution were placed on ice for various times, after which the cell viability was assessed using the colony forming assay.

3.2.2.2 Results and discussion

The standard implantation technique involves preparing a concentrated single cell suspension in balanced salt solution, which is placed on ice to prevent cell clumping while the rats are prepared for intracranial inoculation of 9L cells (Barker 1973, Barker 1976, Wheeler 1981). A potential systematic error is introduced because 9L cells lose viability with time when kept on ice (see figure 3.2.2:1). When many rats are inoculated by one person, the process can take many hours, and the last animals inoculated receive cells which have been on ice several hours longer than those received by the first animals. The result is that the number of viable cells being inoculated into the brain of each rat is not necessarily constant.

To overcome this problem, individual aliquots of cell suspension were prepared and frozen in advance for each rat. A single cell suspension consisting of 2.5×10^7 cells was prepared in freezing solution (10% dimethyl sulfoxide in complete medium), and 80 μ l aliquots were placed into individual cryovials. These vials were kept in liquid nitrogen until just before inoculation.

By using individual aliquots of frozen cells prepared in advance, each rat received the same number of viable cells, resulting in more uniform tumour growth. The viability of 9L cells after cryopreservation is consistently around 80%. The small amount (0.2 μ l) of DMSO injected with the cells appeared to cause no problem for the animal, and was washed away quickly in the aqueous environment in the brain.

Magnetic resonance imaging (MRI) was performed on tumour-bearing rats, and tumour size measured from the images. Figure 3.2.2:2 shows the problem with the standard technique of implantation. Tumour size measurements at various times after tumour implantation are shown for ten rats, five of which were the first rats implanted on a given day, five of which were the last rats done. The last rats received cells which had been on ice for 3 - 4 hours longer than those inoculated into the first rats. The tumours in the "last rat" group are generally smaller than those in the "first rat" group, suggesting that the "last rat" group received smaller numbers of viable cells.

Figure 3.2.2:3 shows MRI tumour size measurements versus time after implantation for tumours implanted with the standard method (cell suspension on ice) and with the new method (individual aliquots of frozen cells). While there is considerable variation in tumour size amongst individual animals for both methods, tumours implanted using the new method are generally more uniform in size and larger, indicating a higher number of viable cells were inoculated.

This technique of preparing individual frozen aliquots of cells for each animal increases efficiency and convenience, since cell aliquots for a large number of animals can be prepared in advance from a single passage, frozen for the duration of the experiments, and thawed for tumour implantation as required. This technique should be suitable for other cell lines that show high viable fractions after cryopreservation.

Figure 3.2.2:1. Effect of incubation on ice on 9L colony forming ability. Cell suspensions in Hank's balanced salt solution were placed on ice for various times, then assayed for survival by the colony forming assay. Error bars represent the standard error of the mean, $n=3$.

Figure 3.2.2:2. Intracranial tumour size measured by MRI at various times after 9L tumour inoculation, using the standard method with the cell suspension in balanced salt solution on ice. The last rat in each inoculation group received cells which had been on ice 3 - 4 hours longer than those inoculated into the first rat in each group. Each point represents one distinct animal. The rate of growth of tumours is greater for the first rats inoculated than for the last rats inoculated, due to loss of viability of cells maintained on ice. (first rat group: slope = $1.25 \text{ mm}^2/\text{day}$, SE = 0.33, $r^2 = 0.83$; last rat group: slope = $0.44 \text{ mm}^2/\text{day}$, SE = 0.20, $r^2 = 0.61$)

Figure 3.2.2:3. Intracranial tumour size versus time after 9L cell inoculation for the two implantation techniques. Each point represents a tumour size measurement from a distinct rat. A comparison of the distribution of tumour sizes after day 13 for the two groups shows that tumours implanted with the new technique (frozen aliquots of cells) are significantly larger ($p=0.003$) than tumours implanted with the standard method (cell suspension on ice).

3.2.3 Development of techniques for MRI of intracranial 9L tumours

3.2.3.1 Purpose

The brain of an adult rat is approximately 9 x 14 x 18 mm in size, and an intracranial 9L tumour is typically 3 mm in diameter. MRI contrast between tumour and surrounding normal brain is often insufficient for delineation of tumour boundaries (Rajan 1990, Bockhorst 1990). Accurate measurement of tumour size by MRI thus requires good image resolution, high signal sensitivity, and optimal imaging techniques for enhancing tumour-brain contrast. This section describes experiments investigating the dependence of tumour-brain contrast on MR image repetition time TR, as well as exploring the use of injectable MR contrast agents to enhance this contrast.

3.2.3.2 Resolution, imaging time, and contrast in MR imaging

While image resolution depends on available gradient strength, in practice it is determined by signal-to-noise and bandwidth considerations, magnet inhomogeneities, and sample motion (Thomas 1986, Cho 1988). Increases in resolution generally cause decreases in signal-to-noise ratio (SNR), since signal is being acquired from smaller volumes of sample, hence the importance of high sensitivity receiver coils. Image SNR can be improved by signal averaging ($\text{SNR} \sim \sqrt{n}$, where n = number of averages), with a resulting increase in total scan time. The total scan time for constant SNR varies as (Wehrli 1986)

$$TS = TR \left\{ \frac{N_x N_y}{d D_x D_y} \right\}^2$$

where TS is the total scan time, TR is the pulse sequence repetition time, N_x (N_y) is the x (y) direction matrix size (usually 256 x 256), d is the slice thickness, and D_x (D_y) is the field of view in the x (y) direction. This relation emphasizes the time penalties paid for increased image resolution; for example, a simultaneous reduction by a factor of two in both field of view and slice thickness would necessitate a 64-fold increase in scan time to preserve the same SNR. Since the maximum time tumour-bearing rats can be reliably maintained in the

magnet is 2-3 hours, SNR places an effective limit on image resolution. Figure 3.2.3:1 shows a high-resolution image of a normal rat brain, with a field of view of 2.0 cm, image matrix 256 x 256 (in-plane pixel size 78 μm) and a slice thickness of 150 μm . With a repetition time of 1800 ms and 22 averages, the scan time was more than 3 hours. For rats burdened with tumours, this amount of time under anesthesia was considered excessive, resulting in increased mortality. Images were normally performed with a field of view of 2.5 - 3.5 cm and a slice thickness of 0.8 - 1.2 mm, with total scan times of approximately 50 minutes.

For accurate measurement of the size of intracranial tumours, contrast is a more important parameter than resolution; the tumour-brain boundary can only be delineated if differences in brightness between tumour and brain can be detected on the image, i.e. if contrast is greater than image noise. Contrast between two regions of an image depends primarily on differences in water proton density, T1 and T2 relaxation times of the tissues, and the image pulse sequence parameters TE (inter-echo time) and TR. It was necessary to keep TE relatively short (22 or 34 ms) in order to minimize diffusion and/or perfusion effects which can cause image artifacts when slice thickness and pixel size are small (Ting 1992, Majumdar 1987). Over the range of magnetic field strengths in use in biomedical NMR, T1 increases with increasing field, whereas T2 is roughly independent of field (Bottomley 1987), or decreases slightly with increasing field (Rajan 1990). The contrast-to-noise ratio (CNR) for two regions of an image is defined as

$$\text{CNR}(i,j) = \text{SNR}(i) - \text{SNR}(j)$$

and the signal from a region of the n th echo image varies as (Wehrli 1986)

$$S(\text{TE}_n) \sim (1 - e^{-\text{TR}/T1}) e^{-\text{TE}_n/T2}$$

where $S(\text{TE}_n)$ is the signal from that region in the n th echo image, TR is the pulse sequence repetition time, T1 and T2 are the spin-lattice and spin-spin relaxation times respectively, and TE_n is the time of the n th echo. While this equation shows that overall signal intensity from one region of an image is maximum when $\text{TR} \gg T1$, it is clear that contrast between two regions of different T1 and T2 depends in a complex way on TR and TE_n . Without precise prior knowledge of the relaxation times of the tissues of interest, it is difficult to predict optimum pulse sequence parameters TR and TE_n , and empirical determination is necessary.

3.2.3.3 Method for experiments to determine optimum TR

Three animals with intracranial 9L tumours implanted 16 days prior to the experiments were used; the tumours were approximately 3.5 mm in diameter. Multiple (8) spin-echo images were acquired for each rat with pulse sequence repetition times ranging from 1165 ms to 8165 ms, keeping the number of signal averages constant at 2. Using the image processing software, the average signal (integral brightness IB) was measured in a square region of interest (0.18 cm^2) in four regions of each image: tumour, contralateral cortex, caudate putamen adjacent to the tumour, and a region outside the rat's head (to estimate the image noise). The contrast-to-noise ratio CNR was calculated for tumour-cortex and tumour-caudate putamen and plotted as a function of TR for each of the three animals.

3.2.3.4 Results and discussion

Figure 3.2.3:2 is the graph of CNR versus TR for the first echo images ($TE = 22 \text{ ms}$) of one of the animals. The CNR for tumour-cortex and tumour caudate reaches a maximum at a TR of 3 seconds. While there is considerable variation between the three animals, all demonstrated a maximum in CNR at a TR of approximately 3 seconds for tumour-cortex contrast. This is in approximate agreement with the findings of Ting and Bendel, who found maximum white/gray matter contrast in the rat at a TR of 3.7 s at 4.7 T (Ting 1992). CNR was highest in the first echo images ($TE = 22 \text{ ms}$); the SNR dropped rapidly in subsequent echo images, due to a decrease in signal due to T2 decay and refocussing errors, and an increase in apparent image noise due to spurious echoes and artifacts (Majumdar 1986).

These experiments show that the optimum contrast-to-noise ratio between tumour and surrounding normal brain is found in first echo images acquired at a TR of approximately 3 seconds. Since values of T1 for brain and glioma at 200 MHz lie in the range 1000 - 1300 ms (Bottomley 1987, Rajan 1990), it is expected that overall signal from these tissues should increase as TR increases up to several seconds. Even at a TR of 3 seconds, signal from brain and glioma at 200 MHz is partially saturated ($1 - e^{-TR/T1} = 1 - e^{-3000/1300} = 0.90$); while these images are predominantly proton-density weighted and T2-weighted in the later echo images of a multi-echo series, some T1 dependence remains.

The relaxation times of 9L gliomas are not consistently different from surrounding normal brain (Rajan 1990, also section 3.2.4), and vary with tumour size, and from animal to

animal. Contrast between tumour and surrounding brain was frequently inadequate to accurately delineate the tumour-brain boundary, a difficulty which has been encountered by other investigators performing MRI of intracranial 9L tumours in rats (Rajan 1990). Of 74 tumour-bearing animals imaged without paramagnetic contrast agent, 25 had tumours which were unmeasurable due to inadequate contrast between tumour and surrounding brain.

