HYPOXIA AND PROLIFERATION IN MURINE TUMOUR MODELS

by

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Gray Laboratory of the Cancer Research Campaign, Mount Vernon Hospital, Northwood, Middlesex.
This thesis considers two of the contributory factors in the local failure of radiotherapy; hypoxia and rapidly proliferating cells. Model systems were developed from SaF murine cells to enable analysis of the effect of the bioreductive quinones, mitomycin C (MMC) and porfiromycin (POR), upon single cells, *in vitro* spheroids, *in vivo* peritoneal spheroids and subcutaneous tumours. A novel hypoxic probe, 7-(4’-(2-nitroimidazole-1-yl)-butyl)-theophylline (NITP) allows quantitation of hypoxia, by bioreductively binding to macromolecules in cells under low O₂ conditions. In cells, NITP displayed progressive binding as oxygen decreased with concomitant increases in the cell kill of both MMC and POR. At extremes of O₂ concentration (air and N₂) MMC and POR had oxic:hypoxic differentials of 1.7 and 3.5 respectively at 10% SaF survival. As spheroids increased in size, (250, 400 and 600μm), their degree of hypoxia increased, (5.0, 15.8, 36.0%). MMC produced high cell kill with pronounced growth delay, whereas POR showed lower cell kill with slight growth delay increasing with spheroid size. Peritoneal spheroids showed little quantitative response to MMC or POR due to the variable nature of this *in vivo* tumour model. Subcutaneous tumours exhibited an exponential dose response, where surviving fraction was strongly correlated with hypoxia for the more hypoxia specific POR in contrast to the poor correlation between hypoxia and the effects of MMC and radiation. Tumour size, geometric mean diameter 4 to 12mm, had no effect on the amount of hypoxia in SaF or Rh tumours. Analysis of each phase of the cell cycle by DNA content showed that although the greatest population of hypoxic cells had G1 DNA content, the highest proportion of hypoxic cells resided in the population with G2/M DNA content. A flow cytometric technique to simultaneously measure hypoxia, proliferation rate (bromodeoxyuridine incorporation) and DNA content in a single sample was developed for murine tumours. This showed that cells recently hypoxic could enter the cell cycle and hypoxic cells tended to accumulate in G2 and G1. This technique could be used as the basis for a clinical test for proliferation and hypoxia in tumours once the NITP hypoxia marker has been approved for clinical evaluation.
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B & S need a mention for their stress relief therapy.

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Abbreviations used in this thesis

- 7-AAD: 7-amino-actinomycin D
- ABC: avidin biotin complex
- BrdUrd: 5-bromo-2'-deoxyuridine
- CAK: p34$$^{cyc}$ activating kinase
- cAMP: cyclic adenosine monophosphate
- cdk: cyclin dependent kinase
- CHART: continuous hyperfractionated accelerated radiation treatment
- CNS: central nervous system
- CV: coefficient of variation
- DAB: 3,3'-diaminobenzidine
- DMSO: dimethylsulphoxide
- DNA: deoxyribonucleic acid
- EDTA: ethylenediaminetetraacetic acid
- FAD: flavin adenine dinucleotide
- FCS: fetal calf serum
- FITC: fluorescein isothiocyanate
- G0: quiescent or resting phase of the cell cycle
- G1: First gap phase of the cell cycle
- G2: Second gap phase of the cell cycle
- H&E: Haematoxylin and Eosin
- hnRNA: heterogeneous nuclear ribonucleic acid
- IL-1α: interleukin 1 alpha
- IUdR: 5-iodo-2'-deoxyuridine
- M: Mitosis
- MEM: minimum essential medium
- MEMS: minimum essential medium for suspension culture
- MMC: Mitomycin C
- mRNA: messenger ribonucleic acid
- MRS: magnetic resonance spectroscopy
- NITP: 7-(4'-(2-nitroimidazole-1-yl)-butyl)-theophylline
- OER: Oxygen enhancement ratio
- PBS: phosphate buffered saline
- PCNA: proliferating cell nuclear antigen
- PE: R-phycoerythrin
- PI: propidium iodide
- PNT: phosphate buffered saline/normal goat serum/Tween 20
- POR: porfiromycin
- RM: relative movement
- RNA: ribonucleic acid
- S: S phase of the cell cycle
- SD: standard deviation
- SEM: standard error of the mean
- SF: surviving fraction
- SOBR: sum of broadened rectangles
- TBS: tris buffered saline
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1.1 GENERAL INTRODUCTION

Human tumours are characterised by their diversity in both biological characteristics and response to treatment. The heterogeneity of response of solid tumours to therapeutic strategies derives from both genetic and physiological factors. Genetic heterogeneity consists of drug resistance, radiosensitivity, karyotype, surface markers and metastatic behaviour. Physiological heterogeneity includes nutritional status, pH of the environment, degree of oxygenation and proliferative status. These physiological factors, caused wholly or in part by an inadequate blood supply, limit the cytotoxic effect of radiotherapy and chemotherapeutic agents on the tumour.

Tumour oxygenation and proliferation are two of the factors which greatly influence tumour response. The poor oxygenation of some tumours may be a limiting factor in the effectiveness of radiotherapy, therefore improvements in identification and quantification of tumour hypoxia are needed. Once hypoxia has been identified within a tumour procedures to selectively kill resistant hypoxic cells, such as bioreductive drugs, can be implemented. In order to study hypoxia and the cytotoxic effect of bioreductive drugs relevant tumour models need to be assessed. Spheroids are a tumour model which produce subpopulations of cells due to the gradients of nutrient penetration into a spherical mass. Hypoxia tends to increase as spheroids increase in diameter, thus differing levels of hypoxia can be achieved. Nutritional gradients in spheroids also lead to changes in proliferative status as spheroids increase in diameter thus proliferative subpopulations can also be examined. These differing characteristics can be used to elicit the effectiveness of bioreductive drugs on different subpopulations of tumour cells. Elucidation of the effectiveness of bioreductive drugs against hypoxic and aerobic cells is necessary to assess which of these numerous compounds should be adopted for use in the clinic.
A priority in the use of bioreductive drugs is to identify the patients which will benefit from their addition to radiotherapy regimes, i.e. those with a high degree of tumour hypoxia. Tumour proliferation is already studied in the clinic thus administration of an hypoxic probe prior to the biopsy could lead to measurement of proliferation and hypoxia in a single procedure. The relationship between hypoxia and proliferation could also be examined.

The aim of this thesis is to study hypoxia and proliferation using novel approaches in a variety of model systems designed to dissect the influence of each factor.

1.1.1 Tumour Microenvironment

Solid tumours are dynamic entities to which cells are constantly being added by cell division; growth results if the rate of cell loss through death or shedding is not balanced with the rate of cell division (Steel 1967). In the evolution of a tumour from single cells, nutrition is initially derived by diffusion from the surrounding tissues. However, for the tumour to grow it has to develop a system of blood vessels to provide nutrients (Folkman 1976). This is achieved through the process of angiogenesis; in which tumour cells release factors, such as vascular endothelial growth factor and basic fibroblast growth factor (Shweiki et al. 1992; Plate et al. 1992; Folkman 1992), which attract endothelial cells into the tumour mass to provide capillaries (Folkman 1974; 1992). The vascular system developed in this way tends to be chaotic, and this leads to cellular heterogeneity; inadequacies in the capillary network produce gradients in oxygen, and in the concentration of nutrient and cellular waste products (Denekamp 1990).

Thomlinson and Gray first suggested the phenomenon of a gradient in oxygen concentration in a paper in 1955 describing the corded structure of a tumour (Thomlinson & Gray 1955) (Figure 1.1). The limit of viable cells in the tumour cords was 180μm from the blood vessel, which represents the diffusible distance of oxygen in tissues (Gray et al. 1953), from where necrosis became evident in the histological sections of human bronchial
carcinomas. This concept of an oxygen gradient can be extrapolated to the other nutrients a tumour cell requires, but oxygen is of primary importance to cancer treatment because lack of oxygen leads to radioresistance, and in some instances chemoresistance (Tannock 1986).

Subsequent to this demonstration of human tumour hypoxia, similar findings were made in murine tumours (Tannock 1968). Tannock found patent blood vessels surrounded by viable tumour cells which degenerated into necrosis with increased distance from the vessel. He hypothesised that the outer cells of tumour cords would become chronically hypoxic as they were being pushed further from the blood vessel, and thus further from the oxygen supply, by proliferation in cells adjacent to the vessel. These cells would have a short life-span, as they would be displaced by newly produced cells pushing them into the hypoxia prone region.

Recently a different population of oxygen depleted cells has been identified (Brown 1979; Chaplin et al. 1986); which are the result of acute
or perfusion limited hypoxia. This occurs where blood vessels shut down for short periods of time, causing a transient loss of oxygen to cells in close proximity to the vessel, and then reopen. Acute hypoxia will still have the same consequences as chronic hypoxia with regard to radiotherapy as it is the oxygen concentration during exposure to radiation which is the crucial factor in determining sensitivity (Gray et al. 1953).

In association with a decrease in tumour oxygenation as distance from blood vessels increases, there is also a decrease in tumour cell proliferation. In general, cell proliferation is most commonly associated with close proximity to viable blood vessels (Kligerman et al. 1962; Tannock 1968; Hirst & Denekamp 1979), although occasionally there are pockets of proliferating cells at a distance from vessels in necrotic areas (Rutgers et al. 1987).

Although there has been much research into the single parameters of tumour oxygen status and proliferation there has been little investigation into their interaction. The majority of work has entailed inducing hypoxia in cells in vitro and monitoring its effect on cell cycle progression by a variety of methods (Merz & Schneider 1982; Shrieve & Begg 1985). These studies demonstrated, in human NHK 3025 and rodent Ehrlichs ascites tumour cells, a prolonged cell cycle time after hypoxia but the effect was phase specific. During hypoxia, cells were inhibited in G1 and accumulated at the G1/S border (under severe hypoxia there was G1 arrest) but when aerobic conditions were re-established cell cycle progression continued as normal. In contrast, S phase cells subjected to hypoxia were inhibited and, even after aerobic conditions were re-established, still maintained a slow progression through the cell cycle (Pettersen & Lindmo 1983). Some tumour cells may not even divide but carry on the cell cycle accumulating higher DNA content (Merz & Schneider 1983). Cells in early and mid-S were completely inhibited but cells in late-S, G2 and M continued the cell cycle and divided (Åmellem & Pettersen 1991). In rodent V79 cells, hypoxia immediately arrested cells in all phases of the cell cycle for over 12 hours, while reaeration of cells led to a slower cell cycle time (Shrieve & Begg
S phase cells were most sensitive to chronic hypoxia with their surviving fraction reduced to 2.5% compared to 20% and 7.6% for G1 and G2/M respectively (Spiro et al. 1984). In vivo experiments have suggested that hypoxic cells in necrotic regions of tumours progress through the cell cycle (Rutgers et al. 1987) and DNA over-replication does not occur to the same extent as in in vitro systems (Young & Hill 1990). There has also been evidence that cells furthest from blood vessels accumulate in G1, which would be in accordance with the other data (Siemann & Keng 1987; 1988). However, studies in Chinese hamster V79 spheroids have shown that hypoxic cells in the inner core die within 6 hours of the spheroids being exposed to fully hypoxic conditions (Franko & Sutherland 1978).

Despite the implications both these parameters have in the response of solid tumours, there is still little known about their interaction in vivo and its consequences in therapy. Each of these contributors to heterogeneity will now be considered individually and their rôle in tumour therapy regimes discussed.

In the last few years interest in the control of proliferation has intensified as the relationship between control of the cell cycle and the initiation of cancer has to some extent been elucidated. The cell cycle, its control and subsequent tumour growth will now be examined.
1.2 THE CELL CYCLE

Control of cellular growth is governed by complex homeostatic mechanisms involving both extracellular and intracellular factors and events. Normal tissues display a balance between cell production and cell loss to maintain an equilibrium. They rarely exhibit a rapid increase in cell number except during embryonal development or regeneration. A common feature of the cancer cell is loss of control of cell proliferation.

There are four compartments in the cell cycle which were defined according to criteria based on cells in culture (Figure 1.2). The cell cycle was first described in detail by the pioneering work of Howard and Pelc (1951) using radioactive phosphorus incorporation in the roots of *Vicia faba*. The four distinct phases were described as G1, the first Gap phase; S, where DNA is Synthesised; G2, the second Gap phase and M, Mitosis which leads to cell division. The early view of the cell cycle was that in G1,
enter it given the correct external stimuli. Various factors such as availability of nutrients, cell size, cell density and the presence of growth factors influence whether the cell remains in G1 or enters G0 (Sorrentino 1989). The second phase is S where DNA is synthesised, doubling the normal amount in the cell. The third phase is G2, the second gap phase, where the cell has twice the DNA and prepares for cell division or mitosis. In mitosis the cell divides producing two daughter cells each with a complete set of chromosomes. From this stage the cell could either carry on through another cell cycle, enter the dormant phase or go on to differentiate into a particular type of cell.

As may be deduced from this oversimplification the cell cycle is subject to a great many controls throughout this process. The cell requires signalling pathways to know when preparation for DNA synthesis is complete, when the DNA genome has been fully duplicated in the S phase and when the cell is ready to divide in mitosis.

In the last few years many of the components involved in this control have been identified but their explicit rôles have yet to be elucidated. Another factor which prevents clarification is that there are differences between cell lines and model systems used for investigational purposes so that what is true for one organism or cell line may not be true in another.