3.2.3.5 Injectable contrast agent for MR imaging

Gadolinium-based paramagnetic contrast agents cause signal enhancement on MR images by reducing the spin-lattice relaxation time (T_1) of extracellular water in tissues (Sobol 1991). Gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA, Magnevist[®], Berlex, Montreal) does not cross the blood-brain barrier unless it is compromised, as in intracerebral tumours (Schmiedl 1990), where it accumulates in the extracellular water compartment (Norman 1989). When imaged using a T_1 -weighted imaging protocol ($TR = 300$ ms), tissues in which Gd-DTPA has accumulated have increased signal compared with other tissues; where Gd-DTPA is present, $T_1 < TR$; where it is not present, $T_1 > TR$ and signal is lost due to saturation.

In the clinical setting, Gd-DTPA is administered by bolus intravenous injection. Since intravenous administration in rats involves surgery to expose a vein (usually the femoral), experiments were undertaken to determine whether the more convenient intraperitoneal (i.p.) route would result in adequate levels of contrast agent in the brain to cause tumour enhancement. Since Gd-DTPA is known to be rapidly excreted by glomerular filtration in humans (Berlex 1990), information on the pharmacokinetics in rats was experimentally determined, i.e. the time of onset after bolus intraperitoneal injection and the duration of tumour enhancement by Gd-DTPA.

3.2.3.6 Method for i.p. Gd-DTPA MRI enhancement experiments

Three rats bearing intracranial 9L tumours (20 days after tumour implantation, tumours 3 - 4 mm diameter) were given a bolus intraperitoneal injection of Gd-DTPA (0.5 mmol/kg, Magnevist[®] 1.0 ml/kg) immediately prior to imaging. Single spin-echo T_1 -weighted images were acquired repeatedly for 70 minutes, with a repetition time $TR = 300$ ms, 4 averages, 5 minute scan time. The initial brightness (IB) from the tumour was measured using the image processing software, as was the IB from a similar region of

normal brain in the contralateral cortex. The ratio of tumour IB to contralateral cortex IB (the "signal enhancement" due to the Gd-DTPA) was plotted as a function of time after i.p. administration of Gd-DTPA.

3.2.3.7 Results and discussion

Figure 3.2.3:3 (upper) shows a T2-weighted image ($TR = 3.0$ s) without contrast agent, demonstrating poor tumour-brain contrast and the difficulty in delineating tumour boundaries. The lower image in figure 3.2.3:3 shows a T1-weighted image ($TR = 300$ ms) of the same 9L intracranial tumour acquired 30 minutes after intraperitoneal administration of Magnevist®. Tumour-brain contrast with Magnevist® is greatly improved compared with T2-weighted imaging, and the tumour-brain boundary is well defined, allowing accurate measurement of tumour size. The correlation between the enhanced region of the image and histopathological identification of the extent of the tumour is investigated in section 3.2.5.

Figure 3.2.3:4 shows the graph of signal enhancement of the tumour as a function of time after intraperitoneal administration of Magnevist®. Tumour signal enhancement occurs rapidly and peaks at 1.6 at 33 minutes following i.p. injection. Enhancement falls off gradually as contrast agent is removed by glomerular filtration, and remains at 1.3 at 70 minutes after injection. Thus there is a broad temporal window during which imaging can be performed for optimum tumour enhancement, extending from about 15 to 40 minutes after intraperitoneal administration of Magnevist®. While the onset of enhancement is somewhat slower for i.p. administration of contrast agent in rats than for intravenous administration in humans, where enhancement of intracranial lesions occurs within a few minutes, the time of onset of maximum enhancement (33 min) corresponds well with the time required to position the rat in the probe, tune, shim, and run the image. Intraperitoneal administration of Magnevist® followed by T1-weighted imaging is clearly an accurate and convenient technique for MR imaging of intracranial 9L tumours.

Additional experiments were performed to evaluate the effectiveness of an injectable ferromagnetic MRI contrast agent. This agent was a dextran-ferrous oxide compound obtained from Biomedical Frontiers Inc. Three rats bearing intracranial 9L tumours were injected intraperitoneally with this agent, and imaged immediately using a repetition time $TR = 300$ ms. Imaging was continued for 3 hours following injection. No enhancement of the tumour was observed with this contrast agent.

Figure 3.2.3:1. High resolution image of normal rat brain. The slice thickness was 150 μm ; the field of view was 2.0 cm; the image matrix was 256 x 256; the in-plane resolution (pixel size) was 78 μm . The repetition time was 1800 ms, 22 averages, total imaging time 181 minutes.

Figure 3.2.3.2. Contrast-to-noise ratio (CNR) versus repetition time for tumour:cortex and tumour:caudate. $CNR = SNR(tumour) - SNR(cortex)$. Each point is based on the mean of six measurements of image intensity at different locations in the tumour, cortex, or caudate. Image noise was estimated from measurements in a region outside the rat's head. Data presented in this graph is from a single animal, but is representative of the data from the three animals.

Figure 3.2.3:3. **Upper:** MR image of a 9L intracranial tumour without contrast agent (T2-weighted, TR = 3.0 s, TE = 22 ms).

Lower: MR image of a 9L intracranial tumour acquired 30 minutes after intraperitoneal administration of Magnevist® (1 ml/kg, Gd-DTPA 0.5 mmol/kg). T1-weighted, TR = 300 ms, TE = 22 ms, 4 averages, scan time 5 minutes.

Figure 3.2.3:4. Enhancement of 9L intracranial tumours on MRI following intraperitoneal injection of Gd-DTPA (Magnevist® 0.5 mmol (1 ml) per kg). Signal enhancement is the ratio of the integral brightness in a region of interest encompassing the tumour to the IB in a similar ROI in the contralateral hemisphere. Images were performed using the first echo of a spin-echo sequence, TR = 300 ms, TE = 22 ms, 4 averages, scan time 5 minutes. Points are the mean of 3 rats, error bars are standard deviation.

3.2.4 MRI characterization of intracranial 9L tumours

3.2.4.1 Purpose

The aim of the experiments described in this section was to characterize the progression of untreated intracranial 9L tumors using MRI. Changes in tumour size, inter-echo signal decay "T2", and "proton density" were measured as tumours grew, in order to determine the approximate rate of growth and changes in appearance on images.

3.2.4.2 Method

Eighteen rats were inoculated with intracranial 9L tumours, and imaged on subsequent days from 7 to 21 days following tumour inoculation. Each animal was imaged once, and sacrificed immediately after imaging by perfusion fixation, to investigate the correlation between MRI and histopathology (see section 3.2.5). Imaging performed with a single slice through the centre of the tumour, multi-echo with TE = 34 ms (8 echoes), TR = 1800 ms, field of view 2.0 cm, slice thickness 0.8 mm, 6 averages, scan time 51 minutes. The area of the tumour was measured from each image, as was the "T2" and "proton density" of the tumour and of a similar region of the contralateral cortex as described in section 2.3.7. "T2" and "proton density" were measured at 6 different locations in the tumour and contralateral cortex; the mean +/- standard deviation of these 6 measurements are plotted. Paramagnetic contrast agent had not yet been obtained and was not used for these images.

3.2.4.2 Results and discussion

Figure 3.2.4:1 shows a graph of tumour size measured from the MR images, versus time after tumour implantation. There is considerable variation in the rate of tumour growth amongst individual animals. This underscores the utility of MRI as a non-invasive technique for pre-treatment measurement of individual tumours in treatment response

studies, since assumptions concerning the size of tumours on a given day are likely to be large sources of uncertainty.

Tumours imaged 7 and 8 days after implantation were sub-millimetre in size, and their measured sizes are only approximate. While a region of abnormality was clearly visible on MRI at the locus of tumour cell inoculation, its small size and irregular shape made accurate size measurement difficult.

Figure 3.2.4:2 (upper) shows a graph of the ratio of the mean apparent T2 measured at various locations throughout the tumours to the mean apparent T2 measured in the contralateral cortex, plotted as a function of time after tumour implantation. While there is a general trend of increasing relative apparent T2 in the tumours with time after implantation, there is considerable variation within each tumour (the error bars) and between individual animals. It is clear from figure 3.2.4:2 that increased apparent T2 in the tumours is not a reliable characteristic of tumour tissue. All of the relative apparent T2 measurements in figure 3.2.4:2 are close to unity, indicating that the contrast between tumour and cortex on multi-echo image series is poor and variable. This emphasizes the utility of injectable contrast agents for improving the contrast between tumour and surrounding brain tissue. The lower graph of figure 3.2.4:2 shows the relative apparent T2 of the tumours plotted as a function of tumour size instead of time after tumour implantation. The correlation is no better, suggesting that the variation in relative apparent T2 is intrinsic to the tumours, and not simply a function of variability in rates of growth of the tumours. The relative apparent T2 is closest to unity for smaller tumours -- this is one reason why accurate and reliable pre-treatment measurements of tumour size could not be made in tumours smaller than 1 - 2 mm in diameter.

A parameter related to the proton density in the tumour and the contralateral cortex was measured by extrapolating the fit of the exponential decay of the multi-echo signal to $TE = 0$. The ratio of this parameter measured in the tumour to that measured in the contralateral cortex is plotted versus time after implantation and tumour size in figure 3.2.4:3. It is clear from the graph that there is no correlation between the relative proton density and either tumour size or time after implantation. While the error bars for each point are large, reflecting variation within each tumour due to the limited number of points used to perform the fit (usually 6 echoes before the signal decayed into noise), the variability between tumours is much greater. This variation in proton density between tumours likely contributes to the variability in tumour-brain contrast observed amongst different tumour-bearing animals. This also emphasizes the utility of injectable contrast agents to provide reliable delineation of the tumour-brain boundary.

Figure 3.2.4:1. Size of untreated intracranial 9L tumours versus time after implantation. Each point represents a single tumour size measurement from a distinct animal. Tumour size (area) was measured from a transverse MR image through the centre of the tumour. There is large inter-animal variation in tumour growth rate.

Figure 3.2.4:2. Upper: Ratio of tumour apparent T2 to contralateral cortex apparent T2, versus time after tumour implantation.

Lower: Ratio of tumour apparent T2 to contralateral cortex apparent T2, versus tumour size. Each point is the mean of 6 measurements in different locations in the tumour of a single animal, error bars are standard deviation.

Figure 3.2.4:3. Upper: Ratio of tumour proton density to cortex proton density versus time after implantation. The correlation coeff. $r^2 = .063$, indicating no correlation. Lower: Ratio of tumour proton density to cortex proton density versus tumour size. The correlation coeff $r^2 = .005$, indicating no correlation. Each point is the mean of 6 measurements at different locations in the tumour of a single animal, error bars are s.d.

3.2.5 Correlation between MRI of 9L ic tumours and histopathology

3.2.5.1 Purpose

Accurate identification of features seen on MR images is often difficult because the resolution of MRI does not yet extend to a cellular level. A region of an image with hypo- or hyperintense signal may be identified as pathological because of asymmetry, but the exact nature of the pathology may be impossible to determine by MRI alone. Even implanted intracranial tumours, where the nature and location is well known, raise questions on MRI because the extent of the tumour can be difficult to identify precisely.

This section describes an investigation of the correlation between morphological features of 9L intracranial tumours from MR images and from microscopic examination of histological sections. In particular, the identification of the spatial extent of the tumours was investigated, i.e. does tumour size measured by MRI correlate with the actual size of the tumour as seen on histological sections. This question is of particular importance in Gd-DTPA enhanced MR imaging of tumours, since there is evidence of blood-brain barrier defect in peritumoural brain tissue (Stewart 1987a) which could lead to enhancement of MR signal in a region larger than the tumour.

3.2.5.2 Method

Before imaging rats, a water phantom of known dimensions was imaged and scaling parameters were adjusted to calibrate the MR system in all three imaging directions. The accuracy of linear measurements taken from MR images was estimated at ± 0.2 mm.

The eighteen tumour-bearing animals used in section 3.2.4 were sacrificed by perfusion fixation immediately following T2-weighted MR imaging and the brains were removed. Histological sections 2 μ m thick through the centre of the tumour were prepared and stained as described in section 2.2.6. The width of the brain at its largest lateral dimension was measured from MRI using the image processing software, and the same width was measured from the histological sections using the micrometer scales on the microscope stage. The width (lateral) and height (dorsal-ventral) of each tumour was measured from MRI, and corresponding dimensions were measured from histological

sections. Three of the eighteen animals had tumours which were unmeasurable on MRI due to poor tumour-brain contrast.

To investigate the accuracy of tumour size measured from Gd-DTPA enhanced MR images, eight tumour-bearing rats (14 - 16 days following tumour implantation) were injected with Magnevist® (1 ml/kg i.p.) and imaged, then immediately sacrificed by perfusion fixation. The brain width, tumour width and tumour height were measured from MRI and from histological sections.

3.2.5.3 Tumour size results: correlation between MRI and histopathology

The width of the brain of rats as measured from histological sections was on average a factor of $0.880 \pm .041$ less than the same width measured from MR images. This was due to shrinkage of formalin-fixed sections, caused by dehydration in graded ethanol solutions after sectioning. This shrinkage of histological sections is well documented (Pearse 1968, Humason 1972) and is usually estimated to be approximately 10%. Since brain width was easily identifiable and could be measured unambiguously from both MR images and histological sections, these measurements provided a good estimate of the degree of shrinkage caused by formalin fixation and sectioning.

Figure 3.2.5:1 shows a graph of tumour diameter measured from histological sections plotted against the corresponding tumour size measured by T2-weighted MRI. The correlation between the two measurement techniques is good, with a correlation coefficient $r^2 = 0.877$. The slope of the regression line is 0.909, which indicates that histological measurement of tumour size is on average 0.909 times that of MRI. This is in agreement with the shrinkage factor of $0.880 \pm .041$ determined from brain width measurements. When shrinkage due to formalin fixing and sectioning is taken into account, identification of tumour boundaries and measurement of tumour size on T2-weighted MR images agrees well with measurements from histological sections.

Figure 3.2.5:2 shows a graph of the correlation between tumour diameter measured Gd-DTPA enhanced MRI and that measured from histological sections. The correlation between the two methods is very good, with a correlation coefficient $r^2 = 0.956$. The slope of the regression line through the data is 0.897, which agrees with the shrinkage factor of $0.880 \pm .041$ determined previously. It is clear from this data that the region of MR images enhanced following ip administration of Gd-DTPA corresponds very well with the

extent of the intracranial 9L tumour as identified by histopathology; signal enhancement does not extend beyond the tumour boundaries. Based on the higher correlation coefficient and slightly better agreement between the slope of figure 3.2.5:2 and the shrinkage factor (0.880), it can be concluded that Gd-DTPA enhanced MR imaging provides more accurate measurement of intracranial tumour size than does T2-weighted MR imaging.

3.2.5.4 Tumour morphology: correlation between MRI and histopathology

The histopathological appearance of the 9L tumour is densely cellular, pseudo-palisading, moderately pleomorphic with a finely fibrillar background. An abundance of mitotic figures is evidence of a high mitotic rate, and occasional multinucleated cells are present. These features are consistent with the classification of the 9L as a gliosarcoma (Wheeler 1981), and are common to both 9L intracranial tumours and 9L cells in culture. Figure 3.2.5:3 shows plateau phase 9L cells in tissue culture, and a histopathological preparation from an intracranial 9L tumour, demonstrating similar morphology in vitro and in vivo. Intracranial 9L tumour cells exhibit little local invasion into surrounding normal brain tissue, but do invade along the subarachnoid space, Virchow Robin spaces surrounding vessels, and occasionally through the ventricular system. These tumours are uniformly cellular and highly vascular, with no evidence of hypoxic regions or necrosis (Barker 1986, Wheeler 1981, Sutherland 1990).

An exception to the otherwise uniform cellularity of 9L intracranial tumours was the presence of acellular cysts in approximately one-quarter of the tumours implanted (see figure 3.2.5:4). These cysts were typically 0.5 mm in diameter, irregular in shape, located in the central region of the tumour, and filled with a proteinaceous fluid (Sutherland 1990). On T2-weighted MRI, the cysts were hypointense compared with tumour and surrounding brain tissue. The ratio of the measured "T2" of the cysts to that of contralateral cortex was $1.17 \pm .06$, while the ratio of the "proton density" of the cysts to that of contralateral cortex was $0.70 \pm .09$. Thus the hypointensity of the signal from the cysts arose from a lower density of protons compared with brain tissue. These cysts also appeared hypointense on Gd-DTPA contrast enhanced MR images, indicating that the contrast agent was not taken up in the proteinaceous fluid filling the cyst.

With the exception of the cysts, untreated 9L intracranial tumours appeared relatively uniform on MR images, with no significant variation in signal intensity which could be

correlated with morphological features on histopathological examination of the tumours. This is likely a reflection of the uniform cellularity of these tumours. When treated with radiation or cisplatin, variation in signal intensity throughout the tumour could often be detected on both T2-weighted and contrast-enhanced MRI, concomitant with the appearance of areas of focal necrosis verified by histopathology. Treated tumours frequently demonstrated the appearance of increased internal structure on MRI, in contrast with the uniformity of signal in untreated tumours. However, in general no clear correlation could be made between regions of hypo- or hyperintense signal on MRI and regions of focal necrosis identified histopathologically. Even radiation-induced necrosis resulting in significant acellular regions of edema, while visible on MRI, could not be unambiguously identified except by later histopathological examination. Neither T2-weighted nor contrast-enhanced MRI were found to be a reliable early indicators of focal necrosis.

Figure 3.2.5:1. Correlation between tumour diameter measured by MRI without paramagnetic contrast agent and histopathology. Animals bearing 9L ic tumours were imaged then immediately sacrificed by perfusion fixation, the brains sliced through the centre of the tumour, and mounted and stained for histological examination. Each point represents measurement of tumour size by the two methods for a single animal.

Figure 3.2.5:2. Correlation between tumour diameter measured by contrast-enhanced MRI (Magnevist®) and histopathology. Animals bearing 9L intracranial tumours were imaged by contrast-enhanced MRI, then immediately sacrificed by perfusion fixation, the brains sliced through the centre of the tumour, mounted and stained for histopathological examination. Each point represents tumour size measurement of by the two methods from a single animal.

Figure 3.2.5:3. Upper: Plateau phase 9L cells growing as a monolayer in tissue culture. Lower: Histopathological section (2 μ m thick) of an intracranial 9L tumour, stained with eosin and hematoxylin.

Figure 3.2.5:4. Upper: T2-weighted MR image of an intracranial 9L tumour containing a proteinaceous cyst approximately 0.7 mm in diameter.

Lower: A histopathological section through the same tumour, showing the cyst.

3.2.6 MRI of radiation injury to normal rat brain tissue

3.2.6.1 Purpose

The effect of radiation on normal rat brain tissue was investigated using MRI to ensure that possible signal enhancement of irradiated brain could be distinguished from irradiated tumour tissue on MR images. In addition, these MRI studies of irradiated normal brain tissue were directed at a specific clinical problem. Many patients who have undergone cerebral radiotherapy experience neurological deficit attributable to the effects of the radiation on normal brain tissue. In one study, of 41 patients receiving cerebral radiotherapy, 21 developed clinical abnormalities due to the radiation; the timescale for appearance of these abnormalities was 6 months to 2 years (Constine 1988). The underlying cause of this late radiation injury to CNS is both small vessel and glial damage, resulting in a demyelinating lesion with white matter necrosis and blood-brain barrier breakdown, though specific mechanisms remain unknown (Burger 1979, Caveness 1980, Larsson 1992, Remler 1986). This injury is progressive and irreversible, often fatal, and represents the major dose-limiting complication in radiotherapy of intracranial tumours (Constine 1988, Pezner 1981). Human brain has a steep dose-response curve between 55 and 70 Gy delivered with conventional fractionation schemes (Leibel 1990). The addition of chemotherapeutic agents to the treatment regime increases the incidence of radiation injury to the brain (Foo 1986).

Changes in MR images accompanying late radiation injury to CNS have been observed clinically (Constine 1988, Valk 1990, Valk 1991, Lindquist 1992) and in animal studies (Bernstein 1990, Lo 1992), but no consensus has emerged concerning the timescale of appearance of MRI changes or the radiation doses required. The purpose of the experiments described in this section was to determine whether injury to the rat brain following 80 Gy single-fraction irradiation produces changes in MRI signal intensity, and to investigate the possibility that MRI might provide an early indication of the development of this injury. This could improve understanding of the time course of radiation-induced brain injury and possibly provide a useful tool in the management of cerebral radiotherapy patients. MR imaging of radiation injury of the brain also has potential applications for monitoring the success of cerebral radiosurgery, where high-dose single-fraction radiation is used for the obliteration of brain pathology such as arteriovenous malformations (AVMs) and gliomas (Lindquist 1992).

Ten adult male Fischer rats were given a single-fraction dose of 80 Gy to a localized region of the brain (caudate putamen and hippocampus), using the technique described in section 2.2.7. Magnetic resonance imaging (TR = 3000 ms, TE = 34 ms, 8 echoes, slice thickness 1mm, FOV = 3.5 cm) was performed at various times from 1 day to 12 months after irradiation. Images were analyzed by measuring the integrated brightness in a region of interest centred on the path of the radiation beam 5.5 mm below the scalp (encompassing d_{max}), and in a similar contralateral region of interest.