1.2.1 Cell Cycle Control

The control of the cell cycle can be divided into two separate integrated parts, mainly occurring in the G1 and G2 phases. Once the cell has entered S or M phase respectively then events are already at too advanced a stage to interrupt or stop. The control of cell proliferation has to be in G1 before any extra DNA has been synthesised to prevent possible reproduction of the incorrect amount of DNA in the cell. In the G1 phase it is extracellular factors which determine whether a cell is in the active G1 or quiescent G0 phase of the cycle. Cell cycle events become largely independent of extracellular factors upon entering S phase.
1.2.1.1 The G1 Phase

There are four stages in the G1 readiness for entry into S (Temin 1971; Stiles et al. 1979; Rossow et al. 1979; Pardee 1989). In the first stage there are changes to chromatin structure and an increase in transport of nutrients through the membrane with concomitant production of novel mRNAs. In the second stage there is synthesis of new macromolecules, an increase in polysomes and glycolytic enzymes. In the third stage there is rapid protein synthesis with enzymes being produced for DNA synthesis, and regulatory proteins are made (Pardee 1987). Finally the enzymes enter the nucleus and are organised into a complex catalysing DNA synthesis (Dingwall 1985).

These four phases of G1 are accompanied by a variety of signalling pathways from the cell membrane to the nucleus. The signalling sequence takes many hours and is generalised in the diagram below (Pardee 1989)(Figure 1.3).

![Diagram](image-url)

Figure 1.3 Events occurring to stimulate entry into the cell cycle. (hnRNA = heterogeneous nuclear RNA)
Growth factors, many of which are small proteins, such as the interferons and tumour necrosis factor, can either stimulate or inhibit growth. These factors combine with specific receptors on the plasma membrane. There can then be either a direct pathway via phosphorylation of tyrosines, serines and threonines on other proteins transmitting the signal or an indirect pathway using second messengers such as Ca^{2+}, cAMP and calmodulin, both pathways rely on protein kinases (Rozengurt 1986). These processes lead to the activation of genes but the exact method is still under investigation. Once the genes are activated new mRNA molecules are produced. There is a general increase in protein in the cell. Proteins such as Fos, Jun, Myc and Krox-20 are produced. Gene transcriptions which occur later in the G1 phase may depend on earlier protein synthesis and require essential amino acids to be brought into the cell by specific transport systems. Parts of this process are repeated, induced by the gene activation. Mid or late G1 mRNAs and proteins are produced. Enzyme activities increase in mid-G1 including transin, ornithine decarboxylase, hydroxymethylglutaryl coenzyme A reductase, p53 and p68 (Kerr et al. 1988; Dafgard et al. 1987). Other enzymes appear in late G1 including the DNA synthesis enzymes; ribonuclease reductase, dihydrofolate reductase, thymidine kinase, DNA polymerase and proliferating cell nuclear antigen (PCNA). Enzymes produced in the cytoplasm are moved to the nucleus, where they form complexes. The cell membrane has to be permeablised for ease of access of the DNA precursor synthesis enzymes and there has to be a change in conformation of the DNA to allow initiation of DNA synthesis before S phase can begin. Mitosis and cell division can then proceed. Accompanying these cell signalling pathways is the process involving START which is fully explained in the G2 section below.

Further control is provided by the tumour suppressor gene p53 (Sager 1989). The phosphoprotein, p53, has been identified as a specific transcriptional factor but it also mediates growth arrest of the cell cycle at G1 (Rotter et al. 1993; Hartwell 1992). It has been suggested that wild-type p53 polices the genome ensuring it is correct; if DNA is damaged p53 will
bind to DNA, accumulate, and switch off replication to allow a further period of DNA repair. If damage is irreparable it is thought to be involved in the switch on of apoptosis. Mutant p53 loses the ability to cause arrest after damage which would explain its presence in many malignancies (Lane 1992).

1.2.1.2 The S Phase

In the S phase of the cell cycle the DNA of the whole genome has to be replicated. The S phase lasts from 6 to 16 hours, depending on the cell type, but within a particular cell type it has a constant length (Dover 1992). This implies that, at the measured rate of base replication for an individual chromosome, replication must start at a number of locations at the same time otherwise replication of the whole genome would be a much more time-consuming process. Initiation of replication on a chromosome must be regulated spatially to obtain complete and precise replication in an optimum time.

In the S phase, not only the DNA, but also the chromatin conformation with nucleosomes and chromosome scaffolds have to be reproduced (Laskey et al. 1989). As the S phase has a constant duration there must be precise regulatory events. Pulse-labelled incorporation of the thymidine analogue bromodeoxyuridine, consistently shows specific regions of chromosomes replicating at different times during the phase (Willard & Latt 1976).

The site of replication is called the replication fork where the DNA is unwound and the two single strands duplicated. The replication fork requires two DNA polymerases, DNA polymerase α and δ. DNA polymerase α has primase activity in 2 of its subunits so is probably involved in the discontinuous synthesis of Okazaki fragments on the lagging strand (Lehman & Kaguni 1989). DNA polymerase δ is probably utilised in the leading strand. PCNA is an essential cofactor of DNA polymerase δ. Other essential proteins in the replication fork are RF-A or RP-A, which binds preferentially to single stranded DNA, whose function
is probably to stabilise the single stranded region for the correct positioning of the polymerase/primase complex. RF-C is required for co-ordination of leading and lagging strand synthesis of the replication fork (Tsurimoto & Stillman 1989). Replication is terminated by the meeting of replication forks not by specific DNA sequences. One theory is that the final helical turns of DNA of two converging forks are unwound by wrapping the two new DNA duplexes around one another. This produces a substrate for DNA topoisomerase II which cuts both strands of a DNA duplex and passes a second duplex through the gap. When a replication fork reaches the end of a linear chromosome a specialised telomere structure replicated by telomerase is employed. Telomerase does not copy the DNA template but adds a DNA repeat sequence to the chromosome ends probably by copying a RNA template constituent in the telomerase molecule (Greider & Blackburn 1989). This telomere structure is thought to decrease in length at each cell division, acting as an indicator of cell age, thus when it is short it programs the cell to die (Allsopp et al. 1992). A probable cause of tumour cells becoming immortal is that they do not shorten the telomere end of the chromosome at replication. The production of nucleosomes closely follows the replication fork but it is unknown at what stage the chromosome scaffolds are reproduced (Worcel et al. 1978).

Cell fusion experiments showed that replicated nuclei of normal cells can not be induced to re-enter S phase until after mitosis has occurred (Rao & Johnson 1970). An hypothesis that upholds experimental data on the subject is that an essential initiating factor of replication can not cross the nuclear membrane to reach the DNA until it disintegrates at mitosis. It could then bind to the DNA to allow replication after mitosis where it would be destroyed in the process of the following replication. No more factor could enter the nucleus until the next mitosis so the DNA could not be duplicated again (Blow & Laskey 1988).
1.2.1.3 The G2 Phase

The regulation of the length of the cell cycle occurs predominantly in the two gap phases of the cycle. Cells which cease proliferation, reversibly such as hepatocytes and lymphocytes, or permanently such as mammalian CNS neurones and muscle, arrest in G1. Progress through G1 requires an adequate nutrient supply and stimulation by an appropriate set of polypeptide growth factors (Unger & Hartwell 1976; Murray & Kirschner 1989). Once past a certain point, the restriction point or START, the cell is committed to divide and removal of either the nutrients or growth factors can not stop entry into S phase (Pardee 1974). Both becoming committed to start the cell cycle and entry into mitosis is dependent on the activation of a specific protein kinase containing cyclins and p34cd2 (Draetta & Beach 1988; Hunt 1989; Nurse 1990). The protein, p34cd2, remains constant throughout the cell cycle but its protein kinase activity rises steeply at the onset of mitosis (Solomon 1993). The increase depends on the formation of a complex with the proteins, cyclins (Draetta et al. 1989; Pines & Hunter 1989; Solomon et al. 1990). A and B cyclins are a regulatory subunit of CDC2n kinase (Pines & Hunter 1990). Histone H1 kinase increases abruptly at the G2/M transition indicating that association with cyclin alone does not activate p34cd2. A further level of regulation is added by the phosphorylation/dephosphorylation control of p34cd2. At the start of the cell cycle p34cd2 is mainly unphosphorylated and monomeric (Draetta & Beach 1988; D’Urso et al. 1990). There have been three phosphorylation sites identified in p34cd2, of which Tyr15 and Thr14 phosphorylation inactivates p34cd2 (Norbury et al. 1991) and Thr161 phosphorylation activates p34cd2 (Soloman et al. 1992). wee1 acts as an inhibitory tyrosine kinase phosphorylating Tyr15 and possibly Thr14 (Parker & Piwnica-Worms 1992) and p34cd2-activating kinase (CAK) phosphorylates Thr161 (Figure 1.4). As cells progress through S and G2 p34cd2 develops more phosphorylation and becomes complexed with other proteins, including the G1 cyclins. Binding of cyclin to p34cd2 induces Thr161 phosphorylation and concomitantly strengthens binding. Activation
Figure 1.4  p34\textsuperscript{cd2} regulation by phosphorylation/ dephosphorylation.

to generate a functional H1 kinase involves substantial de-phosphorylation of the tyrosine and threonine of p34\textsuperscript{cd2} at the G2/M transition (Morla \textit{et al.} 1989). The phosphorylation that remains is necessary for H1 kinase activation. The inhibitory phosphorylations of p34\textsuperscript{cd2} at Tyr15 and Thr14 are designed to prevent premature activation of the kinase until an appropriate level of the inactive cyclin/p34\textsuperscript{cd2} complex has accumulated. Removal of the inhibitory phosphorylations by cdc25, acting as a phosphatase (Kumagai & Dunphy 1991), permits the sudden, large rise in kinase activity which triggers mitosis. The p34\textsuperscript{cd2}/cyclin B complex phosphorylates lamins to cause the disassembly of the nuclear lamina during mitosis. p34\textsuperscript{cd2} kinase activation also produces induction of cyclin proteolysis (Murray \textit{et al.} 1989). Cyclin B degradation during anaphase leads to inactivation of p34\textsuperscript{cd2} and is essential for exit from mitosis.

The initiation of the cell cycle with DNA synthesis also has a role for p34\textsuperscript{cd2} at START (Murray 1992). Mitogen-stimulated T-lymphocytes need p34\textsuperscript{cd2} at the G1/S transition as quiescent cells have low levels but the level rises dramatically just before S phase (Furukawa \textit{et al.} 1990). G1 cyclins, C, D and E, are implicated in progression from G1 into S. Cyclin C is probably of major importance as it peaks at the G1/S transition, but their exact rôles have yet to be elucidated (Sherr 1993). This suggests that START
also depends on kinase activation as does the onset of mitosis but there is much less protein phosphorylation involved. The precise differences between S and M phase initiation are as yet undiscovered.

Recent work has shown that D cyclins act as growth factor sensors to aid the decision to divide and pass the G1 restriction point. Furthermore, the cyclin D1 gene has been identified as the \( bcl \) oncogene, overexpression of which leads to B cell lymphoma and maybe a number of other cancers. Excess cyclin D1 would lead to uncontrolled proliferation of cells, a known facet of cancer. Cyclins D2 and D3 have also been found to block cell differentiation, another hallmark of cancer cells (Marx 1994).

1.2.1.4 Mitosis

In mitosis eukaryotic cells ensure the equal division of their replicated chromosomes (McIntosh & Koonce 1989). In traditional cell division nomenclature the G1, S and G2 phases are grouped together as interphase and mitosis is subdivided into the following according to the processes taking place. Prophase - condensation of chromosomes to make them suitable for transport. Prometaphase - disruption of the nuclear envelope followed by the positioning of the chromosomes. Metaphase - alignment of centromeres in the centre of the cell. Anaphase - separation of each chromosome into two identical chromatids and movement towards opposite poles of the cell. Telophase - reformation of two nuclei and the chromosomes decondense gradually. Cytokinesis - division of the cytoplasm into two individual daughter cells.

There are a number of checkpoints in mitosis preventing the progression into the next phase of the cell cycle (Hartwell & Weinert 1989). When DNA synthesis is inhibited chromosome condensation, elaboration of mitotic spindle and cytokinesis is prevented and spindle pole body or centromere duplication at metaphase is arrested.

Initiation of DNA synthesis is inactivated by DNA single strand lesions. In the genetic disease, \textit{ataxia telangiectasia}, the dependency of replication on an intact template is lost, and by forcing replication to occur
on a damaged template the effect of radiation-induced lesions is exacerbated resulting in radiation sensitivity (Murray 1992).

As mentioned previously, for there to be exit from mitosis, cyclin proteolysis has to be in operation. If this degradation does not occur and $p34^{cd2}$ is not deactivated then the cell stays in a constant mitotic state (Murray & Kirschner 1989). The negative feedback of cyclin degradation is thought to be linked to spindle function. A further feedback control is thought to be in the microtubule organising centre where the presence of the centrosome is monitored.

### 1.2.2 Tumour Proliferation and Cell Cycle Kinetics

Tumours grow because they contain a population of cells which increase in number as a result of cell division. The essential difference between a tumour and normal cell population is that tumours lack normal homeostatic controls which would maintain the appropriate cell number. The rate a tumour grows depends on the cell cycle time of the tumour, the fraction of cells proliferating in the tumour and the rate of cell loss from the tumour by either cell maturation, death or shedding (Figure 1.5).

![Cell Cycle Diagram](image)

*Figure 1.5  Methods of cell production and cell loss from a tumour.*
1.2.2.1 Tumour Growth

Tumour growth is determined in murine models by measurement of tumour volume or mean geometric diameter as a function of time. Subcutaneous tumours are measured using callipers on three orthogonal dimensions. Tumour volume plotted against time usually produces a curve displaying an exponential relationship. Exponential growth occurs if the rates of cell production and cell loss or death are proportional to the number of cells present in the population (N). The differential equation (1) describes tumour growth mathematically

\[
\frac{dN}{dt} = (K_p - K_L) N
\]  

(1)

where \(K_p\) and \(K_L\) are rate constants for cell production and loss. Integration of this equation (2) gives a time scale of increase in cell population between time 0 and t.

\[
N = N_0 \exp\left( (K_p - K_L) t \right)
\]  

(2)

Tumour volume is also related to time in an exponential manner. Exponential growth implies that the time for a tumour to double is constant thus the doubling time \(T_D\) for the tumour volume is when \(N = 2N_0\) at time \(t = T_D\) thus from (2)

\[
2N_0 = N_0 \exp\left( (K_p - K_L) T_D \right)
\]  

(3)

and (3) can be rearranged so that doubling time is directly related to cell production and loss.

\[
\left( 0.693 \right) \left( \frac{0.693}{K_p - K_L} \right) = \frac{\log 2}{K_p - K_L} = T_D
\]  

(4)
From this assumption the growth equation below using tumour volumes can be derived

\[ V = V_0 \exp \left( -\frac{0.693 t}{T_D} \right) \]  

(5)

where tumour volume (\( V \)) at time \( t \) is related to the initial tumour volume (\( V_0 \)) and the tumour doubling time (\( T_D \)).

1.2.2.2 Growth Fraction

The growth fraction in a tumour population is the ratio of proliferating to non-proliferating cells. This can be denoted by the index of proliferation (\( I_p \))

\[ I_p = \frac{P}{P + Q} \]  

(6)

where \( P \) are the proliferating and \( Q \) the quiescent cells in the tumour. If the growth fraction is one and all the cells are proliferating then the cell cycle time will be equal to the population doubling time, \( T_D \). The figure usually quoted is the potential doubling time, \( T_{pot} \), defined as the doubling time taking into account dividing and non-dividing cells in the absence of cell loss. Within a tumour the growth fraction will vary, being higher close to the blood vessels and lower near the necrotic areas.

1.2.2.3 Age Distribution

Experimental measurements of the duration of cell cycle phases often use the mitotic or labelling indexes. The mitotic index, MI, is the proportion of cells in a tissue which are in mitosis at any given time, where \( T_M \) is time in mitosis and \( T_C \) is cell cycle time.

\[ MI = \frac{T_M}{T_C} \]  

(7)

Using an S phase marker the proportion of labelled cells within a tissue at
an interval after injection can be quantified, where $T_s$ is time in S phase.

$$LI = \frac{T_s}{T_c}$$ (8)

These equations both assume that the cell population is in a steady state. In an exponentially growing population of cells, with no cell loss, each mitosis leads to two G1 cells. The number of G1 cells in the population will be double that of the mitotic cells so the age distribution will be biased (Figure 1.6a). If a tissue had a steady state with random loss from each cell cycle phase then the distribution would be as in Figure 1.6b.

![Figure 1.6](image)

**Figure 1.6** Age distribution of cells (a) Exponential cell population, no cell loss (b) Steady-state cell population, random cell loss.

In real terms the situation is usually between these two extremes so a constant, $\lambda$, which is dependent on the nature of the cell population is used.

$$LI = \lambda \frac{T_s}{T_c}$$ (9)
The labelling index can also be calculated using $T_{pot}$, the potential doubling time, but it will be lower because of the non-proliferating cells ($T_{c}$ is usually less than $T_{pot}$).

1.2.2.4 $T_{pot}$: measurement and clinical relevance

$T_{pot}$ can now be readily measured in patients due to the advent of the halogenated pyrimidines, bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IUDR). Initially used in the clinic as radiosensitizers, they are thymidine analogues which are incorporated into DNA during synthesis, thus act as S phase markers. Monoclonal antibodies produced against BrdUrd and IUDR (Gratzner 1982) led to development of a dual flow cytometry technique simultaneously measuring BrdUrd uptake and DNA content (Dolbeare et al. 1983). This in turn enabled measurement of labelling index and duration of S phase ($T_{s}$) to be calculated from a single observation, therefore allowing calculation of the potential doubling time ($T_{pot}$) of the cell population (Begg et al. 1985a). BrdUrd is administered intravenously to the patient, usually as 200mg in 20ml saline over 5 minutes this acts as a pulse label due to a short half-life. A tumour biopsy or resection is taken several hours later; the exact time being dependent on the tumour type (Wilson 1993).

Several methods have now been developed to calculate $T_{pot}$ since the original one by Begg and colleagues (White & Meistrich 1986; White et al. 1990; Terry et al. 1991) due to the inherent assumptions in the technique. The Begg method assumed that the distribution of BrdUrd labelled cells immediately after labelling was uniform throughout the S phase, therefore the mean DNA content would be in the middle of S phase. "Relative Movement" (RM) described this population of cells, calculated by dividing the DNA profile into G1, S and G2 populations and finding their mean relative fluorescence.

$$RM = \frac{x_{BrdUrd} - x_{G1}}{x_{G2} - x_{G1}}$$  \hspace{1cm} (10)
RM at time zero would be 0.5 and RM reaches 1.0 when all the BrdUrd labelled cells have entered G2 or divided providing a time scale RMt. The next assumption is that progression through S phase is linear, thus cells in early S when labelled must have transversed the whole of S phase when RM is equal to one, so $T_s$ may be calculated from a single observation

$$T_s = \frac{1.0 - 0.5}{RM - 0.5} \times t$$  \hspace{1cm} (11)$$

where $t$ is time between injection and biopsy and RMt is the relative movement at that time.

To obtain a labelling index for $T_{pot}$ calculation, a correction is required to account for cell division, thus the number of BrdUrd labelled cells in G1 at the time of biopsy is halved and subtracted from the total number of labelled cells and total cell number. Once this is accomplished the $T_{pot}$ can be calculated as below

$$LI = \lambda \frac{T_s}{T_{pot}}$$  \hspace{1cm} (12)$$

where $\lambda$ is a function of the age distribution of the cell population, which can vary between 0.693 and 1.38 as described by Steel (1977). Different groups may use alternative constant values of $\lambda$ but results can still be compared as only the absolute values are changed, the ranking is still the same. Use of the original or subsequent methods of measurement of $T_{pot}$ has yet to be standardised, meanwhile both values can be calculated as they are derived from the same primary data.

The use of $T_{pot}$ as a prognostic parameter or predictive test to determine the optimal therapy regime is currently being investigated in clinical trials. Radiotherapy will perturb the proliferation of tumour cells and $T_{pot}$ may indicate potential for proliferation during radiotherapy. An advanced head and neck EORTC trial is comparing accelerated fractionation versus conventional radiotherapy with incorporation of IUdR (Begg et al. 1990; 1992). A trend for tumours with short $T_{pot}$ values (less
than 4.6 days) to do worse with conventional radiotherapy is indicated, whereas there is no discernable effect with the accelerated radiotherapy, in the first evaluable 60 patients. In trials using CHART (continuous, hyperfractionated, accelerated radiation treatment) there was no significant difference in this accelerated regime, although there was a trend for slower proliferating tumours \( (T_{pot} \text{ above 4.3 days}) \) to do worse as measured by local recurrence (Lochrin et al. 1992). An ongoing randomised trial of conventional radiotherapy versus CHART will provide further data on the prognostic value of \( T_{pot} \).

1.2.2.5 Cell loss

In any tumour there is evidence of cell loss from extensive necrosis within the tumour, the ability of tumours to shed cells and form metastases, and cell death via apoptosis. Cell loss (\( \theta \)) can be quantified as

\[
\theta = 1 - \frac{T_{pot}}{T_D}
\]

(13)

In normal tissues cell loss is 100% indicating a steady state of neither growth nor regression. Cell loss in animal tumours can be from 0 to over 90%, tending to be small in small tumours and increase with increasing tumour size. There is also a trend for cell loss to be large in carcinomas and smaller in sarcomas. In human tumours the cell loss tends to be greater than 50%.

With respect to irradiated tumours there are three categories of cell loss, by normal untreated tumour processes, by direct cytotoxic action and by indirect action of radiation related to tissue injury and irritation. In the normal progression of a tumour, cells are lost as they are pushed further from the vasculature away from required nutrients towards necrotic areas where cell proliferation has exceeded stromal growth (Cooper 1973). Cells may also be lost by metastasis or exfoliation if a tumour is adjacent to a body cavity. Individual cells may die from apoptosis (programmed cell death) a regulatory function controlling the overall population size (Sarraf
Apoptosis may be induced by ionizing radiation in a possible linkage with p53. p53 monitors the integrity of DNA prior to division and arrests the cell in G2 until repairs are made, inhibiting replication. Radiation damage of DNA causes accumulation of p53, blocks DNA replication to allow restoration of complete DNA, and if repair fails, p53 may trigger cell suicide by apoptosis (Lane 1992).

Mitotic-linked cell death is the most common form of direct cell kill in tumours from irradiation (Denekamp 1986). When tumours are irradiated, proliferating cells arrest at the G1/S boundary but primarily the G2/M boundary (Wilson et al. 1994). On release a higher proportion of chromosome aberrations are present leading to cell death (Kovacs et al. 1976).

In tissue damaged by radiation there is an acute inflammatory response. Phagocytic inflammatory cells such as granulocytes and mononuclear phagocytes resorb a large proportion of directly killed cells. Activated inflammatory cells such as macrophages and associated humoral mediators such as tumour necrosis factor and activated oxygen may have an additional cytotoxic effect (Brammer & Jung 1993).

Cell loss is an important parameter when discussing proliferation, as increased proliferation will only become significant if cell loss remains constant or decreases. Treatment will theoretically increase cell loss and therefore reduce the size of a tumour mass.

This concludes the relevant details of proliferation and tumour growth. The second important factor in the biological response of tumours to radiotherapy examined in these studies is tumour oxygenation. This involves both identification and subsequent quantification of tumour hypoxia and one method of killing this subpopulation of cells. The method used to selectively target hypoxic cells and cause cytotoxicity in these studies are the bioreductive drugs, mitomycin C and porfiromycin.
1.3 TUMOUR OXYGENATION

1.3.1 Influence of Tumour Oxygenation on Therapy

The effect of hypoxia on the radiation response of tumour cells can readily be shown by survival curves of cells irradiated in air and in nitrogen (Figure 1.7). The cells irradiated under hypoxic conditions exhibit radioresistance relative to well oxygenated cells. This would mean that a greater number of hypoxic tumour cells would survive a specific dose of radiation than better oxygenated cells in the same tumour, thus reducing therapeutic benefit. This is defined as the oxygen enhancement effect.

![Figure 1.7 Survival curve of mammalian cells in hypoxic and oxic conditions.](image-url)
In radiobiological terms the difference in cell survival between oxic and hypoxic cells is measured by the oxygen enhancement ratio (OER). It is the ratio of radiation dose in hypoxia to the dose in air that causes the same biological effect. The full OER for many cell types with x-rays is approximately 3. The OER is directly related to the oxygen status of cells. As the partial pressure of oxygen is increased up to 20mm Hg there is a steep increase in radiosensitivity, and thus OER, which then rises only slightly up to the oxygen concentration in air (155mm Hg). The partial pressures of oxygen in the blood system range from about 40 to 100mm Hg so are located on the gradual slope of the curve (Figure 1.8).

![Graph showing the relationship between OER and partial pressure of oxygen.](image)

**Figure 1.8** Relationship between OER and partial pressure of oxygen.

The enhancement of radiation damage by oxygen is described by the oxygen fixation hypothesis. Free radicals (R•), produced either directly in DNA by radiation, or indirectly by reaction of DNA with hydroxyl radicals formed by the radiolysis of water, may react in the presence of oxygen to produce RO₂• and can subsequently become chemically fixed to species such as ROOH. In the absence of oxygen, or in the presence of a reducing species e.g. RSH or glutathione, the free radical R• may react to form RH restoring its original status.
1.3.2 Measurement of Tumour Hypoxia

The importance of hypoxia in tumours as a determinant of tumour response to radiotherapy has led to use of a variety of methods to quantify hypoxia. The ideal method would be quick, non-invasive and reliable, but current methods do not match these criteria.

The effect of hypoxic regions in tumours can be demonstrated in murine models by irradiating tumours in air-breathing mice and nitrogen asphyxiated mice, which should be fully hypoxic (Chapman 1984; Moulder & Rockwell 1984). Survival curves tend to exhibit large differentials between oxic and hypoxic conditions (Figure 1.9).

![Figure 1.9 Survival curve of murine CaNT tumours irradiated under oxic and hypoxic conditions (R.J. Hodgkiss, unpublished data).](image-url)
In air-breathing mice the curve is theoretically biphasic, as at low radiation doses a large proportion of aerobic cells are killed, but as the dose increases the curve becomes dominated by the remaining resistant hypoxic cells, so will parallel the hypoxic curve. The hypoxic fraction can be calculated from the two curves once they are parallel and is related to the vertical separation between them by the equation

$$\text{Hypoxic fraction} = \exp (\ln \text{aerobic SF} - \ln \text{hypoxic SF})$$

where SF = surviving fraction.

The hypoxic fractions of experimental tumours range from below 1% to over 90% (Moulder & Rockwell 1984).

Each technique for measuring radiobiological hypoxic fractions in experimental animal models involves biological and mathematical assumptions which may compromise the validity of the calculated hypoxic fractions. Different methods of calculation may provide different values for the same tumours. The three methods for experimental systems are described below.

1. The paired survival curve method which compares surviving fractions of disaggregated tumour cells in a colony forming assay from animals irradiated under normal aeration and acute hypoxia (Figure 1.9).

2. The clamped tumour control dose (TCD\textsubscript{50}) assay which compares the radiation doses necessary to control tumours irradiated under normal aeration and acute hypoxia (clamped).

3. The clamped tumour growth delay assay which compares the growth of tumours which were clamped or unclamped at the time of irradiation.

The assumptions which accompany these experimental systems are as follows:-

1. Survival curves for naturally and artificially hypoxic cells have exponential regions at large radiation doses and these curves have the same slope and intercept.

2. Induction of artificial hypoxia produces complete hypoxia and no regions of radiobiological oxygenation persist.
3. The viability of unirradiated hypoxic cells is the same as normally aerated cells. This can be checked by the plating efficiency of paired survival curves.