Magnevist® Gd-DTPA contrast agent became available 21 weeks following irradiation; subsequent imaging sessions included T1-weighted contrast-enhanced imaging. To investigate possible early enhancement of damage in irradiated brain by Gd-DTPA, an additional 3 rats were irradiated and imaged using Magnevist® at 1, 3, 10, and 15 days following irradiation. Animals were sacrificed by perfusion fixation for histopathological examination.

3.2.6.3

Results and discussion: MRI findings

Figure 3.2.6:1 shows a graph of the ratio of image signal intensity from the irradiated region to that from a contralateral region, plotted versus time after irradiation, for non-contrast enhanced images. Each line on the graph represents an individual rat. There is a long-term trend towards lower signal intensity in the irradiated region. A paired t-test was performed to compare the distribution of signal ratios at time T after irradiation with the pre-irradiation distribution of signal ratios; the signal ratio became significantly different ($p < .05$) from pre-irradiation controls at 130 days (19 weeks) after irradiation.

Contrast-enhanced MRI performed at 21 weeks following irradiation showed no enhancement of the irradiated regions. At 49 weeks, irradiated regions were clear on Gd-DTPA enhanced images, corresponding to areas of massive radiation necrosis evident on histopathological examination. These regions of necrosis were detectable earlier using non-contrast enhanced MRI than with Gd-DTPA enhanced MRI. The three animals imaged with Gd-DTPA at 1, 3, 10, and 15 days after irradiation showed no enhancement of the irradiated region. Blood-brain barrier breakdown has been detected using other techniques 14 weeks after 60 Gy irradiation of rat brain (Remler 1986), suggesting that there are more sensitive techniques than Gd-DTPA MRI for detecting BBB breakdown (e.g. leakage of

horseradish peroxidase (Bernstien 1990)). From these findings, it appears that Gd-DTPA enhanced MRI offers no advantage for detecting radiation damage to brain tissue.

3.2.6.4 Histopathological findings

Animals were sacrificed at 34 and 49 weeks after irradiation for histopathological analysis. At 34 weeks, demyelination was evident throughout the irradiated region, and many small areas of focal necrosis were present. These areas of focal necrosis could not be correlated with specific features on the MR images, but as noted above there was an overall decrease in signal in the irradiated region compared with a contralateral region on non-contrast enhanced images at this time. At 49 weeks, large acellular areas were evident in the irradiated region, indicating extensive radiation necrosis with macrophage infiltration. Figure 3.2.6:2 shows a non-contrast enhanced MR image of a rat 49 weeks after irradiation; the oblong feature in the right hemisphere corresponds with the irradiated region. Figure 3.2.6:3 shows a histological slice of the same animal; this feature is seen to be an acellular region of massive radiation necrosis. Brain regions at the periphery of the beam show demyelination and focal necrosis, giving the brain an open, spongy appearance. The areas of maximum demyelination and radiation necrosis do not correspond exactly with the distribution of dose in the radiation beam, suggesting that the sensitivity to radiation varies in different parts of the brain; the caudate putamen is apparently more sensitive than either the hippocampus or the cortex.

While it is clear that MRI is capable of detecting radiation damage once significant demyelination and radiation necrosis has occurred, the results of these experiments show that MRI is not a sensitive technique for providing an early indication of radiation damage. Contrast-enhanced MRI was not performed between 15 days and 21 weeks following irradiation (due to unavailability of Magnevist® and MR system); it is possible that early radiation damage is detectable during this period. Other MR techniques, such as localized spectroscopy and diffusion-weighted imaging, may also provide an opportunity for early detection of radiation injury of the brain.

Figure 3.2.6:1. Change in MR signal intensity of irradiated rat brain with time after irradiation, measured from non-contrast enhanced MR images. Signal intensity is expressed as a ratio of the integral brightness in the irradiated region to that in a similar contralateral region. One side of the rat brain was irradiated to 80 Gy using a Co-60 beam 5.1 mm in width. Each line represents an individual rat.

Figure 3.2.6:2. T2-weighted MR image of rat brain 49 weeks after 80 Gy irradiation, showing development of radiation-induced necrosis.

Figure 3.2.6.3. Histopathological sections of rat brain, 49 weeks after irradiation with 80 Gy. Acellular regions of massive radiation-induced necrosis coincide with the depth of maximum dose, while regions of brain at the edges of the radiation beam show hemorrhage, demyelination and macrophage infiltration.

3.2.7 Intraperitoneal injection of cisplatin

3.2.7.1 Purpose

The normal route of administration for cisplatin in the clinic is slow intravenous infusion (CPS 1991). This is not a convenient route of administration in rats, since it requires surgery to expose the femoral vein or the tail vein. In animals burdened with intracranial tumours, this additional surgery was considered to be undesirable, adding further stress and anesthesia in addition to the anesthesia necessary for radiation treatment and MR imaging. The purpose of this experiment was to determine whether the more convenient intraperitoneal administration of cisplatin resulted in adequate levels of drug in the intracranial tumours, and to determine the approximate rate of uptake and clearance of the drug in the tumours.

Other investigators have measured levels of cisplatin metabolites in the tissues of experimental animals following intraperitoneal cisplatin administration: kidney, plasma, urine, and liver of rats (Mistry 1989); peritoneal tumour nodules in rats (Los 1991). However, no data have been published on cisplatin levels in experimental intracranial tumours following intraperitoneal administration.

3.2.7.2 Materials and methods

Nine rats were inoculated with intracranial 9L tumours as previously described. Each animal was imaged at day 14 to ensure that a tumour of approximately 3 mm diameter was present. One rat was rejected due to a small and poorly defined tumour. The remaining 8 animals were each given a bolus intraperitoneal injection of cisplatin (Platinol) at a dose of 6 mg/kg. Two animals were sacrificed at each of the following timepoints after cisplatin injection: 0.5 h, 1 h, 2 h, and 4 h. Animals were sacrificed by injecting 100 mg/kg sodium pentobarbital intraperitoneally (Somnitol 1.5 ml/kg). Upon the onset of heavy surgical anesthesia, animals were decapitated live with a guillotine, the scalp and skull were quickly resected, and the tumours removed and frozen.

The tumour samples were analyzed for elemental platinum levels by the Division of Chemistry, National Research Council, using inductively coupled plasma flameless atomic

absorption spectroscopy (Beaty 1978, Gaffin 1979, Delves 1986). A sample of normal brain tissue from a rat not injected with cisplatin was included as a control.

3.2.7.3 Results and Discussion

The control sample was found to have an insignificant level of Pt (<20 ng/g). The data from the other 8 samples is plotted in figure 3.2.7:1 as a function of time between i.p. cisplatin injection and sacrifice. Each point represents the mean of two animals, with the range represented by the error bar.

The results from this experiment confirm that platinum accumulates in 9L intracranial tumours following bolus intraperitoneal injection of cisplatin. The tumour Pt level peaks approximately 2 hours after injection at $1.1 \mu\text{g/g}$, and by 4 hours after injection the Pt level has decreased to one-third of its peak level. These data agree with results for various tissues by other investigators: Total Pt levels in rat kidney after bolus i.p. administration were found to peak at 1 hour and decline gradually at 6 hours (Mistry 1988); Rapid i.v. administration in rats of 6 mg/kg cisplatin gave Pt levels in hind-leg mammary tumours of approximately $1 \mu\text{g/g}$ (Jakowatz 1991); Clearance from peritoneal fluid after i.p. administration of cisplatin in rats occurred with a half-time of 78 minutes (Los 1991).

Atomic absorption spectroscopy for elemental platinum gives total levels of Pt in tissue, providing no information about the location of this platinum (extracellular, intracellular, bound to DNA, etc.) or the chemical form of the platinum (native cisplatin, aquated, bound to protein, etc.). Hence atomic absorption spectroscopy provides only a crude measure of cisplatin distribution. For example, HPLC analysis of plasma ultrafiltrate in rats by other investigators has shown that 1 hour after iv cisplatin administration, over 50% of cisplatin is bound to protein (Daley-Yates 1986), a form which has been shown to be non-nephrotoxic (Cole 1981).

The in vitro experiments of section 3.1.2.6 give some information about the reduction of anti-tumour activity of cisplatin with time. In these experiments, cisplatin was applied to plateau-phase 9L cells and incubated for various times. The cisplatin-containing medium from these cells was then aspirated and applied to fresh plateau-phase cultures for 1 hour. These cells were then assessed for survival by the colony-forming assay. It was found that after 2 hours of incubation on cells, the cisplatin had lost about 20% of its cytotoxicity. Since the results were the same whether the cisplatin was originally incubated on plateau-phase cells, or simply in depleted media, it is likely that this loss of cytotoxicity is a result of

cisplatin binding to serum proteins. Binding to proteins in the intraperitoneal fluid and to plasma proteins likely results in a similar loss of cisplatin toxicity en route from the peritoneal cavity to intracranial tumours.

A common pharmacokinetic measure of drug exposure is the area under the concentration-time curve (AUC). From figure 3.2.7:1, the AUC of Pt in intracranial tumour tissue can be estimated at approximately $2.8 \mu\text{g/g} \times \text{h}$, or $860 \mu\text{M} \times \text{min}$. The in vitro cisplatin exposure used to inhibit PLDR in the experiments of section 3.1.3 was $6 \mu\text{g/g} \times \text{h}$, or an AUC of $1200 \mu\text{M} \times \text{min}$. Clinical values for Pt AUC in plasma are about $700 \mu\text{M} \times \text{min}$ (Stewart 1989). While it is expected that Pt AUC in tumour tissue might be less than Pt AUC in plasma, it appears that intraperitoneal injection in rats of cisplatin at 6 mg/kg results in tumour Pt concentration which is clinically relevant but somewhat lower than that which causes PLDR inhibition in vitro. Hence it was decided to increase the cisplatin dose in rats from 6 mg/kg to 8 mg/kg .

The goal of this experiment was not to undertake a detailed pharmacokinetic study of intracranial tumour cisplatin levels following i.p. administration, which would have necessitated a large commitment of animals and time. From this limited experiment, it can be concluded that intraperitoneal administration of cisplatin in rats does result in clinically relevant levels of Pt in intracranial tumours, and the peak concentration occurs approximately 2 hours after injection.

Figure 3.2.7:1 Elemental platinum concentration in 9L intracranial tumours at various times after intraperitoneal injection with cisplatin (6 mg/kg Platinol). Each point is the mean of two rats; error bars represent the range of the measurement.

3.2.8 Size measurements of treated tumours

The purpose of this experiment was to use MRI to measure the size of intracranial 9L tumours before and at various times after treatment, to monitor the growth or regression of the tumours in response to treatment. Four treatment groups were investigated: radiation treatment only, cisplatin treatment only, radiation concurrent with cisplatin, and untreated controls (discussed in section 3.2.4).