4. All cells are fully oxygenated or fully hypoxic, although a more likely situation is a continuous distribution of oxygen tensions and therefore a range of radiosensitivities.

Problems inherent in the systems are if a significant proportion of the tumour cells are of intermediate oxygen tensions, and thus sensitivities, the calculated hypoxic fractions will be misleading. In addition, in an excision assay, a higher hypoxic fraction could be produced by excision rescuing cells that would have died in situ. Excision assays also assume complete disaggregation of tumour samples so that no artificial selection of oxic or hypoxic cells occurs. There are obviously many factors which have to be taken into consideration when using experimental models for hypoxic fraction measurements.

A number of methods have been used to assess hypoxia in the clinic. A useful method is to assess the vascularity of tumours, as chronic hypoxia is directly related to distance from the blood vessel. Estimation of tumour vascularity can be achieved by measuring the intercapillary distances from biopsy specimens (Awwad et al. 1986) or the vascular density (Siracka et al. 1988; Revesz et al. 1989) and these figures have been shown to predict treatment outcome. Breast tissue vascular densities have shown the reverse correlation i.e. dense vasculature and poor prognosis (Fox et al. 1993).

One of the most useful techniques now available is the oxygen electrode, as measurements can be made in situ. Large numbers of measurements are made on one tumour so that an indication of the heterogeneity of the tumour oxygen status is gained. This method is only practical on tumours which are easily accessible to the electrode. The electrode samples a volume of tumour rather than individual cells, due to the 300μm diameter of the microelectrode, and all the data is processed by computer. Measurements of oxygenation status can be taken before, during and after treatment so that the oxygen tensions can be related to therapeutic outcome (Gatenby et al. 1988; Vaupel et al. 1991; Höckel et al.)
It is very important to obtain a convenient and rapid measurement of tumour hypoxia so that patients whose tumours exhibit significant hypoxia can be identified and treated more appropriately. The work of Gatenby and colleagues showed the significance of hypoxia in radiotherapy in squamous cell carcinoma metastases by using an oxygen electrode (1988). Treatment outcome was classified as complete responders (CR), partial responders (PR) and non-responders (NR). In the CR group mean \( \text{pO}_2 \) was 20.6±4.4 mm Hg whereas it was only 4.7±3.0 mm Hg in the NR group, which was highly significant. Preliminary results in 31 patients with cancer of the uterine cervix, treated with primary radiotherapy or irradiation as part of multimodality therapy, also showed significantly lower survival and recurrence-free survival for patients with a median \( \text{pO}_2 \) of ≤10 mm Hg compared to those with better oxygenated (median \( \text{pO}_2 > 10 \) mm Hg) tumours (Höckel et al. 1993). Patients with low oxygen measurements could have their treatment modified to try and combat these resistant hypoxic cells and thus improve their prognosis.

A promising method under development to study hypoxia is to utilise the binding of oxygen-sensitive metabolites of nitro aromatic compounds within cells at low oxygen tensions. Reductive metabolism has mainly centred on compounds such as nitroimidazoles (Chapman et al. 1981; Raleigh et al. 1985), nitrofurans (Olive & Durand 1983) and other nitroaromatics (Begg et al. 1983; 1985b; Olive 1984; Hodgkiss et al. 1991a). Detection of these markers has primarily been by isotopes, with \(^{18}\text{F}\) for positron emission tomography (Rasey et al. 1989), \(^{19}\text{F}\) for nuclear magnetic resonance spectroscopy (MRS) (Raleigh et al. 1986; Maxwell et al. 1989) or autoradiography of tumour sections. \(^{3}\text{H}\)-Misonidazole has already been administered to patients with various tumours to mark hypoxic regions (Urtasun et al. 1986). A less time consuming method is to locate the marker immunologically and quantify by histological or flow cytometric analysis (Raleigh et al. 1987; Hodgkiss et al. 1991b). This method can be used to both quantify hypoxia rapidly (flow cytometry) and display the hypoxic regions.
with respect to tumour architecture (histology).

Other methods either in development or as future possibilities for investigating hypoxia include MRS, electron paramagnetic resonance, luminescence quenching and intrinsic hypoxic markers (for review see Hodgkiss & Wardman 1992).

1.3.3 Modification of Therapy to Counteract Hypoxia

There are two directions therapy has taken towards overcoming the radioresistance conferred on tumours by hypoxia. One is to provide more oxygen to the tumour thus reducing hypoxia physiologically. The alternative approach is to specifically target the hypoxic cells, exploiting the reduced oxygen environment and possibly even increasing hypoxia to obtain a greater effect with this approach.

The blood flow, and therefore oxygen supply, to the tumour has been demonstrated to increase by using angiotensin II, noradrenaline, calcium antagonists, anaesthetics, hyperthermia and nicotinamide but these agents, except nicotinamide, have also proved to decrease blood flow under different conditions (Reviewed by Horsman 1993). Another technique to improve tumour oxygenation is to increase the oxygen carrying capacity of blood. This can be achieved by oxygen or carbogen breathing under normobaric or hyperbaric conditions (Dische 1985; Kjellen et al. 1991), or by using perfluorocapchemical emulsions (Rockwell 1985). Alternatively, the affinity of haemoglobin for oxygen can be modified to increase the ease with which haemoglobin releases oxygen (Siemann et al. 1989). These methods all target the chronically hypoxic population of cells. The leading compound to combat acutely hypoxic cells is nicotinamide which is thought to reduce transient fluctuations in tumour blood flow which would normally cause acute hypoxia (Chaplin et al. 1990; Horsman et al. 1990a).

Combination of agents against acute and chronically hypoxic cells with radiation has shown a therapeutic benefit in experimental systems. These include radiation with oxygen/carbogen and nicotinamide, heat and nicotinamide and fluosol DA/carbogen/nicotinamide (Kjellen et al. 1991;
Horsman et al. 1990b; Chaplin et al. 1991). These combination regimes are now being developed in a variety of clinical trials for different solid tumours where the aim is to increase tumour oxygenation during radiation therapy.

1.3.4 Exploitation of Hypoxia in Therapy

Specific targeting to deplete the hypoxic cells has primarily utilised bioreductive drugs, although hyperthermia is also known to be more cytotoxic to hypoxic cells. Hyperthermia is selective for chronic hypoxia as it is the lowered pH and nutrient deficiency within the cell which causes sensitization rather than the lack of oxygen. Bioreductive drugs are metabolised in any cell with a reduced oxygen tension so are cytotoxic to both acutely and chronically hypoxic cells.

This alternative approach to exploit hypoxia within tumours can be enhanced by a number of methods, generally in reverse of the previous techniques to improve tumour oxygenation. The oxygen-carrying capacity of haemoglobin can be reduced by carbon monoxide breathing (Grau et al. 1992) or by addition of a drug such as BW12C which increases the affinity of oxygen binding to haemoglobin so that it is less likely to release the oxygen (Adams et al. 1986). There are also agents which decrease physiological tumour blood flow (e.g. hydralazine (Chaplin & Acker 1987), 5-hydroxytryptamine (Knapp et al. 1985) and glucose (Hiraoka & Hahn 1990)) or permanently damage vasculature (e.g. flavone acetic acid (Evelhoch et al. 1988), tumour necrosis factor (Kallinowski et al. 1989), photodynamic therapy (Star et al. 1986) and hyperthermia (Song 1984)). The former will have an effect of only minutes to hours whereas the latter group has a more prolonged effect of several days.

Once hypoxia has been induced by the above agents, therapy can be improved by addition of bioreductive drugs or applying hyperthermia to increase toxicity within the tumour. Bioreductive drugs have been combined with hydralazine (Chaplin & Acker 1987), 5-hydroxytryptamine (Chaplin 1986), tumour necrosis factor, flavone acetic acid (Sun & Brown
1989), photodynamic therapy and heat to increase the antitumour effects in experimental systems. This method of exploitation of hypoxia is also about to undergo clinical trials for a number of different tumours.

Although clinical trials including agents targeted against hypoxia in radiotherapy regimes are an encouraging acknowledgement of the hypoxia problem, one of the criteria for trial entry should be evidence of hypoxic cells so that the population of patients that may benefit can be identified. The availability of a method for stratifying patients on the basis of their tumour oxygenation would also facilitate statistical analysis of clinical trials where hypoxia may influence treatment outcome.

1.4 BIOREDUCTIVE DRUGS

Bioreductive drugs are a relatively new phenomenon in cancer therapy, although drugs now recognised to be in this class have been used for many years, both experimentally and in the clinic. The field of bioreductive drugs originated from investigation of radiosensitizers which would increase the sensitivity of the hypoxic portion of the tumour towards that of the better oxygenated cells (Lin et al. 1972). It was observed that some of these radiosensitizers were differentially cytotoxic to the hypoxic cells and thus developments were taken a stage further and drugs specifically targeted against these hypoxic cells were produced. An oxic:hypoxic differential in drug toxicity was first observed in multicellular spheroids where metronidazole was more toxic towards central non-cycling cells compared with outer cycling cells of the spheroid (Sutherland 1974). Over the last twenty years interest in drugs which exhibit an oxic:hypoxic differential has grown due to the growing evidence that hypoxia is a potent predictor of radiotherapy outcome.

There are three main classes of bioreductive drugs that may be of clinical use; benztriazene-di-N-oxides (e.g. SR 4233), dual function alkylating nitroimidazoles (e.g. RSU 1069 and its prodrug RB 6145), and quinones (e.g. mitomycin C). The activity of a bioreductive drug is
dependent on its redox potential, so that the higher the redox potential, the more toxic it will be for both hypoxic and oxic conditions (Adams et al. 1980). The drugs require one or two electron reduction to produce toxic metabolites, as shown below for a nitro-containing compound.

\[
\begin{align*}
RNO_2 & \xrightarrow{O_2} RNO_2^* \xrightarrow{O_2} \text{CYTOTOXIC METABOLITES}
\end{align*}
\]

The equilibrium of the reaction will be affected by drug concentration, redox potential and cellular oxygen tension (Stratford 1992). Progression of each of these reaction steps requires a variety of reductive enzymes. Different drugs utilise different enzymes. Thus enzymology of the tumour is also a variable which needs to be taken into account when assessing the activity of a bioreductive drug. Compounds from each of the three classes are now in clinical trial in combination with radiation. However this thesis has focused on the quinone group.

1.4.1 Bioreductive Quinones - Mitomycin C

The prototype bioreductive quinone is the naturally occurring antitumour antibiotic mitomycin C. It was first isolated in 1958 from *Streptomyces caespitosus* (Crooke & Bradner 1976). It is closely related to mitomycins A and B, but mitomycin C has the greatest activity against tumours. Mitomycin C occurs as blue-violet crystals which dissolve in water to produce a purple solution. In solution it is inactivated by visible light, but not ultraviolet (Powis 1987). The structures of mitomycin C and its N-methyl aziridyl analogue porfiromycin are shown below (Figure 1.10).

![Structure of two bioreductive quinones](Figure 1.10)
Mitomycin C has been used since the 1960's in the clinic, but it is only recently that it has been exploited specifically for its bioreductive qualities rather than for its general cytotoxicity (Doroshow 1992). Mitomycin C is an important component in the chemotherapeutic approach to treatment of cancers of the lung, breast, bladder and gastrointestinal tract (Crooke & Bradner 1976). New trials involve mitomycin C, mainly in combination with radiation, to selectively kill the hypoxic cells remaining after a dose of radiation, or as an additional drug in chemotherapeutic combinations. Mitomycin C has only a modest oxic:hypoxic differential so a variety of structurally related compounds, such as porfiromycin and EO9, are also being incorporated into clinical trials as they have a greater oxic:hypoxic differential and reduced toxicity to normal tissues.

1.4.1.1 *Mitomycin C: Mechanism of action and enzymology*

Quinones can be reduced to form semi-quinones, hydroquinones or both by cellular enzyme systems. Quinone reductases include DT-diaphorase, NADPH:cytochrome P-450 reductase, NADH dehydrogenase and xanthine oxidase (Butler & Hoey 1987). Quinones can be reduced by one or two electron reduction, depending upon the enzymes present and the oxygen availability. Aerobic reduction produces toxicity from one electron reduction by NADPH:cytochrome P-450 reductase as it forms the semi-quinone free radical leading to production of toxic oxygen species by auto-oxidation in a futile cycle (Workman et al. 1989). Further one electron reduction of the semi-quinone or straightforward two electron reduction of quinones by DT-diaphorase leads to more stable hydroquinones, which can then be detoxified via conversion to glucuronide and sulphate conjugates ready for excretion (Figure 1.11). Reduction by the obligate two electron donor DT-diaphorase avoids the toxic semi-quinone radicals and thus DT-diaphorase probably has a role in the cellular defence against oxidative stress (Ernster 1987). This has been confirmed as inhibition of DT-diaphorase by dicoumarol leads to increased toxicity from oxygen radical species.
Mitomycin C is teratogenic, carcinogenic, and becomes cytotoxic on metabolism by reductive enzymes; acting as an alkylating agent. One electron reduction is sufficient to activate mitomycin C, but cytotoxic species also result from two electron reduction (Andrews et al. 1986). Mitomycin C has several potential biologically active groups, the C-1, C-2 aziridine ring, the C-10 carbamate group and the quinone moiety. It forms mono- and bi-functional DNA adducts and crosslinks (Iyer & Szybalski 1963; Szybalski & Iyer 1964; Tomasz & Lipman 1981), and at higher doses mitomycin C blocks DNA synthesis (Matsumoto & Lark 1963). The aziridine ring and C-10 carbamate are where crosslinks may result from their displacement by nucleophilic groups in opposite strands of DNA. Both in vitro and in vivo crosslinking increases with increasing guanine and cytosine content of DNA (Iyer & Szybalski 1964). Activation is rapid and intermediates are unstable and therefore DNA must be present during reduction. The site of covalent bonds is probably the O6 position of guanine, as this could occur without distortion of the double helix (Tomasz et al. 1974). Production of specific crosslinks and adducts is dependent on whether oxygen is present, as different species are produced under hypoxic conditions. Mitomycin C induces chromosome aberrations, requiring passage through S phase where chromosome breaks are produced from
cells reaching mitosis without fully completing DNA replication. A series of cell lines has been developed displaying a spectrum of reactivity to mitomycin C showing the importance of DNA excision repair in reversing mitomycin C toxicity. From least to most resistant the Chinese hamster ovary cells are (i) proficient in mitomycin C bioreduction and deficient in DNA excision repair; (ii) partially deficient in mitomycin C bioreduction and deficient in repair; (iii) bioreduction and repair proficient and; (iv) bioreduction deficient and repair proficient (Dulhanty et al. 1989).