The tumours were treated when they were approximately 2 - 3 mm in diameter (day 14-16 after tumour implantation). The objective of treatment was not to eradicate these tumours; treatment at approximately day 9-10 after implantation is necessary for tumour eradication (Kimler 1992, Barker 1973). At that early stage, tumours are less than 1 mm in diameter, and difficult to measure accurately using MRI. The objective was to administer treatments which would result in tumour response measurable by MRI.

3.2.8.1 Radiation treatment only

3.2.8.1.1 Method

Ten animals bearing intracranial 9L tumours were treated with radiation to a dose of 80 Gy single fraction, using the method described in section 2.2.7. The mean (s.d.) day of treatment was 15.9 (2.1) days. The day of treatment for individual animals varied due to availability of the MRI system, and difficulty in resolving the tumour in some animals. Animals were not treated until a tumour of 2-3 mm diameter could be clearly distinguished and accurately measured from the image, to give a pretreatment baseline measure of tumour size. After treatment, animals were imaged as previously described at 1, 3, 5 etc. days following treatment (treatment was on day 0), or whenever the MRI system was available. Animals were sacrificed at the first symptoms of intracranial pressure or distress. Whenever possible, animals were imaged immediately prior to sacrifice, then perfusion fixed and the brains removed for later histological examination.

Figure 3.2.8:1 shows the tumour size measurements for the 10 animals treated with radiation only. The upper graph is the raw tumour size measurements; in the lower graph, the tumour size has been normalized to the pretreatment (day 0) value for each animal, to show the fractional change in size, independent of the initial pre-treatment tumour size. Tumour size was interpolated to day 3 for those animals imaged after but not on day 3, to provide a common time point for comparison with the other treatment groups. The mean normalized tumour size at day 3 following radiation treatment only is 1.82 ± 0.47 ($n=8$).

Even at a dose of 80 Gy, no tumour regression occurred in the animals treated with radiation only. This confirms the *in vitro* findings that 9L cells are very radioresistant. Histological analysis of irradiated tumours shows good correlation with MRI size measurements, with focal necrosis apparent 3-4 days following irradiation. Figure 3.2.8:2 shows a photograph of a tumour which received only partial irradiation, because a portion of the tumour had grown beyond the usual region of irradiation. Cell death is evident in the irradiated portion, but not in the portion which escaped irradiation. Tumour necrosis following irradiation was observed to increase with depth up to approximately 3 mm below the skull, corresponding to the depth of dose maximum of Co-60; tumour regions close to the surface show little necrosis.

The region of interface between tumour and normal brain is very clearly defined in untreated 9L tumours, showing very little infiltration of tumour cells into the surrounding brain (except along subarachnoid spaces and Virchow-Robin spaces). However, in the irradiated animals this interface takes on an open, spongy appearance 3-4 days following irradiation, with spaces in the normal brain tissue, possibly due to the onset of radiation demyelination (Larsson 1992). The corpus callosum region of the brain showed this damage most frequently. This structure is directly under the tumour and is close to the depth of dose maximum. The corpus callosum appears to be more sensitive to radiation damage than the adjacent caudate putamen, which also lies close to d_{max} , but did not show evidence of demyelination. The corpus callosum, being white matter, contains a higher proportion of myelin than the caudate putamen. Brain tissue adjacent to the tumour appears to be more sensitive to radiation, possibly due to vasogenic edema (Valk 1991).

Increased infiltration of tumour cells into surrounding normal brain is evident in irradiated animals compared with unirradiated animals (see figure 3.2.8:3). This increased infiltration could be a factor in the apparent increase in tumour size following irradiation. This effect has been observed clinically in glioma patients treated radiosurgically with large single fractions of radiation (Colombo 1986, Steiner 1992).

3.2.8.2 Cisplatin treatment only

3.2.3.2.1 Method

Eight animals with intracranial 9L tumours were treated with intraperitoneal injection of cisplatin as described in section 2.3. The mean (s.d.) day of treatment was 14.0 (0) days after tumour inoculation. The dose used was 6 mg/kg body weight. Animals were imaged for tumour size measurement immediately prior to treatment, then at three or four days after treatment. Only one animal was imaged after 4 days, due to unavailability of the MRI system. All animals were perfusion fixed and their brains removed for histological analysis.

3.2.8.2.2 Results and discussion, cisplatin treatment only

Figure 3.2.8:4 shows a graph of tumour size versus time after treatment with cisplatin only. The upper graph is the raw tumour size data; in the lower graph, tumour size has been normalized to the pre-treatment size for each animal, to show the fractional change in tumour size following treatment. Normalized tumour size measurements were averaged at day 3 for comparison with the other treatment groups. The mean tumour size at day 3 following treatment with cisplatin was 1.87 ± 0.37 (n=8).

None of the animals showed any adverse effects or symptoms attributable to the cisplatin treatments. The tumour size data shows no evidence of tumour regression in 3-6 days following cisplatin administration. Figure 3.2.8:5 shows a photo of a histological section through a tumour 4 days following cisplatin administration. A small amount of focal necrosis is evident, though not as much as following irradiation. The cisplatin treatment appears to have no effect on normal brain tissue surrounding the tumour, and the tumour-brain boundary appears no different on histological sections following cisplatin treatment.

3.2.8.3 Combined radiation plus cisplatin treatments

3.2.8.3.1 Method

Seven rats with intracranial 9L tumours were treated with combined radiation and cisplatin using the methods previously described. The mean (s.d.) day of treatment was 16.25 (3.06) days after tumour inoculation. The radiation dose was 80 Gy single fraction, the cisplatin dose was either 6 or 8 mg/kg; the data from the two doses was pooled. There was no significant difference in the tumour size results between the two doses. The cisplatin dose was increased when the results from the experiments of section 3.2.7 became available, in an attempt to achieve tumour concentrations of platinum similar to clinical levels (Mistry 1989, Stewart 1988, Los 1991).

Intraperitoneal injections of cisplatin were administered either 10 minutes before irradiation (3 rats), or 2 hours before irradiation (4 rats). The time of administration was changed to 2 hours before irradiation when the results of the experiments of section 3.2.7 became available, showing peak tumour Pt levels 2 hours following i.p. injection. There was no significant difference in the results between the two times of administration. The animals were imaged immediately prior to treatment to obtain a measurement of pretreatment tumour size, then at 3 and 5 days following treatment, or whenever the MRI system was available.

3.2.8.3.2 Results and discussion, combined radiation plus cisplatin treatments

Figure 3.2.8:6 shows the graph of tumour size versus time after treatment with radiation plus cisplatin. The upper graph shows the raw tumour size data; in the lower graph, tumour sizes for each animal have been normalized to the pretreatment size. Normalized tumour sizes were averaged at day 3 to allow comparison between the treatment groups. The mean normalized tumour size at day 3 following combined radiation plus cisplatin treatments is 1.68 ± 0.45 ($n=7$).

The animals treated with radiation and cisplatin at 8 mg/kg all showed some adverse systemic effects following treatment, including weight loss and diarrhea. This dose is probably approaching the maximum tolerated systemic dose; the LD₁₀ dose in rats is reported to be 6 mg/kg (Ward 1976), and the LD₅₀ dose in guinea pigs is 9.7 mg/kg (Merck 1983). Gastrointestinal effects (nausea, anorexia) lasting up to one week after cisplatin administration are common clinical side effects (CPS 1991)

Two animals in this treatment group showed tumour regression: one imaged at day 8 showed a 23% reduction in tumour size from the maximum at day 5; the other imaged at day 11 showed a 27% reduction in tumour size from the maximum at day 5. Subsequent images of both these animals were unable to resolve the tumour well enough to make a measurement of tumour size. Histological analysis (see figure 3.2.8:7) of these animals on day 8 and day 11 showed massive tumour necrosis in the irradiated region, with macrophage infiltration and only a few remaining viable tumour cells around the periphery of the main tumour. Spread of tumour cells through the subarachnoid and Virchow Robin spaces allowed some cells to escape the irradiated region. At the time of sacrifice, there was considerable infiltration throughout the brain via these routes, even though the main tumour contained very few viable cells.

Figure 3.2.8:8 shows the MR image corresponding to the histological section of figure 3.2.8:7 taken 8 days following treatment with radiation plus cisplatin. Healthy, actively growing tumours normally have uniform signal slightly brighter than surrounding normal brain on T2-weighted images, whereas this tumour has slightly non-uniform signal intensity, giving the appearance of some structure within the tumour. No direct correlation could be made between regions of signal variation in the tumour on MRI and features of the tumour visible on histological sections. However, MR images of treated tumours containing significant amounts of focal necrosis or open regions following massive cell death consistently showed increased signal variation or structure within the tumour compared with untreated tumours. This observation holds for both T2-weighted MR images and gadolinium contrast enhanced images, and could be a useful indicator of tumour response to treatment.

3.2.8.4 Comparison between treatment groups

3.2.8.4.1 Method

Comparison of tumour size between the treatment groups was complicated by variations in the time of measurements of tumour size. The plan was to measure tumour size on day 0 (pre-treatment), day 3, 5, 7 etc. Due to unavailability of the MRI system and the unreliability of adequate contrast between tumour and normal brain necessary for accurate measurements of tumour size, many measurements were taken at other times or missed altogether. This made detailed statistical comparison between the groups awkward.

In the interests of minimizing the use of animals and maximizing the information derived from each animal, tumour size data for the untreated group was compiled from images of animals used for establishing correlations between MRI and histopathology, and for MRI characterization of 9L intracranial tumours. In general, untreated animals were imaged only once, then sacrificed for histological analysis to allow direct comparison between MRI and histopathology. In this way the untreated group differs from the treated groups, in which individual animals were imaged several times each in the days following treatment. The untreated group consisted of 36 animals, usually imaged only once, anywhere from 14 to 26 days after tumour inoculation. It is assumed that the pooled data from all these animals gives an accurate representation of the change in tumour size with time for an untreated population of animals.

A qualitative comparison between the treatment groups was made by averaging the normalized tumour size data of figures 3.2.8:1,4,6 for each day, linearly interpolating between data points where necessary to fill in missing measurements. A quantitative comparison between the different treatment groups was made by calculating the mean and standard deviation of the normalized tumour size at day 3 for each animal (interpolating to day 3 if measurements existed before and after but not on day 3). A two tailed t-test was then performed on these mean normalized day 3 tumour size data, to determine whether significant differences existed between the groups. For the untreated group, day 0 was taken to be 14 days after tumour inoculation.