Mitomycin C damage results in a reduction in the rate of DNA replication and a dose dependent delay in cell cycle progression (Sognier & Hittelman 1986). Transient delays in S phase and a G2 delay becomes a G2 block (possibly combined with a G1 block) with increased exposure and concentration of mitomycin C (Barlogie & Drewinko 1980).

DT-diaphorase (NADPH: quinone oxidoreductase, E.C. 1.6.99.2) is predominantly a cytosolic enzyme consisting of two equal sized subunits and two molecules of FAD. Humans have multiple forms of the enzyme, so it is quite possible that these isoenzymes have different activities, some may bioactivate and others may detoxify mitomycin C. There is a correlation between enzyme content, including DT-diaphorase activity and cytotoxicity of mitomycin C in EMT6 tumour cells. Use of HT-29 and BE human carcinoma cells with high and low DT-diaphorase activity respectively, showed that two electron reduction by DT-diaphorase of mitomycin C caused a greater cytotoxicity in HT-29 cells (Siegel et al. 1990). A selection of 10 colon cancer cell lines, including HT-29 and BE, showed large variations in DT-diaphorase gene expression, also demonstrating gene expression directly reflected enzyme activity, except in the BE cells which displayed a mutation in the DT-diaphorase gene (Traver et al. 1992). Mitomycin C resistant CHO cell lines were found to be deficient in DT-diaphorase (Dulhanty & Whitmore 1991) but this only affected cytotoxicity under aerobic, not hypoxic, conditions. This finding was confirmed in non-transformed human skin fibroblasts from a cancer prone family, whose cells were deficient in DT-diaphorase (Marshall et al. 1991). Conversely, it
was found that "quinone resistant" cell lines L5178Y/HBM2 and L5178Y/HVBM10 had increased DT-diaphorase activity compared to the parental line L5178Y with a concomitant increase in mitomycin C cytotoxicity (Begleiter et al. 1989). A criticism of much of the work examining DT-diaphorase activity is the use of dicoumarol as an inhibitor, as it is not particularly specific. Dicoumarol inhibits other related enzymes and may leave some enzymes active that can metabolise mitomycin C. Thus no precise conclusions can be made using dicoumarol as a DT-diaphorase inhibitor. Nevertheless, in hypoxic cells dicoumarol has been shown to enhance mitomycin C cytotoxicity and decrease aerobic cytotoxicity (Keyes et al. 1985a) so that it is now being combined with mitomycin C and radiation therapy in a clinical trial (Sartorelli et al. 1993).

NADPH:cytochrome P-450 reductase is membrane bound and for optimal activity with mitomycin C requires NADPH, phospholipid, cytochrome P-450 and hypoxia (Kennedy et al. 1982) (Figure 1.12).

\[
\text{NADPH} \rightarrow \text{FLAVOPROTEIN (ox)} \rightarrow \text{SEMIQUINONE} \rightarrow O_2 \\
\text{NADP}^+ \rightarrow \text{FLAVOPROTEIN (red)} \rightarrow \text{QUINONE} \rightarrow O_2^-
\]

Figure 1.12 Components of Mitomycin C activation by NADPH: cytochrome c (P-450) reductase.

One electron reduction by this enzyme in the presence of oxygen transfers an electron to oxygen, creating a superoxide, but as mitomycin C metabolism by this pathway is inhibited by oxygen, the toxicity is predominantly from DNA alkylation. NADPH:cytochrome c reductase (NADPH:ferricytochrome oxidoreductase, E.C. 1.6.2.4) is a flavoenzyme which utilises cytochrome P-450 as an electron acceptor in its reaction with mitomycin C, as substitution of cytochrome P-450 with cytochrome C leads to a decrease in the rate of activation and metabolism. Although the
nuclear activity of the reductase is 10% that of the microsomes, it may be crucial to toxicity as reduction occurs in close proximity to the target molecule|DNA (Kennedy et al. 1982).

NADH:cytochrome b5 reductase (E.C. 1.6.2.2) also activates mitomycin C, reduction being 1.5 times greater under hypoxic rather than aerobic conditions (Hodnick & Sartorelli 1993). Dicoumarol inhibits the activity of this enzyme.

Xanthine oxidase (E.C. 1.2.3.2.) acts via single electron reduction and, as NADPH:cytochrome P-450 reductase, activates MMC under anaerobic conditions (Komiyama et al. 1979). Pan et al. demonstrated in hypoxia the formation of a mitomycin semiquinone free radical intermediate and subsequently both mono- and bi-functional alkylating species (Pan et al. 1984; 1986). Aerobically xanthine oxidase and NADPH:cytochrome P-450 reductase generate reactive oxygen species.

Xanthine dehydrogenase (E.C. 1.1.1.204) metabolises mitomycin C, dependent on the presence of NADH, forming alkylating intermediates including 2,7 diaminomitosen under hypoxic and aerobic conditions, but does not generate reactive oxygen species when compared to xanthine oxidase under aerobic conditions (Gustafson & Pritsos 1992a). This indicates that xanthine dehydrogenase can activate via one but preferentially two electron reduction. Dicoumarol potentiates mitomycin C metabolism to 2,7 diaminomitosen, producing three and four fold increases in aerobic and hypoxic depletion (Gustafson & Pritsos 1992b).

Due to several enzyme systems being involved in mitomycin C metabolism, each differing in intracellular distribution, there is probably a large variation in site- and tissue-specific effects of mitomycin C (Gustafson & Pritsos 1992a). Alterations in resistance to mitomycin C may be related to expression of these enzyme systems leading to inactivity of mitomycin C in many human solid tumours. Tumour tissue with low concentrations of enzyme could lead to resistance (Marshall et al. 1991). Marshall has shown that cells derived from an afflicted member of a cancer-prone family are deficient in DT-diaphorase activity and six times more resistant to
mitomycin C and porfiromycin. There was no difference in NADPH:cytochrome c reductase or glutathione content between the cancer-prone derived cells and cells derived from a normal volunteer. It has been hypothesised that this lack of an enzyme system could be a causal effect in why the family is cancer-prone as DT-diaphorase acts in detoxifying many naturally occurring quinones.

It has been suggested that tumours should be "enzyme profiled" before initiating treatment so that the selection of bioreductive drug will depend on whether there are specific enzymes present to metabolise the drug (Riley & Workman 1992). For example, DT-diaphorase tends to have high expression in non-small cell lung cancer cells and lower more varied expression in breast cancer cells. Relevant bioreductives could exploit high DT-diaphorase levels causing greater cytotoxicity (Stratford et al. 1994; Robertson et al. 1994).

1.4.2 Modifying Factors of Bioreduction

The main factor modifying bioreductive metabolism besides type and concentration of intra-cellular reductases within the tissue is oxygen status. It has been shown to influence bioreduction of mitomycin C as the drug is more cytotoxic in hypoxic conditions (Rauth et al. 1983; Kennedy et al. 1980a,b; Bremner et al. 1990). This differential toxicity is a direct result of which enzymes are activated under varying conditions and therefore to which species the drug is activated. This species can either be directly cytotoxic or cause toxicity through further chain reactions, or it may be metabolised to a harmless product. The oxygenated pathway of enzyme metabolism leads to free radical production and thus cell kill from bioreductive drugs can be modified by the levels of superoxide dismutase, glutathione peroxidase, catalase, glutathione-S-transferase, cellular thiols and DT-diaphorase. Potential pathways for the bioactivation of mitomycin C are shown below (Figure 1.13).

A second factor in the enzymatic reduction of drugs is the cellular pH (Andrews et al. 1986). DT-diaphorase metabolism of mitomycin C is markedly pH dependent, detectable at pH 5.8, 6.4, 7.0, 7.4, but not at 7.8
Figure 1.13 Potential pathways for the bioactivation of mitomycin C.

(Siegel et al. 1990). A dose dependent production of DNA crosslinks by mitomycin C has been observed, with a lower pH enhancing DNA-DNA crosslinking, (pH 5.7, 6.4, 7.5), suggesting intracellular and/or extracellular pH is important in aerobic toxicity of EMT6 tumour cells (Kennedy et al. 1985). Both NADPH:cytochrome P-450 and xanthine oxidase are pH dependent, with DNA alkylation increasing as pH increases from 6.5 to 8.5 (Pan et al. 1986). Xanthine dehydrogenase formation of mitomycin intermediates with alkylating ability, increases with increasing hypoxia and a decrease in pH from 7.4 to 6 (Gustafson & Pritsos 1992a). NADH:cytochrome b5 reductase has increased enzymatic reduction at pH 6.6 compared to 7.6 (Hodnick & Sartorelli 1993). These data again illustrate that reduction of mitomycin C will be enzyme dependent as different enzymes function optimally at different environmental pH values.

1.4.3 Porfiromycin: Mechanism of action

Porfiromycin is an analogue of mitomycin C with a methyl group replacing a hydrogen on the aziridine ring (Figure 1.10). Porfiromycin has a similar hypoxic cytotoxicity to mitomycin C but it has a much lower cytotoxicity to aerobic cells; thus a greater oxic:hypoxic differential (Keyes
Porfiromycin generates oxygen radicals to a far lesser extent than mitomycin C and as a result has lower aerobic toxicity (Pritsos & Sartorelli 1986; Gustafson & Pritsos 1991). The rate of uptake of porfiromycin by EMT6 cells is directly correlated with its cytotoxicity under hypoxia and aeration. Uptake of porfiromycin into hypoxic cells is more rapid than uptake into aerobic cells, and hypoxic cells also accumulate porfiromycin in excess of the concentration in the extracellular medium (Keyes et al. 1987). This is probably due to less activation and covalent binding in aerobic cells to produce DNA crosslinks, mono- and bis-adducts (Fracasso & Sartorelli 1986; Keyes et al. 1991a; Sartorelli et al. 1993). Aerobic cells maintain the intra/extracellular equilibrium for porfiromycin, whereas under hypoxic conditions binding of porfiromycin to cellular nucleophiles prevents equilibrium, resulting in evermore higher intracellular concentrations.

Keyes and colleagues found that crosslinking of DNA is a major lesion in the cytodestructive action of the mitomycins regardless of oxygenation (Keyes et al. 1991a), but different lesions were produced by mitomycin C and porfiromycin under aerobic and hypoxic conditions. Neither porfiromycin nor mitomycin C produce single strand breaks (Hughes et al. 1991). The crosslinks can be repaired as cytotoxicity of mitomycin C and porfiromycin is reduced 24 hours after exposure (Fracasso & Sartorelli 1986). A number of adducts have been identified including N²-(2'deoxyguanosyl)-7-methylaminomitosene, a mono functional porfiromycin-guanine adduct linked to C-10 and a porfiromycin crosslinked dinucleotide (porfiromycin crosslinked to two deoxyguanosines at their N² positions) (Pan 1990; Tomasz et al. 1991). The ratio of mono-adducts to crosslinked product differed due to the environment, 1:1 under hypoxic and 2:1 under aerobic conditions (Tomasz et al. 1991). Bioreductive enzymes are also important in porfiromycin metabolism as DT-diaphorase deficient cells were porfiromycin resistant (Marshall et al. 1991). DT-diaphorase metabolism of porfiromycin, like that of mitomycin, is also pH dependent (Siegel et al. 1991).
1.4.4 Use of Mitomycin C and Porfiromycin as Bioreductive Agents

In murine models Rockwell and colleagues have shown an enhancement of effect with combining radiation with either mitomycin C or porfiromycin. As the result of combining these two modalities was supra-additive the increased effectiveness was thought to reflect differential toxicities to different sub-populations rather than radiosensitization (Rockwell et al. 1988). Addition of dicoumarol was shown to protect against aerobic toxicity but increased hypoxic toxicity in tumour cells. However, dicoumarol did not protect well oxygenated marrow cells in vivo, possibly because the DT-diaphorase concentration of marrow was <1% that of the EMT6 tumour cells, even though it showed the same toxic characteristics towards the in vivo tumour (Rockwell et al. 1988).

In vivo the cytotoxic effects of porfiromycin were complete within 1 hour of injection. At 24 hours cell survival increased indicating either a slow repair of potentially lethal damage or loss of porfiromycin killed cells from the tumour (Rockwell et al. 1988). Distribution of porfiromycin in mice reached a peak at 15 minutes, exhibiting higher labelling in the liver and small intestine. In tumours the highest labelling was around necrotic areas i.e. where most bioreduction had taken place (Keyes et al. 1991b).

A difference was shown between the response of young and old mice, the latter having a greater hypoxic fraction. Thus cytotoxicity was greater in old mice whether mitomycin C or porfiromycin was used alone or with radiation (Rockwell et al. 1991; Keyes 1991c). Post irradiation administration of mitomycin C also enhanced cell kill in another murine model (Adams et al. 1992). Combination of mitomycin C or porfiromycin with Fluoso-DA/Carbogen/radiation at 5Gy showed a 2 and 1.6 fold difference in cytotoxicity respectively, which was reduced at 10 and 15Gy (Holden et al. 1990). Addition of mitomycin C to a combined regime of radiation and 5-fluorouracil was shown to be beneficial in the clinic. However murine solid tumour models demonstrated that combinations of single doses of mitomycin C with x-rays was more effective than the same regimen with 5-fluorouracil; thus it was probably the mitomycin C
providing the therapeutic benefit not 5-fluorouracil alone (Rockwell 1993).

Potentiation of the bioreductive effects of mitomycin C have also been produced by inducing hypoxia with hydralazine, IL-1α and tumour necrosis factor (Brown 1987; Bremner et al. 1990; Adams 1989; Braunschweiger et al. 1991; Edwards et al. 1991), but combination with flavone acetic acid showed no improvement specifically in RIF-1 and KHT tumours (Edwards et al. 1991).

In the clinic mitomycin C can be nephrotoxic although the main cause of discontinuing administration is delayed and cumulative myelosuppression, usually thrombocytopenia but also leukopenia where the recovery time is about three weeks (Crooke & Bradner 1976; Baker et al. 1976). Extravasation causes mild and infrequent anorexia, nausea, vomiting and diarrhoea. Infrequent but potentially lethal side effects include haemolytic uremic syndrome, interstitial pneumonitis and cardiac failure (Verweij & Pinedo 1990). Human pharmacokinetic studies have shown wide distribution of mitomycin C to the kidney, tongue, muscle, heart but none to the brain. Tissues from man known to inactivate mitomycin C are the liver, spleen, kidney, brain and heart but excretion is primarily by glomerular filtration and it is cleared from serum by metabolism.

Mitomycin C is not given as a single agent due to only short remission times, but is used in active combinations with proven activity in breast, stomach, colorectal, head and neck and pancreatic carcinomas (Verweij & Pinedo 1990).

Results from one of the few clinical trials where mitomycin C has been added for its bioreductive qualities look promising. Mitomycin C was administered intravenously on the fifth day after irradiation with a $1.8/2.0$ Gy daily dose, 5 days a week, up to a total dose of 56Gy in cancers of the head and neck. Actuarial disease free survival at 5 years was 49% in the radiotherapy alone arm and 75% in the radiotherapy + mitomycin C arm (p<0.07). However local recurrence free survival was 66% for radiotherapy and 87% for radiotherapy + mitomycin C (p<0.02), thus a significant
improvement was found with the addition of mitomycin C (Weissberg et al. 1989). Updating of this trial with larger patient numbers and up to 10 year survival times has shown greater significance for mitomycin C addition with local recurrence free survival (p<0.01) (Haffty et al. 1992). Ongoing clinical trials with the same group have seen the addition of dicoumarol to the protocol, just before mitomycin C administration. Although there are not enough patients recruited to compare survival with and without dicoumarol, addition of dicoumarol has not been detrimental to disease free survival rates. The efficacy of the treatment is being examined further by the use of porfiromycin with its greater oxichypoxic differential. Phase I, dose escalation trials are underway with regard to comparison of addition of mitomycin or porfiromycin to the regime (Fischer et al. 1993).

Use of radiation treatment with the addition of mitomycin C and 5-fluorouracil for anal canal cancer produced a 5 year cause specific survival of radiation alone 68%, radiation and 5-fluorouracil 64% and radiation, 5-fluorouracil, mitomycin C 76%, with significance differences between radiation and radiation/5-fluorouracil/mitomycin C (p<0.14) and radiation/5-fluorouracil and radiation/5-fluorouracil/mitomycin C (p<0.02) (Cummings & Keane 1993). At the same centre, head and neck cancers showed no difference in survival with the addition of the two drugs but a European group showed evidence of slight improvement where a different radiation regime was used (Dobrowsky et al. 1993).

Another analogue of mitomycin C, BMY-42355, has been shown to have a greater oxichypoxic differential than either porfiromycin or mitomycin C when used alone or combined with radiotherapy. It is also less toxic than porfiromycin with LD50 values in mice of mitomycin C 8.1μg/g, porfiromycin 43μg/g and BMY-42355 54μg/g; therefore a larger therapeutic ratio is produced (Rockwell et al. 1989). This may also be introduced into clinical trials in the future. The indoloquinone, EO9, is also entering clinical trials in Europe as a bioreductive drug (Stratford et al. 1994).
The future of bioreductives in clinical trials looks promising, but inclusion of bioreductives needs to be on a firm prospective, randomised basis so that results can be properly assessed with regard to improved outcome.

1.5 EXPERIMENTAL TUMOUR MODELS

In order to assess the response of different tumour cell populations to bioreductive drugs four tumour models were used. A model tumour system has to fit a number of criteria. Paramount is that the chosen system will have the ability to provide the answers required by the investigator. This dictates which system is best suited for the question. A number of systems are available with varying degrees of complexity, each with inherent advantages and disadvantages when extrapolating data to human tumours. The tumour models in these studies consisted of single cells, in vitro spheroids, in vivo peritoneal spheroids and subcutaneous tumours derived from the same tumour cell line.

1.5.1 Transplantable Tumours

Murine transplantable tumours are primarily obtained from spontaneously arising or chemically-induced tumours which can then be excised and transplanted, usually subcutaneously, on the backs of syngeneic mice. After a latent period the tumour appears and all should have comparative histology and biological characteristics. As one donor tumour can be transplanted to many recipients large quantitative studies of radiotherapy or chemotherapy regimes can be tested. Advantages of subcutaneous, dorsal tumours are their accessibility and their progress can be monitored visually unlike internal tumour models. Major tests developed using transplantable tumours include tumour growth measurements, tumour cure rate, in vivo toxicity testing and in vivo/in vitro colony forming assays. A major drawback in mouse models is the accuracy with which mouse data can be extrapolated to humans. There is some
controversy as to whether spontaneously arising tumours are a better model than repeatedly transplanted tumours, but these spontaneous tumours are not as readily available so large scale experiments would not be feasible. The other alternative is to use transgenic mice where oncogenes have been injected into mouse embryos and the resulting mice will eventually develop tumours. These tumours occur reliably and are usually restricted to one or a few specific tissues (Kingsman & Kingsman 1988).

The study of Rockwell and Moulder (1990) compared rapidly growing transplanted tumours to xenografts of human tumours in the estimation of radiobiological hypoxic fraction. There was no significant systematic difference in oxygenation between a range of murine tumours and human xenografts. The transplantation history (i.e. number of passages) of the murine tumours was also compared and this had no effect on hypoxic fraction (Rockwell & Moulder 1990).

1.5.2 Human Xenografts

Human xenografts in immune-deficient mice are thought by some to be a more authentic model, in comparison with transplantable murine tumours, for human tumours. They retain human karyotypes through serial passages and maintain some of the responses of the original human tumour from which they were derived. A further advantage is that xenografts tend to keep the chemotherapeutic and radiation response characteristics of the tumour class from which they are derived. Disadvantages of this model are the immune reactions by the host so that sometimes the tumour is rejected thus measurements using local tumour control as an endpoint may not be objective. Human tumour cells may also undergo kinetic changes and cell selection when transplanted into mice, as tumour volume doubling times are shorter in mice than humans. Histological characteristics of the tumour may be maintained but the stromal tissue and vasculature is of murine origin, thus for vascular based therapies xenografts are only as valid as established murine transplants (Rofstad 1989).
Again, comparisons of the percentages of hypoxic cells within different human tumour xenografts and rodent tumours showed there was a wide range, from <1 to 90%, in both human and rodent tumours (Guichard 1989).

1.5.3 Cell Lines

Cells are the easiest system to use and manipulate experimentally but there is a lack of interaction between cells which would be found in a solid tumour. Cellular heterogeneity exhibited in a tumour is also lost, as are the pharmacokinetics of drug delivery. Thus monolayers and cells in suspension can not be representative of a tumour system responding to drugs or radiation although trends in response may be highlighted for further investigation. A "sandwich technique" using monolayer culture has been developed, where diffusion of nutrients is limited and gradients in cellular proliferation are generated so this produces a more realistic tumour environment (Hlatky & Alpen 1985). An advantage of cell lines are that they are relatively easy to transfer from one laboratory to another so are a good system for scientists to compare results of similar treatments.

1.5.4 In vitro Spheroids

Experimentally induced aggregates of animal cells were used as early as 1944 by Holtfreter (Historical review by Mueller-Klieser 1987). In the 1950's work by Moscona looked at reaggregation, proliferation and differentiation in embryonic and malignant cells, which led to study on the induction and growth of multicellular aggregates in suspension and development of "Moscona's method" of aggregate initiation. Studies on the response of cellular aggregates to radiation were instigated by Sutherland in the 1970's (Sutherland et al. 1970). His development of a spinner method of culture led to production of a large number of spherical aggregates and thus the term "multicellular spheroid" was adopted. The spheroid resembled nodular carcinomas in structure and radiation experiments found similar cell survival curves between in vitro spheroids and solid
tumours (Sutherland et al. 1970; 1971; Inch et al. 1970). Subsequently, multicellular spheroids were considered a good in vitro model for cancer cells in vivo. Further work with spheroids has diversified with studies in radiobiology, drug cytotoxicity, drug penetration and cellular heterogeneity and its effects with a variety of treatment regimes (Sutherland & Durand 1976; Nederman & Twentyman 1984).

Spheroids can be grown from some cell lines by preventing the cells from attaching to the surface of the culture vessel by either agar coating or continuous stirring (Carlsson & Yuhas 1984; Sutherland & Durand 1984). The spheroid develops from one or more cells aggregating, upon cell division the daughter cells do not detach but remain as a clump and continuous division leads to a spherical mass. As the spheroid develops it provides cell to cell contact as well as cellular heterogeneity brought about by the growth of the spheroid outstripping the diffusion limits of nutrients and oxygen. A central core of necrotic tissue surrounded by hypoxic cells recreates the environment of a tumour cord but in reverse. Proliferative diversity is also created in the spheroid as the outer rim contains asynchronous oxygenated cells, proliferation decreases with increasing distance from the surface until cells become hypoxic and non-cycling (Allison et al. 1983; Durand 1990a).

Many human cell lines can be grown as spheroids, although the ability to form spheroids is not a general property of tumour cells. Spheroids can be initiated directly from surgical specimens, consequently treatment of the primary tumour can be mimicked in vitro and patient outcome compared with spheroid radiosensitivity or drug resistance (Jones et al. 1982; Rofstad 1986). Morphological studies show that spheroids maintain many of the characteristics of the original line and some cell lines such as the moderately well-differentiated human colon adenocarcinoma Co112 display signs of differentiation within the spheroid (Sutherland et al. 1986). Techniques that elicit information concerning heterogeneity have developed, including sequential trypsinisation and cell sorting by Hoechst 33342 to divide the cellular environment into individual layers and use of
micro-electrodes to measure pO₂ and nutrient values at increasing depths into the spheroid (Olive et al. 1985; Sutherland et al. 1986). These have enabled studies in drug penetration (Kerr et al. 1988), synergism of combinations of drugs in spheroid subpopulations (Durand 1990b) and sequencing of drugs and radiation (Sutherland 1988). Simpler studies have examined drug toxicity and radiation effects in spheroids compared to monolayers, disclosing a cell-contact effect reducing radiosensitivity in spheroids (Durand & Sutherland 1973; Durand & Olive 1992) and the ability of drugs such as vincristine, cisplatin and adriamycin to inhibit shedding of cells from the exterior of the spheroid. Generation of a hypoxic central core in larger spheroids has led to them being used extensively in radiobiology as a model for the effect of hypoxia on different treatments and in development of improved hypoxic cell sensitizers (Shibamoto et al. 1987). A proviso for work with spheroids is that there are large cell-type dependent variations in growth rate, cell morphology and thickness of viable cell layers causing drugs to have different penetration patterns and sensitivities (Carlsson & Nederman 1989).

The spheroid system is simpler, more reproducible, more economical and easier to manipulate than in vivo tumours. These attributes make it an excellent model for studying treatments in a heterogeneous cell system.

1.5.5 In vivo Spheroids

Lord et al. first injected EMT6 spheroids in the peritoneum of mice to study the infiltration and cytotoxicity of host immune cells to the tumour (1979). Spheroids have since been injected into various sites including the kidney, brain and iliacus muscle as well as the peritoneum of syngeneic mice (Krajewski et al. 1986; Coomber et al. 1988; Lord 1980; Zwi et al. 1990). Krajewski et al. found that tumour pieces grew at the same rate as spheroids once transplanted into the kidney and became vascularised. The vasculature of astrocytoma spheroids transplanted into the brain and iliacus muscle was the same in both transplant sites but significantly different from vessels from the host tissues (Coomber et al.
1988). The Zwi model demonstrated that some EMT6 spheroids injected into the peritoneum became attached to internal tissues and developed vasculature whereas others remained free floating and avascular (Zwi et al. 1990). This raised the interesting possibility that spheroids could be used as a model to study the influence of vascularisation in relation to chemotherapeutic agents. The vascular network of a tumour could be exploited by manipulation either closing down the vessels resulting in greater hypoxia or by using targeted drugs against tumour vessels. Response between \textit{in vitro} spheroids and spheroids exposed to an \textit{in vivo} environment can be compared according to their vascularity.

1.6 AIMS OF THE STUDY
The aims of this thesis were:-

a) To develop spheroids from a panel of Gray Laboratory tumours.

b) To develop a murine model for implanted peritoneal spheroids.

c) To investigate the action of mitomycin C and porfiromycin in SaF cells, spheroids, peritoneal spheroids and subcutaneous dorsal tumours.

d) To relate hypoxia and cell survival after administration of mitomycin C and porfiromycin.

e) To develop a flow cytometry triple staining technique for simultaneous measurement of hypoxia, proliferation and DNA content.

f) To investigate the interactions of proliferation and hypoxia in an \textit{in vivo} tumour model.
CHAPTER 2 MATERIALS AND METHODS

The suppliers of materials used in these studies are listed in Appendix 1.