3.2.8.4.2 Results and discussion, comparison between treatment groups

Figure 3.2.8:9 shows the graph of mean normalized tumour size as a function of time after treatment for the untreated, radiation only, cisplatin only, and radiation plus cisplatin treatment groups. Table 3.2.8:1 shows the results of t-test comparison between the treatment groups. The only significant ($p < .05$) difference is between the untreated and cisplatin only groups: tumours treated with cisplatin only are significantly larger at day 3 than tumours left untreated.

From figure 3.2.8:9, it is clear that radiation or cisplatin treatment individually have little effect on the measured tumour size. In fact, the data show these treatments cause an early increase in tumour size when compared with the untreated group. This could be an artefact of the experiment arising from the different method of selecting animals for the untreated group, as discussed above. However, transient increases in brain tumour size have

been observed clinically following large single fractions of radiation (Colombo 1986, Steiner 1992), hence it is possible that the data are showing a similar effect. A possible mechanism for this transient increase in tumour size is a reduction in intracranial pressure (allowing expansion of the tumour) following irradiation due to the onset of radiation-induced demyelination in surrounding irradiated normal brain tissue. This early demyelination is due to direct cellular injury to oligodendroglial cells and is not secondary to capillary damage (Rosander 1992).

When cisplatin and radiation treatments are combined, figure 3.2.8:9 shows a long-term decrease in tumour size. However, the points at 8 and 11 days following radiation plus cisplatin treatment are based on only two animals -- the rest were sacrificed after 5 days due to discomfort or distress. The radiation plus cisplatin tumour size data in figure 3.2.8:9 shows a slower increase in tumour size than either radiation or cisplatin treatments alone, but except for the points at 8 and 11 days, the tumours in this group remained larger than those in untreated controls.

Table 3.2.8:1. Results of t-test comparison of normalized day 3 tumour size between the four treatment groups.

Comparison between	p-value	significant difference?
"untreated" and "radiation only"	.10>p>.05	no
"untreated" and "cisplatin only"	p<.05	yes
"untreated" and "radiation plus cisplatin"	p>.10	no
"radiation only" and "cisplatin only"	p>.10	no
"cisplatin only" and "radiation plus cisplatin"	p>.10	no

The measurements of tumour size were subject to several possible errors. The tumours were imaged using a transverse slice through the brain, positioned according to a coronal pilot scan used to locate the hole in the skull from the tumour inoculation procedure. The accuracy of tumour area measurements was estimated at 10%, based on repeated positioning and imaging of the same rat and subsequent area measurements from the images. However, errors in fixing the rat's head horizontally in the probe, possible motion of the rat, and occasional difficulty in locating the inoculation hole could lead to errors in positioning the slice through the central part of the tumour.

It was assumed that tumour area measured from a single transverse image could be used as a surrogate for tumour volume. This assumption was necessary due to the technical difficulties outlined in section 2.3.8. However, significant departure from spherical tumour geometry would likely invalidate the assumption of tumour area as a surrogate for tumour volume, leading to errors when comparing tumour sizes from different animals.

During this project the technical difficulties outlined in section 2.3.8 were solved. The introduction of Gd contrast reagent, in addition to improving tumour contrast, also reduced imaging times, enabling the acquisition of several orthogonal images in one session without undue stress to the rat. New linear RF amplifiers and improved selective pulse shapes allowed contiguous slices to be acquired without serious penalty in image signal-to-noise ratio. However, in the interests of minimizing the use of experimental animals, it was decided to maintain the original protocol in order to be able to make use of the data already collected. Future experiments involving the measurement of intracranial tumour size should use Gd contrast reagent to enhance contrast and reduce imaging time, contiguous slices through the tumour volume, and either three-dimensional volume reconstruction or a simpler summation of tumour areas multiplied by the slice thickness to calculate tumour volume (Kim 1992).

Figure 3.2.8:1. Graph of tumour size versus time after treatment for the group of rats treated with radiation only. The upper graph shows the raw tumour size measurements for each rat. In the lower graph, tumour size has been normalized to the pre-treatment size for each animal, to show the fractional change in size after treatment.

Figure 3.2.8:2. Histological section of a tumour receiving radiation treatment (80 Gy) to one region of the tumour. The left side of the photograph shows significant tumour necrosis in the area which received the radiation; in the upper right is a region of the tumour which escaped the radiation treatment. The convoluted structure in the ventricle in the centre of the photograph is the choroid plexus, which secretes cerebrospinal fluid.

Figure 3.2.8:3. Upper: histological section of an untreated tumour, showing the clearly defined boundary between tumour (left) and normal brain (right). Tumour cells invade along Virchow Robin spaces surrounding vessels, but there is little local infiltration into brain parenchyma. Lower: irradiated tumour with poorly defined boundary and increased infiltration of tumour cells into brain parenchyma. The irradiated brain adjacent to the tumour has an open, spongy appearance.

Figure 3.2.8:4. Graph of tumor size versus time after treatment for the group of rats treated with cisplatin only. The upper graph shows the raw tumour size measurements for each rat. In the lower graph, tumour size has been normalized to the pre-treatment size for each animal, to show the fractional change in tumour size after treatment.

Figure 3.2.8:5. Histological section of an intracranial 9L tumour 4 days after intraperitoneal injection of cisplatin. A small amount of focal necrosis is visible in the tumour, while the surrounding brain parenchyma appears normal.

Figure 3.2.8:6. Graph of tumour size versus time after treatment for the group of rats treated with combined radiation and cisplatin. The upper graph shows the raw tumour size measurements for each rat. In the lower graph, tumour size has been normalized to the pre-treatment size for each animal, to show the fractional change in size after treatment.

Figure 3.2.8:7. Histological section of an intracranial 9L tumour 8 days after combined treatment with radiation plus cisplatin. Massive necrosis is evident in the tumour (lower left).

Figure 3.2.8:8. Magnetic resonance image corresponding to the histological section of figure 3.2.8:7, showing an intracranial tumour 8 days after combined radiation and cisplatin treatment. The lower boundary of the tumour is indicated by the arrow. The massive necrosis evident in the histological section appears in MRI as a subtle non-uniformity in signal throughout the tumour. Untreated tumours typically have uniform signal throughout the tumour.

Figure 3.2.8:9. Mean normalized tumour size as a function of time after treatment for the 4 treatment groups: untreated, radiation only, cisplatin only, and radiation plus cisplatin. Measurements of tumour area from MRI have been normalized to the pre-treatment tumour area for each animal.

3.2.9 Survival analysis of treated and untreated rats with 9L tumours

3.2.9.1 Introduction

Survival is a traditional endpoint in experimental oncology, and many survival studies have been performed on intracranial 9L tumours (Leith 1975, Wheeler 1980, Henderson 1981, Kimler 1992). Since survival studies require that every animal suffers pain and discomfort prior to death, one of the goals of this project was to demonstrate that MRI measurements of tumour size could be used as an endpoint in place of survival, thus minimizing the suffering of the animals.

Measurement of tumour size by MRI was the desired endpoint of the experiments involving treatment of intracranial 9L tumours in rats, not the survival of the animals. The approved application to the Animal Care Committee of the National Research Council of Canada for this project made clear that animals would be sacrificed at or preferably before the time of first appearance of symptoms of intracranial pressure (ataxia, somnolence, periorbital bleeding) or other distress. However, for those animals which developed these symptoms unexpectedly quickly, the time of appearance of these symptoms contains potentially valuable information about the effectiveness of the treatments, and constitutes a valid endpoint for performing survival analysis.

Large increases in lifespan were not expected for animals bearing intracranial tumours treated at approximately 2 - 3 mm diameter. Eradication of tumours would have required treatment earlier, when tumours were too small to be accurately measured using MRI.

3.2.9.2 Method of survival analysis

Animals were implanted with intracranial 9L tumours, imaged, and treated as previously described. The preferred endpoint was always tumour size measurement by MRI -- any animals which for any reason could not be imaged further were sacrificed, whether or not symptoms of intracranial pressure were present. To minimize pain and discomfort, animals were not kept alive for the sole purpose of measuring the time until appearance of such symptoms. However, sufficient numbers of animals developed these symptoms that survival analysis was feasible. A few treated animals did actually die; for the purposes of the survival analysis, these animals were considered to have developed

observable symptoms of intracranial pressure one day prior to death (Wheeler 1980a, Barker 1973, Wheeler 1981).

Any animals which were sacrificed prior to the development of observable symptoms were included in the survival analysis as "censored observations", that is, subjects which were removed from the study for reasons unrelated to the effect under investigation. Censored observations contain information useful in the analysis, because while their actual survival time is not known, it is known that they survived at least up to the time of their removal from the study (Everitt 1989).

Table 3.2.9:1 contains information on the number of animals in each group included in the analysis. All of the radiation doses were 80 Gy single fraction. Cisplatin doses were either 6 mg/kg or 8 mg/kg; for the survival analysis, these were pooled together. The group of animals (n=8) which received cisplatin alone were not included in the analysis because of the large number (7) of censored observations.

Survival time for each animal is defined as the time from tumour inoculation to the time of first observation of symptoms of intracranial pressure (in days). The time of

TABLE 3.2.9:1 Treatment groups for survival analysis.

Treatment group	Number of animals	Number of censored obs.	Median survival time (days)
untreated	57	30	21.5
radiation only	14	2	24.5
cisplatin only	8	7	
radiation + cisplatin	8	0	22.0

treatment varied somewhat, for reasons previously discussed, but was generally 14 or 16 days after tumour inoculation.

Estimates of the actuarial survival curves including censored observations were constructed according to the method of Kaplan and Meier (Kaplan 1958). The survivor function $s(t)$ is an estimate of the probability that an individual survives longer than time t . The median survival times quoted in the table above are the times at which $s(t) = 0.5$.

The survival curves were compared using the log-rank test (Peto 1972), which tests the null hypothesis that there is no difference between two survival curves. Two commonly used variations of the log-rank test, the generalized Wilcoxon test and Gehan's generalized log-rank test, were not used since the Wilcoxon test weights early survivors more heavily (which cannot be justified in this case), and Gehan's generalization has been seriously criticized (Prentice 1979). The log-rank test involves constructing a test statistic T , which, if the null hypothesis is true, should be distributed as a χ^2 variable (Matthews 1988).

3.2.9.3 Results and discussion

The survival curves for the three groups analysed (untreated, radiation alone, and radiation plus cisplatin) are shown individually for clarity in figures 3.2.9:1-3.2.9:3, and the combined graph is shown in figure 3.2.9:4. Table 3.2.9:2 below summarizes the results of the log-rank test, showing the values of the test statistic T and its corresponding p -value from standard tables of χ^2 variables.

Table 3.2.9:2. Summary of results of log-rank test comparing K-M survivor functions.

Groups compared	Test statistic T	p -value
"untreated" and "radiation only"	1.312	$p = .24$
"untreated" and "radiation plus cisplatin"	0.401	$p > .25$
"radiation only" and "radiation plus cisplatin"	0.007	$p > .25$

The median time to appearance of symptoms of intracranial pressure for untreated rats, from Table 3.2.9:1 is 21.5 days. This compares well with values reported in the literature for median lifespan (which is approximately one day more than time to appearance of symptoms of ICP), which range from 18 to 25 days, depending on the location of implantation in the brain (Wheeler 1980a, Barker 1973, Henderson 1981, Kimler 1992).

The log-rank test shows no significant differences in survival between the three groups analysed, despite apparent small differences in the median survival times. One limitation of the analysis stems from comparing groups of different *n*. The log-rank test compares the two survival curves on a day by day basis until the survivor function of one of the groups drops to zero, at which point the analysis stops. The existence of long survivors in the other group thus has little influence on the test statistic. In the case of the radiation plus cisplatin treatment group, two individuals survived to 30 and 56 days. The different *n* and different numbers of censored observations of the treatment groups is a result of the fact that survival was not the primary endpoint of the experiments.

The poor response of these tumours to radiation treatment can be attributed to several factors. The 9L is clearly a very radioresistant tumour, as demonstrated by the *in vitro* studies. In addition, the tumours were large when treated (approximately 2.5 - 3 mm), a limitation imposed by the abilities of the MRI techniques to visualize the tumours well enough to obtain accurate pre-treatment measurements of tumour size. Tumours of that size undergoing one or two post-irradiation doublings can cause symptoms of intracranial pressure, before tumour regression occurs. Tumours of this size (~3mm) can be termed late tumours (Wheeler 1981). Reports in the literature of radiation treatment of late intracranial 9L tumours also cite minimal increase in lifespan compared with untreated controls (Weizsaecker 1981), whereas radiation treatment of early tumours (7-12 days following inoculation) results in significant increased lifespan (Henderson 1981, Wheeler 1980, Kimler 1992).

The poor results of the combined cisplatin and radiation treatments are likely due to inadequate concentrations of cisplatin accumulating in the tumour. The experiments of section 3.2.7 showed more than 1 µg of elemental platinum per gram of tissue accumulating in intracranial 9L tumours approximately 2 hours following intraperitoneal injection of cisplatin at 6 mg/kg. While this is roughly equal to the 2 ppm levels achieved in tissues after *i.v.* administration in patients (Coughlin 1989), it corresponds to less than 3 µg/ml in tissue culture, based on the area under the free cisplatin concentration-time curve (Stewart 1988, Stewart 1989). The *in vitro* results of section 3.1.3 showed that this concentration in tissue culture is not adequate to cause significant inhibition of PLDR following radiation. Attempts to increase the dose of cisplatin given to rats to 8 mg/kg administered

intraperitoneally resulted in increased systemic toxicity (diarrhea, weight loss). The LD₅₀ for guinea pigs is 9.7 mg/kg (Merck 1983).

The experiments of section 3.2.7 measured total elemental platinum, by flameless atomic absorption. The results show that platinum is getting to the tumour, but show nothing about the Pt species present. HPLC analysis of cisplatin metabolites in plasma ultrafiltrate show that six hours after intravenous administration of cisplatin in rats, approximately half is bound to protein and inactive (Mistry 1989). Intraperitoneal administration results in a higher rate of protein binding, as determined by HPLC analysis of plasma ultrafiltrate. Three hours after i.p. administration of cisplatin in rats, 95% of total plasma Pt is protein bound (Daley-Yates 1986).

Significant increase in lifespan has been reported with combined radiation and cisplatin administered intracranially (injected directly into the tumour) to rats bearing early 9L tumours (Kimler 1992). This route bypasses the blood brain barrier and results in high peak concentrations of drug in the tumour. However, intracranial chemotherapy resulted in severe central nervous system toxicity (Kimler 1992), or early death due to the chemotherapy (Kimler 1990). Encouraging results were obtained in animal (Jakowatz 1991) and clinical studies using intraarterial administration of cisplatin in glioma patients (Stewart 1987, Watne 1991). This route of administration is prohibitively difficult in rats; intraperitoneal administration remains the most convenient route.

Figure 3.2.9:1. Kaplan-Meier survival plot for the untreated group of rats with intracranial 9L tumours.

Survival plot for rats with 9L intracranial gliomas treated with radiation

Figure 3.2.9:2. Kaplan-Meier survival plot for the group of rats with intracranial 9L tumours treated with radiation only (80 Gy).

Survival plot for rats with 9L intracranial glioma treated with radiation and cisplatin

Figure 3.2.9:3. Kaplan-Meier survival plot for the group of rats with intracranial 9L tumours treated with radiation and cisplatin.

Survivor functions for rats with 9L intracranial gliomas treated with radiation, radiation + cisplatin, and untreated.

Figure 3.2.9:4. Combined survival plots for rats with 9L intracranial gliomas: untreated, treated with radiation only, and treated with radiation and cisplatin.

4.0 CONCLUSIONS

4.1 In vitro radiation and cisplatin interactions

The results of section 3.1.1 indicate that the 9L cell line is highly radioresistant, with a D_0 of 1.94 Gy. The radiation survival curve is distinguished by its pronounced shoulder, characterized by a large quasi-threshold dose D_q of 5.5 Gy, and a large α/β ratio of 3.4 Gy. These parameters indicate a large capacity to accumulate and repair radiation damage.

The 9L cell line is proficient at repair of potentially lethal damage, with a recovery ratio of 18.1 at a dose of 18 Gy (section 3.1.3). The extent and kinetics of this recovery are almost identical to that of the human malignant glioma cell line U-87-MG at the iso-survival dose of 9 Gy. Exposure to cisplatin for the first hour of the recovery period following irradiation results in inhibition of PLDR in a cisplatin dose-dependent manner. Cisplatin at 3 $\mu\text{g/ml}$ has no effect on PLDR; 4.5 $\mu\text{g/ml}$ has little effect; 6 $\mu\text{g/ml}$ causes significant inhibition of PLDR (table 3.1.3:1). The concentration of cisplatin found in tumours following clinical intravenous administration is equivalent to approximately 7 $\mu\text{g/ml}$ in tissue culture (Stewart 1982); intracarotid administration results in increased concentrations in brain tumours (Stewart 1987, Eapen 1990).

These results indicate that the concentration of cisplatin in the cells is critical for effective inhibition of PLDR, since there exists a quasi-threshold for PLDR around 4.5 - 6 $\mu\text{g/ml}$. This dose corresponds to the position of the shoulder in the 9L cisplatin dose-response curve. This may reflect the increasing probability of interaction between intrastrand Pt-DNA crosslinks and radiation-induced single-strand breaks at cisplatin doses beyond the shoulder region of the cisplatin dose-response curve, as predicted by the linear-quadratic model of platinum-radiation interactions (Chadwick 1976).

The 9L cell line is relatively resistant to cisplatin, as shown by the comparison of the cisplatin response of various cell lines in table 3.1.2:1. The significant inhibition of PLDR by cisplatin seen in 9L cells appears to contradict the hypothesis of Coughlin and Richmond that mechanisms of radiation potentiation by cisplatin operate only in cells inherently sensitive to cisplatin (Coughlin 1989). The origins of cisplatin resistance have not been clearly established, but increased removal of Pt-DNA adducts (Micetich 1983) and increased capacity for DNA repair (Meijer 1990, Eastman 1988, Canon 1990) have been implicated. The radioresistance of 9L cells, due to a large capacity for repair of radiation-induced DNA damage, might suggest that this increased capacity for DNA repair is linked to their cisplatin resistance. However, figure 3.1.6:3 and the work of other investigators

(Barot 1985, Dewit 1987, Drewinko 1975) show that the mechanisms for repair of cytotoxic cisplatin lesions operate on a much longer timescale than the mechanisms of repair of potentially lethal radiation damage (figure 3.1.3:1). These two repair phenomena are likely different, and the mechanisms of resistance to cisplatin and radiation are likely different (de Pooter 1991).

Maximum inhibition of PLDR by cisplatin occurs when cells are exposed to cisplatin immediately after irradiation (figure 3.1.6:1 and 3.1.6:2), resulting in peak cisplatin levels when recovery is most rapid (figure 3.1.3:1). Inhibition of PLDR is reduced considerably if one-hour cisplatin exposure occurs 6 or 12 hours before irradiation. These results suggest that the cellular lesion responsible for cisplatin inhibition of PLDR is rapidly formed upon exposure to cisplatin, and is removed by plateau-phase cells with a time-scale of several hours. In contrast, the cytotoxic cisplatin lesion is not repaired quickly, if at all, since plateau-phase cells exposed to cisplatin for 1 hour then incubated for up to 20 hours before plating show no significant increase in survival with time (figure 3.1.6:3). Recovery from the cytotoxic effects of cisplatin occurs on a much longer timescale, possibly 24 - 60 h (Barot 1985, Dewit 1987), than recovery from the PLDR inhibitory effects of cisplatin. These results show that the lesions responsible for cisplatin cytotoxicity are likely different from the lesions responsible for cisplatin inhibition of PLDR following irradiation.

The clinical implication of these results is that combined cisplatin-radiation treatment schedules should be designed so that cisplatin administration causes peak drug levels at the tumour during or immediately after irradiation in order to achieve maximum interaction between the two modalities. Separation of irradiation and cisplatin administration by even a few hours significantly reduces the PLDR inhibitory effect of cisplatin.

Future *in vitro* experiments on cisplatin-radiation interactions could be directed at generating radiation survival curves in the presence of cisplatin at various doses, in order to establish the dependence on cisplatin dose of the coefficients α and β in the linear quadratic model. These data could then be used to evaluate the validity of models of cisplatin-radiation interactions (Chadwick 1976, Begg 1987). Additional data are also required concerning the hypothesis of Coughlin and Richmond that mechanisms of radiation potentiation by cisplatin operate only in cells inherently sensitive to the cytotoxic effects of cisplatin (Coughlin 1989). The experiments on 9L cells described in sections 3.1.2,3,4 suggest the alternative hypothesis that radiation potentiation by cisplatin occurs in cells resistant to the cytotoxic effects of cisplatin, provided the dose of cisplatin is beyond the shoulder region of the cisplatin dose-response curve. This hypothesis could be examined by experiments on cell lines with cisplatin resistant variants such as the human ovarian carcinoma 2780CP.

The inoculation of intracranial 9L rat brain tumours was improved considerably by the technique of using individual frozen aliquots of cells, as described in section 3.2.2. This new method avoids the loss of cellular viability as the inoculation suspension is held on ice (figure 3.2.2:1), with the concomitant variation in tumour size (figure 3.2.2:2). A higher and more constant number of viable cells is inoculated into each animal with the new technique, resulting in tumours which are generally larger and more uniform between animals than was achieved using the standard inoculation method (figure 3.2.2:3). The new technique offers two additional advantages: a large number of aliquots of cells can be prepared and cryopreserved at the beginning of an experimental project, ensuring that each animal receives cells from the same passage; and advance preparation of the cells speeds the tumour inoculation process. This new technique should work well for any cell line which has a high viability after cryopreservation.