2.1 CELL LINES AND CULTURE CONDITIONS

2.1.1 Cell Culture Media

Monolayer cultures were grown in Minimum Essential Medium (MEM) with Earle's salts containing 20mM sodium bicarbonate solution, 1.4mM L-glutamine, 100mg streptomycin sulphate, 200,000 units of benzylpenicillin sodium and 10% fetal calf serum. This was modified for suspension cultures (MEMS) by reducing the fetal calf serum content to 7.5% and containing 1mM Mg$^{2+}$ compared to 1.8mM Ca$^{2+}$ and 0.8mM Mg$^{2+}$ in the full medium.

2.1.2 Cell Lines

2.1.2.1 SaF

The SaF is a rapidly growing anaplastic sarcoma that spontaneously arose in a CBA strain mouse in the Gray Laboratory animal colony in 1957. It has a latent period of about 6 days and a volume doubling time of 2.1 days. The tumour cells grow readily in culture, but the plating efficiency can be increased by providing a lethally irradiated feeder layer of Chinese hamster V79-379A cells. The doubling time of the cells in culture is approximately 22 hours.

2.1.2.2 Murine derived tumour cell lines

The origins of the other murine tumour cell lines used in these studies are detailed in Table 3.1. All are maintained at the Gray Laboratory in the on site mouse colony. There are both sarcomas and carcinomas, varying in degree of differentiation, latency and volume doubling time. The monolayer culture conditions for CaNT, SaNeo and CaRD have been established. The conditions required for the in vitro growth of the other tumour cell lines have yet to be established as they are only used for in vivo studies at present.
2.1.2.3 V79-379A

V79-379A Chinese hamster lung fibroblasts were originally isolated in 1958, from lung tissue of a mature Chinese hamster (Cricetulus griseus), for use in genetic studies and designated the V strain. They were later subcloned and redesignated V79. A sub-clone was donated to Dr. L. Revesz at the Karolinska Institute, Stockholm and an aliquot given to Dr. O.C.A. Scott of the Gray Laboratory in 1968 which is the V79-379A strain.

2.1.3 Cell Maintenance

2.1.3.1 Storage of cell lines in liquid nitrogen

Cells were stored frozen in liquid nitrogen at a concentration of $1 \times 10^6$ cells/ml in a mixture of MEM and 10% dimethylsulphoxide (DMSO) in 2ml aliquots in cryovials. The vials were frozen slowly to avoid any damage by placing above liquid nitrogen overnight, and then stored in liquid nitrogen until required.

2.1.3.2 Recovery of cell lines from liquid nitrogen

Frozen aliquots of cells were removed from liquid nitrogen and thawed rapidly using a 37°C waterbath. The cells were then added to a T-150 flask with 30ml of MEM and allowed to attach for at least 4 hours. The MEM was then replaced with 50ml of fresh medium to remove DMSO derived from the frozen aliquot.

2.1.3.3 Maintenance of murine derived tumour cells

Monolayer murine tumour cells were kept in T-75 tissue culture flasks in 20ml of MEM in a 37°C, 5% CO$_2$, 5% O$_2$ incubator. Cells were trypsinised twice a week and reseeded at a density of $2 \times 10^5$ cells/flask.

2.1.3.4 Maintenance of V79-379A cells

V79-379A cells were kept in suspension in a spinner vessel at 37°C. Cells were counted and diluted daily to a density of 1-2 $\times 10^5$ cells/ml with the addition of fresh MEMS.
2.1.3.5 Preparation of feeder layer

V79-379A cells were used as a feeder layer for the SaF cell line. They were irradiated with 30Gy from a Pantak x-ray set operating at 250kVp and 15mA (1.7Gy/min dose rate) at room temperature (18-25°C) under aerobic conditions. Following irradiation, the 25ml of cells from a pancake spinner pot were centrifuged at 1000rpm for 10 minutes, resuspended and counted. For colony forming assays, 2 x 10⁵ lethally irradiated feeder cells in 9ml of MEM were plated per 9 or 10cm petri dish and allowed at least 2 hours to attach before addition of the required number of tumour cells.

2.2 SPHEROID TECHNIQUES

2.2.1 Spheroid Development

Two main methods of developing spheroids from cell lines were studied. These were the Yuhas method of liquid-overlay culture (Yuhas et al. 1977) and the Sutherland spinner method (Sutherland et al. 1971). Two other methods of producing cellular aggregates with regard to implantation in syngeneic mice were also investigated; microcarrier culture and calcium alginate beads.

2.2.1.1 The liquid-overlay method

T-75 tissue culture flasks were aseptically coated with an autoclaved 2% solution of tissue culture grade agar and left to cool and solidify in a flowhood. Initially, 25ml of mixtures of MEM and MEMS (detailed later) were used to start cultures. In subsequent experiments 40mM HEPES buffer was added to keep a stable pH in the flasks. Known numbers of cells were added and the contents mixed and incubated at 37°C, 5% O₂ and 5% CO₂ up to 2 weeks. The degree of aggregation was assessed daily and the medium was changed if required.

2.2.1.2 The spinner method

Bellco 100ml hanging bar spinner vessels containing 40ml of media mixture (detailed later) were seeded with a known number of cells and the
media gassed with 5% CO$_2$, 95% air prior to being put on a magnetic stirrer pad at 100rpm in a 37°C warm room. Small aliquots were removed each day and observed under the microscope for aggregation unless clumps could be detected by eye. The medium was changed as required and the vessels gassed every day.

2.2.1.3 Validation of methods

Chinese hamster V79 cells have been grown successfully as spheroids by both methods; in spinner flasks (Inch et al. 1970) and in suspension over agar (Carlsson & Yuhas 1984). These cells were used to develop the basic skills of growing and manipulating spheroids. The media mixtures used were MEM or MEMS alone or combinations of both (ratios of 1:2, 1:1 or 2:1) to vary the amount of fetal calf serum and calcium and magnesium ions in the medium. The mixtures of media were initially tested on their own but subsequently it was decided that the addition of a buffer may be beneficial to prevent fluctuations in pH. 10, 20, 30, 40 and 50mM HEPES buffer were tried with V79-379A spheroids in their optimum medium. Spheroids grown in 40mM HEPES increased most in size over 14 days.

2.2.1.4 Gray Laboratory tumour lines

A series of spontaneous tumours were grown in vivo (section 2.3.1) until they reached a mean diameter of 6-8mm. They were excised and disaggregated (section 2.3.2) to produce spheroids using the Yuhas method. Several variations in media and cell density were tested on each tumour cell line using this approach. The characteristics of the tumours are shown in Table 3.1. If cells remained after setting up the liquid-overlay cultures, the Sutherland approach was also tested. The Yuhas method proved to be the most successful in developing spheroids (see Table 3.2) and as such was the method of choice for routine production.
2.2.2 Use of Microcarrier Beads to Produce Cellular Aggregates.

2.2.2.1 Microcarrier beads

Microcarrier beads are generally used to produce high yields of anchorage-dependent cells. In microcarrier culture cells grow as monolayers on the surface of small spheres which can be suspended in culture medium by gentle stirring. The rationale for using microcarrier beads to produce spheroids was that tumour cells reaching confluence on the surface of the bead would pile up on top of one another developing a spheroid with a bead for the core.

Two types of microcarrier bead were selected to investigate which provided the best matrix for cell attachment.

2.2.2.2 Cytodex 1

Cytodex 1 microcarriers are based on a cross-linked dextran matrix which is substituted with positively charged N,N-diethylaminoethyl groups to a degree which is optimal for cell growth. The charged groups are found throughout the entire matrix of the microcarrier. Physical characteristics are summarised in Table 2.1.

2.2.2.3 Cytodex 3

Cytodex 3 microcarriers are based on a different principle. Unlike Cytodex 1 which has a specific density of small charged molecules to promote attachment and growth of cells, Cytodex 3 has collagen as a cell culture substrate. Cytodex 3 consist of a surface layer of denatured collagen covalently bound to a matrix of cross-linked dextran. The denatured collagen is derived from pig skin type I collagen which is extracted, denatured by acid treatment, concentrated and purified by an ion exchange step and steam sterilised before being coupled to the microcarrier matrix. Physical characteristics are summarised in Table 2.1.
Table 2.1  Physical characteristics of Cytodex 1 and 3

<table>
<thead>
<tr>
<th></th>
<th>Cytodex 1</th>
<th>Cytodex 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density * (g/ml)</td>
<td>1.03</td>
<td>1.04</td>
</tr>
<tr>
<td>Size * $d_{50}$</td>
<td>180</td>
<td>175</td>
</tr>
<tr>
<td>Size * $d_{5,95}$</td>
<td>131-220</td>
<td>133-215</td>
</tr>
<tr>
<td>Approx. area * (cm$^2$/g dry weight)</td>
<td>6,000</td>
<td>4,600</td>
</tr>
<tr>
<td>Approx. number microcarriers/g dry weight</td>
<td>$6.8 \times 10^6$</td>
<td>$4.0 \times 10^6$</td>
</tr>
<tr>
<td>Swelling * (ml/g dry weight)</td>
<td>18</td>
<td>14</td>
</tr>
</tbody>
</table>

Note: Size is based on diameter at 50% of the volume of a sample of microcarriers ($d_{50}$), or the range between the diameter at 5% and 95% of the volume of a sample of microcarriers ($d_{5,95}$).

* In 0.9% NaCl

2.2.2.4 Preparation of microcarriers

A weighed amount of dry Cytodex beads were added to a siliconized glass bottle and 100ml/g of Ca$^{2+}$, Mg$^{2+}$-free PBS added and left for over three hours with occasional gentle mixing. The supernatant was decanted and the microcarriers washed once in PBS for a few minutes with gentle shaking. The PBS was discarded and replaced with fresh PBS (50ml/g Cytodex) and the microcarriers autoclaved at 110°C for 11 minutes. Before use the microcarrier beads were allowed to settle, the PBS removed and the microcarriers rinsed in warm culture medium (50ml/g Cytodex). This allowed medium to replace PBS between and within the beads. The medium was discarded and the beads resuspended in a small volume of culture medium. The microcarrier beads were then ready for use in cell culture.

2.2.2.5 Initiation of microcarrier culture

$1 \times 10^6$ Cytodex 1 beads were hydrated and sufficient CaNT cells added to provide 10 cells per bead. A small amount of medium was added and stirred gently for 3 hours then topped up to 40ml. Three days later there was little evidence of cell growth with only the occasional cell found
attached to a bead; the rest were free floating in the medium.

Cytodex 3 beads were hydrated and mixed with sufficient CaNT cells for 10 cells per bead. To try and improve cell attachment prior to the initiation of stirring, cultures were left either 6 or 24 hours in agar-coated T-75 flasks before being stirred. Another flask was set up to investigate whether growth could occur with static beads. There was still no evidence of cell growth on any of the beads after 7 days.

The above experiments were repeated with Chinese hamster V79-379A cells which were not subject to trypsinization; there was still very little growth on the beads. It was therefore decided not to pursue this culture system any further.

2.2.3 Alginate Gels

Alginate is a transparent polymer of guluronic and mannuronic acids that provide a favourable microenvironment for cell growth. A solution of sodium alginate is liquid but when it comes into contact with calcium an exchange of ions results in a solid gel of calcium alginate. Biologically inert alginate has been used in animal models for diverse applications such as cell encapsulation and immobilisation (Li et al. 1992), drug carriers and controlled release drug delivery (Downs et al. 1992), protective coating on frozen transplanted rodent embryos (Adaniya et al. 1993) and in patients as a packing material in wound healing (Dawson et al. 1992) and a dressing to prevent bleeding in surgical techniques (Basse et al. 1992). Single tumour cell suspensions encapsulated in calcium alginate have been injected into mice to study angiogenesis (Plunkett & Hailey 1990) but these cells did not have the intimate contact and structure of spheroids displaying the response of a solid tumour mass. Consequently, if it proved useful in developing spheroids it could be readily implanted into a host mouse without adverse toxicity and without surgical procedures disrupting any neovascularisation.
2.2.3.1 Alginate preparation

An alginate gel containing V79-379A cells was produced by dropping a 0.8% solution of low viscosity sodium alginate containing 1 x 10^6 cells/ml into stirred calcium chloride using a fine 25g needle. It formed solid agar-like beads of 5-7mm diameter. The beads were immediately transferred to MEM and incubated at 37°C in 5% O_2 and 5% CO_2. In an attempt to reduce the size of the beads, a spray unit was used to produce an aerosol of the sodium alginate solution into calcium chloride. This reduced the average size of the beads to 3-5mm in diameter.

2.2.3.2 Alginate beads

Daily observation of the beads with twice weekly medium changes showed that some cells continued to proliferate forming colonies within the porous beads. This method could lead to colonies of cells being implanted into the peritoneum within the inert alginate or further \textit{in vitro} growth to develop the colonies into spheroid-like structures before implantation.

This methodology was discontinued when there was successful production of SaF spheroids by conventional means, which would not have the obstacle of alginate possibly slowing down drug penetration or disrupting the flow of biochemicals to and from the spheroid.

2.2.4 Routine SaF Spheroid Culture

SaF cells were routinely obtained by excision from subcutaneous dorsal tumours. Tumours were aseptically removed, minced finely with scissors then mixed with 10ml of an enzyme cocktail containing 0.2% collagenase and 0.02% DNase in serum free Eagle's MEM. This mixture was rotated at 37°C for 30 minutes. 15ml of MEM with 10% FCS was added, it was centrifuged for 10 minutes at 1000rpm and the supernatant decanted. The cells were resuspended in medium and counted. To initiate spheroid growth, 1 x 10^6 cells were inoculated into 25ml of medium (a mixture of MEM 2 : 1 MEMS with 40mM HEPES buffer) in a T-75 agar coated flask and placed in an incubator with 5% CO_2, 5% O_2 at 37°C.
During the growth of the spheroids, the medium was changed on day 3 when the spheroids were thinned to 100 per flask; then twice weekly.