The technique of irradiation of intracranial tumours in rats described in section 2.2.7 resulted in well localized radiation dose delivered to the tumour-bearing region of the brain. Other areas of the brain were spared, as were the radiosensitive salivary glands. This localized cranial irradiation technique was well tolerated by the rats, with no adverse reactions at tumour doses as high as 90 Gy.

Intraperitoneal injection of cisplatin was found to be a simple and reasonably effective route of administration for intracranial tumours in rats (section 3.2.7). Platinum levels in intracranial 9L tumours were found to peak at 1.1 $\mu\text{g/g}$ approximately 2 hours after intraperitoneal injection of 6 mg/kg cisplatin, and declined to 1/3 of the peak level 4 hours after injection (figure 3.2.7:1). While ip injection of 6 mg/kg resulted in clinically relevant Pt levels, they were lower than comparable in vitro levels required for PLDR inhibition. This observation, together with the poor in vivo tumour response observed in animals treated with 6 mg/kg ip cisplatin, led to an increase in the in vivo dose to 8 mg/kg ip. However, animals treated with 8 mg/kg experienced adverse systemic reactions (diarrhea, weight loss) which might be attributable to the cisplatin. In conclusion, intraperitoneal cisplatin injection results in clinically relevant Pt concentrations in experimental intracranial tumours, but 6 mg/kg is close to the maximum tolerated dose which can be delivered by this route. The tumour Pt levels achieved at this dose are less than optimum for inhibition of PLDR; the more difficult (in rats) intravenous or intraarterial routes might result in higher tumour Pt levels with lesser adverse effects.

Investigations were carried out to determine optimum MR imaging methods for detecting and measuring the size of 9L intracranial tumours in rats (section 3.2.3). Because of the long T1 relaxation times of tumour and brain at 4.7 T, maximum contrast-to-noise ratio between tumour and surrounding brain in non-contrast enhanced images was found to occur at long repetition times (TR ~ 3 seconds). The apparent T2 of 9L intracranial tumours as measured from the decay of signal in multi-echo images (8 echoes, TE = 34 ms) was found to increase slightly with time after inoculation and with tumour size, but this increase was not significant and could not be used to reliably distinguish between tumour and normal brain. The apparent T2s of tumour and normal brain were close to equal; approximately one-third of animals imaged using a non-contrast enhanced imaging technique had tumours which could not be measured due to inadequate contrast between tumour and surrounding brain. Tumour-brain contrast was generally better in larger tumours, which is one reason why treatments were performed on tumours 2 - 3 mm in diameter, when pre-treatment size could be more reliably measured. When contrast was adequate for tumour delineation, there was excellent correlation between tumour size measured from non-contrast enhanced MR images and from histopathological sections (figure 3.2.5:1). No connection could be established between poor tumour-brain contrast and histopathological observations; it remains unclear why some tumours had adequate contrast with surrounding brain, and others did not.

The injectable MR contrast agent Gd-DTPA (Magnevist®) was found to greatly increase the contrast between tumour and surrounding brain on T1-weighted images (TR ~ 300 ms). Intraperitoneal administration of Gd-DTPA (0.5 mmol/kg, Magnevist® 10 ml/kg) quickly enhanced intracranial 9L tumours, with peak enhancement occurring 30 minutes after ip injection (figure 3.2.3:4). Enhancement was substantial when images were acquired anywhere from 15 to 40 minutes following i.p. administration of Magnevist®, after which the contrast agent is removed by nephric excretion.

A comparison was made between the spatial extent of the enhanced region on Gd-DTPA MR images and the extent of the tumour as determined from histopathological examination. When shrinkage of histopathological sections due to formalin fixation and ethanol drying was accounted for, there was excellent correlation between tumour size measurements from the two techniques (figure 3.2.5:2). For the 9L tumour, which has minimal local invasion into surrounding brain, Gd-DTPA causes enhancement of the entire tumour, where the blood-brain barrier is disrupted, but does not enhance regions of brain surrounding the tumour. This finding may have clinical implications -- in human brain

tumours, where local invasion is generally greater but detailed comparison between MR images and histopathological sections is rarely possible, the extent of contrast enhancement is Gd-DPTA dose dependent and does not correlate well with tumour extent determined from T2-weighted images (Yuh 1992). In 9L intracranial tumours, intraperitoneal administration of Gd-DPTA followed by T1-weighted imaging within 15 - 40 minutes is the most reliable and accurate method of detection and measurement of the size of the tumours.

The rate of growth of intracranial 9L tumours was found to vary considerably from animal to animal (fig. 3.2.4:1). This emphasizes the utility of MRI in experimental studies using intracranial tumour models, since non-invasive pre-treatment measurement of tumour size allows each animal to serve as its own control. This has been possible in subcutaneous sites through caliper measurements, but the inaccessibility of intracranial tumour models has limited their utility and necessitated averaging over large numbers of animals to compensate for animal to animal variability. By allowing the use of each animal as its own pre-treatment control, MRI helps to minimize the number of animals used. Furthermore, the usual endpoint for intracranial tumour studies has been survival of the animal; these studies are increasingly difficult to justify to animal care committees. MRI allows tumour size to be used as an endpoint, minimizing animal discomfort.

4.4 MR imaging of radiation injury to normal brain

Imaging was performed on rats without tumours following localized single-fraction 80 Gy brain irradiation, to determine whether MRI could provide an early indication of radiation injury to normal brain tissue (section 3.2.6). The irradiated region of the brains of these animals showed a time-dependent decrease in signal intensity on non-contrast enhanced images, which became significantly different from pre-irradiation controls at 130 days following irradiation (figure 3.2.6:1). Comparison with histopathological findings indicated that MRI signal changes occurred only after significant radiation necrosis had occurred. MRI did not provide an early indication of the onset of the demyelinating lesion associated with the development of late radiation injury to brain tissue. The use of injectable contrast agent Gd-DTPA did not provide any advantage in the early detection of this lesion.

4.5 Cisplatin and radiation treatments of intracranial 9L tumours

Serial MRI measurements of the size of intracranial 9L tumours in rats treated with radiation, cisplatin, and combined radiation and cisplatin were performed, to determine whether *in vitro* cisplatin inhibition of potentially lethal damage repair translated into enhanced therapeutic gain *in vivo*. Tumours treated with radiation alone (80 Gy single fraction) showed no tumour size regression, confirming the radioresistance of the 9L tumour model. Histopathological examination of irradiated tumours revealed the onset of focal necrosis 3 - 4 days following irradiation. The occurrence of demyelination in irradiated brain tissue surrounding the tumour resulted in an open, spongy morphology which appeared to be more susceptible to local invasion by 9L tumour cells. This observation is potentially significant in the treatment of human malignant gliomas, since resistant cells which survive radiation therapy could show increased infiltration of surrounding radiation-injured brain tissue.

Treatment with cisplatin alone did not result in tumour regression or significant growth delay, but a small amount of focal necrosis was evident upon histopathological examination 3 - 4 days following treatment. Combined cisplatin and radiation treatments resulted in a slight growth delay which was not statistically significant when compared with the other treatment groups. Two animals (of seven) receiving the combined treatment showed tumour regression and increased survival time; histopathological examination of these animals revealed massive necrosis in the tumours. Log-rank analysis of the Kaplan-Meier survival curves showed no significant difference in survival between the treatment groups.

Untreated 9L intracranial tumours had relatively uniform signal intensity throughout the tumour, on both non-contrast enhanced and Gd-DTPA contrast enhanced images. An exception was the appearance of acellular, proteinaceous cysts, found in a quarter of the tumours implanted, whose MRI manifestation was a hypointense region approximately 0.5 mm in diameter. Tumours treated with radiation and/or cisplatin frequently demonstrated increased variation in signal throughout the tumour, on both non-contrast enhanced and contrast enhanced images, concomitant with the development of focal necrosis. This appearance of increased structure within the tumour was clearly connected with cellular damage due to the treatment, but detailed correspondence between particular regions of focal necrosis identified by histopathological examination and signal variations on MRI could not be established. The determination of the spatial and temporal relationship between MRI signal variations and the appearance of focal necrosis would require very thin MRI slices

and excellent spatial correlation with histological sections. This was beyond the capacity of the experimental protocols of this project.

The inhibition of potentially lethal damage repair by cisplatin observed *in vitro* did not translate into tumour regression observable by MRI *in vivo*. Histopathological analysis did show substantially increased cellular necrosis in tumours treated with cisplatin and radiation compared with either treatment alone, but this did not result in regression observable by MRI. One reason was the difficulty in distinguishing between viable tumour, necrotic tumour and edema on MRI. MR techniques sensitive to the metabolic status of cells, such as phosphorus-31 spectroscopic imaging and lactate-edited spectroscopic imaging, could make this distinction. These techniques have been developed at the National Research Council and are currently being applied to the monitoring of radiation and cisplatin treatments of intracranial 9L tumours.

In addition, the *in vivo* treatments were initiated when the intracranial tumours were 2 - 3 mm in diameter, to ensure accurate pre-treatment tumour size measurement. At this point, the tumours were likely too large to respond to treatment. Residual post-irradiation cell division, or edema resulting from radiation damage to microvasculature, likely caused sufficient increase in intracranial pressure to terminate the experiment before regression could occur. Imaging techniques have now been refined to allow accurate measurement of smaller intracranial tumours, and 3D imaging techniques allow accurate measurement of the tumour volume (Johnson 1987). Future experiments using imaging to monitor the effects of treatment of intracranial 9L tumours should initiate treatments when tumours are about 1 mm in diameter (approximately 10 days after tumour implantation).

Clinical implementation of combined radiation and cisplatin treatments for radioresistant and repair-proficient tumours such as malignant gliomas will depend on the development of techniques for achieving high intratumour drug concentrations during or immediately after the administration of radiotherapy fractions, within the limits of acceptable systemic toxicity. These techniques could include intra-arterial cisplatin administration (Stewart 1987), direct intratumour cisplatin injection (Kimler 1992), the use of less toxic cisplatin analogues such as carboplatin (Coughlin 1989), or intratumour implantation of time-release drug delivery gels (Brem 1991, Duerloo 1990). Better understanding of the mechanisms of interaction of drugs and radiation is required in order to improve treatment for patients with these resistant tumours. Hopefully the work described in this thesis has contributed in some way to a greater knowledge in this area.

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Appendix: Accounting of live animals (rats) used in this project

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PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

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