2.3 IN VIVO TECHNIQUES

All *in vivo* procedures were carried out to Home Office standards under a personal licence.

2.3.1 Implantation of Tumours

Tumour fragments stored in liquid nitrogen in MEM and 10% DMSO were thawed quickly and washed twice in 0.9% saline solution. Mice were anaesthetised by inhalation of metofane, the back shaved and wiped with alcohol. A small piece of tumour was then implanted subcutaneously on the dorsum using a trocar. Subsequently tumours from donor animals were processed into a cell suspension by mincing finely with scissors, and were resuspended in 10ml of 0.9% saline. The suspension was put through a series of needles with the gauge decreasing in size, from 19g to 25g. A 0.05ml aliquot of this suspension was then injected subcutaneously onto the shaved and alcohol wiped backs of anaesthetised, recipient mice.

2.3.2 Excision Assay

Tumour bearing mice were sacrificed by cervical dislocation and doused in 70% alcohol in a flowhood to minimise contamination from bacteria and fungi. Tumours were used when they reached a mean diameter of 6 or 10mm, depending on the particular experiment, and were weighed prior to disaggregation. The tumour was minced finely with scissors and 10ml of enzyme cocktail (0.2% Collagenase, 0.02% DNAase in serum free medium) was added. The universal was clamped to a rotating wheel in a 37°C warm room for at least 30 minutes when the suspension was checked for presence of single cells. Large pieces of tumour were broken up by repeated pipetting of the suspension which was then filtered through a 35μm nylon mesh in a Swinnex filter and washed through with
15ml of medium containing serum to neutralise the enzymes. The suspension was centrifuged at 1000rpm for 10 minutes, the supernatant discarded. For cell culture, the pellet was resuspended in 5ml of fresh medium, topped up to 25ml with MEM, the suspension was then counted on a Coulter counter and the appropriate number of cells plated. For flow cytometry, the pellet was resuspended in 200µl of PBS and 20ml of 70% ethanol added for fixation. The cells were then counted on a haemocytometer and stored at 4°C.

2.3.2.1 Colony forming assay

A pellet of centrifuged cells was resuspended using a 21g needle and 5ml syringe in 5ml of MEM, then topped up to a total of 25ml. Cells were counted on a Coulter counter and serially diluted such that an appropriate aliquot of cell suspension could be added to 9ml of MEM with a feeder layer of \(2 \times 10^5\) lethally irradiated V79-379A cells. The cells were incubated at 37°C in 5% \(O_2\), 5% \(CO_2\) for at least 7 days to allow colonies to develop. The resulting colonies were stained with 0.2% crystal violet in 70% ethanol, counted and the surviving fractions calculated from the number of colonies on the control and treated dishes.

2.3.3 Preparation and Administration of Drugs

2.3.3.1 Bioreductive drugs

A 2mg vial of mitomycin C powder was dissolved in 5ml of sterile water. Further dilutions were carried out by addition of sterile water. Porfiromycin was weighed, sterile water added, the solution sonicated and warmed to 37°C to aid the drug to dissolve. All vials containing the drugs were wrapped in foil to prevent light reaching the solution. Concentrations of drug, for \textit{in vivo} and \textit{in vitro} use, were calculated such that equal volumes of solution were administered to all the mice or all vessels. Control vessels had equivalent volumes of sterile water added. Drugs were injected \textit{in vivo} using a 25g needle by intraperitoneal route after weighing the mice. Control mice had equivalent volumes of 0.9% saline injected.
2.3.3.2 2-Nitromidazole-theophylline (NITP)

The *in vivo* formulation of the hypoxic probe, NITP, was 7mg NITP powder, 0.05ml DMSO and 0.45ml peanut oil (warmed to 37°C). NITP and DMSO were sonicated together until the solid was dissolved as fully as possible. Peanut oil was added and the mixture kept at 37°C in a waterbath wrapped in foil to protect the drug from light until ready for use. The mixture was vortexed immediately before injection. For a 35g mouse, 0.38ml of the NITP mixture was injected intraperitoneally; equivalent to 0.45µM. Equimolar doses were given to all mice, dependent on weight, via a 23g needle.

*In vitro*, a final concentration of 100µM was required, achieved by sonicating the powder in PBS until fully dissolved.

2.3.3.3 5-Bromo-2'-deoxyuridine (BrdUrd)

5-Bromo-2'-deoxyuridine was weighed, made up to 10mg/ml with sterile water and injected intraperitoneally to a final concentration of 100mg/kg.

*In vitro* a final concentration of 20µM was required, achieved by dissolving the powder in a little PBS and adding either medium or further PBS.

2.3.4 Spheroid Implantation

EMT6 spheroids were first implanted into the peritoneum by Lord *et al.* (1979) to investigate which cells would infiltrate and destroy the foreign tumour masses. Zwi then implanted spheroids to investigate their vasculature following attachment to surrounding organs in comparison with those which remained free floating and non-attached (Zwi *et al.* 1989). We were interested to use this system to investigate the influence of neovascularisation on proliferative and hypoxic characteristics of spheroids. The first experiment was carried out using SaNeo spheroids but subsequent experiments used SaF spheroids.
2.3.4.1 Pilot spheroid implantation

As a pilot experiment, 10 SaNeO spheroids, between 150 and 350μm diameter, were implanted using a 19g needle into the peritoneum of anaesthetised WHT female mice. Three mice were randomly selected every week for 6 weeks, sacrificed and the peritoneum examined macroscopically for spheroids or any tumour cell growth. Free floating and attached spheroids were recovered and placed immediately in neutral buffered formalin. Sections were stained with haematoxylin and eosin.

Ten SaF spheroids between 200 and 300μm in diameter were selected and injected using a 19g needle into the peritoneum of CBA female mice. The SaF is a more rapid growing tumour than the SaNeo so 3 mice were examined daily from day 3 to day 10 after implantation. Only attached spheroids were recovered from the peritoneum with a very low yield.

2.3.4.2 Routine SaF spheroid implantation

Optimisation of the technique is described in Chapter 3. Twenty SaF spheroids between 250 to 350μm in diameter were selected from in vitro cultures in aseptic conditions by a 19g needle into a 1ml syringe. CBA male mice were anaesthetised with metofane and their abdomen wiped with 70% alcohol. The spheroid containing syringe was flicked a number of times to dislodge any spheroids from the syringe wall and the spheroids injected into the left side of the peritoneum. The syringe was then refilled with 0.9% saline and a further injection given to the mouse into the right side of the peritoneum to rinse any remaining spheroids from the syringe and needle.

The spheroids were recovered 7 days later, from cervically dislocated mice, by a central incision to remove the skin which was peeled outwards disclosing any spheroids growing between the subcutaneous tissue and peritoneal wall. Spheroids attached to the peritoneal wall were excised and the peritoneum exposed. The peritoneal cavity and organs were then examined for any attached or free tumour masses, which were
subsequently excised. The fate of the excised spheroids was dependent on the experimental protocol. Spheroids were either fixed in neutral buffered formalin or snap frozen in liquid nitrogen for immunohistochemical staining or placed in PBS in preparation for disaggregation into a single cell suspension (described later).

2.4 FLOW CYTOMETRY

Flow cytometry is a technique which allows the rapid quantitation of several measurements simultaneously on individual particles such as single cells or nuclei. The particles need to be in suspension such that individual characteristics can be measured, using laser light excitation, based on light scatter and fluorescence, either intrinsic or added immunologically. The flow cytometer is now widely used and has numerous applications in medicine and molecular and cellular biology due to its easy use and rapid analysis of samples.

The accuracy of the flow cytometer often relies predominantly on the state of the sample preparation and staining technique not on the performance of the flow cytometer. The prerequisite for good flow cytometry is obtaining a decent single cell suspension. Techniques which can be used vary from mechanical disaggregation with scissors, enzymic disaggregation or lysing of the cells to produce nuclei. The staining method then has to be adjusted to obtain the maximal differentials between positively and negatively stained cells. This is achieved by manipulation of the titre of the staining reagents and using substances to suppress background fluorescence; in the case of antibodies this would be serum from the species in which the fluorescent reagent was raised. In addition it is important to include appropriate controls such that the specific fluorescence of the measurement being made can be accurately quantified.

The main attributes of flow cytometry are its sharpness of measurement, ability to make several measurements simultaneously, its rapidity and quantitative power. These combine to make the technique very attractive in both the experimental and clinical setting.
2.4.1 The FACScan

The FACScan is an automated cell analyzer attached to a computer. It is capable of measuring five optical parameters simultaneously: forward scatter, side scatter, and three spectral regions of fluorescence. It has three high performance photomultipliers with band pass filters of 530nm (FITC), 585nm (PE/PI) and >650nm (red fluorescence).

The FACScan has an air-cooled 15 milliwatt Argon-ion laser with a single excitation wavelength of 488nm. The optical layout is shown in Figure 2.1. The fluidics system provides a laminar, single-file procession of cells through the optical sensing region. The sample flow rate is a function of sample capillary tube diameter, sample fluid viscosity and differential pressure. The high flow rate is 60μlmin⁻¹ and the low flow rate is 12μlmin⁻¹, this provides a higher resolution as it has a smaller sample core with more precise cell positioning. Particle velocity in the flowcell is 6msec⁻¹.

When individual cells enclosed within a pressurized saline solution pass through the orifice of the flowcell five signal pulses are generated simultaneously from the optical detectors. These pulses are first converted to analog electronic pulses, then to digital data by the A/D converters which can then be stored and processed by the computer system. The software provided with the FACScan allows data acquisition, real-time displays, instrument control, status monitoring and file management facilities. Application software includes single and multiple parameter analysis using single or multiple histograms, dot plots and contour plots with statistics, histogram overlays and DNA cell cycle analysis.

2.4.1.1 Fluorochromes

In the flow cytometry experiments described in this thesis the probes for hypoxia and proliferation have been labelled indirectly with a primary antibody followed by secondary antibodies conjugated to fluorochromes, and then counterstained with a DNA stain. The most commonly used DNA stain is propidium iodide. The phenanthridinium, propidium, intercalates between the bases in double stranded nucleic acids, binding
Figure 2.1  The FACScan optical layout.
stoichiometrically such that it can be used to measure relative DNA content. Propidium iodide (PI) does not freely cross the intact plasma membrane so cells have to be fixed or permeabilized before binding takes place. The excitation and emission spectra of PI are shown in Figure 2.2A and B respectively, it has a broad excitation range with peaks in UV and visible, its emission maximum is 639nm (Rabinovitch et al. 1986). In two colour flow cytometry, the fluorochrome of choice to combine with the red-emitting propidium iodide is the green-emitting fluorescein derivative fluorescein isothiocyanate (FITC)(Figure 2.2B). This fluorochrome has an emission maximum of 520nm and thus there is little spectral overlap between the fluorescence signals produced by the two fluorochromes.

For simultaneous triple staining, the three fluorochromes used were green FITC, orange/red R-phycoerythrin (PE) and the DNA stain 7-aminoactinomycin D (7AAD). As can be seen from Table 2.2, PE has an emission peak of 576nm thus in conjunction with PI there is a large degree of overlap between emissions. This overlap can be reduced by using 7AAD, which also binds specifically and stoichiometrically with DNA but has an emission peak of 650nm in the far-red.

Table 2.2 The maximum emission of 4 fluorochromes

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Emission Maximum (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein isothiocyanate (FITC)</td>
<td>520</td>
</tr>
<tr>
<td>R-phycoerythrin (PE)</td>
<td>576</td>
</tr>
<tr>
<td>Propidium iodide (PI)</td>
<td>639</td>
</tr>
<tr>
<td>7-Aminoactinomycin D (7AAD)</td>
<td>650</td>
</tr>
</tbody>
</table>

2.4.1.2 Doublet Discrimination Mode

The FACScan has a pulse processing facility which allows the selection of two parameters for doublet discrimination. It uses variations on normal parameter measurements to differentiate between two cells.
Figure 2.2  (A) Absorption and (B) fluorescence emission spectra of fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), propidium iodide (PI) and 7-aminoactinomycin D (7AAD).
clumped together and one cell with a high level of fluorescence (e.g. a G0/G1 doublet and a G2/M singlet). Normally, when fluorescence is measured it is expressed as pulse height (in FSC, SSC, FL1, FL2 or FL3). With doublet discrimination turned on the pulse width and area can also be measured. The area of the light pulse is a better indicator of total fluorescence than pulse height. The pulse width of a particle will increase as the particle is larger because it takes longer to cross the laser beam. This will allow discrimination between a G0/G1 doublet and a G2/M singlet, which have the same amount of DNA so would appear at the same position in a DNA histogram, but a doublet would have a greater width signal. Doublets are therefore removed by gating around a singlet population on an area versus width plot of the DNA channel.

2.4.1.3 Lysys II - Data Acquisition and Analysis System

Lysys II is a software system which allows the collection and analysis of the light emission data from the FACScan. It employs a variety of pull-down menus and option windows for selection of the required information.

In acquisition mode specific regions of the data can be selected by gating procedures. Data can then be collected for storage on discs, usually in the order of 10,000 events from each sample. The data can be analysed at a later date as the specific pulses recorded for each event are stored. In analysis mode any combination of the collected data may be visualised as histograms or dot plots and numerical and statistical analyses carried out. Specific regions may be selected and examined for particular characteristics and trends.

2.4.1.4 Cellfit - Data Analysis

Cellfit is a comparative data package to Lysys II but in this case was only used for analysis of DNA profiles. DNA histograms were used to determine the distribution of cells within the cell cycle and provide numerical values for the percentages of cells in G1, S and G2/M. They also provided the DNA index of the samples which is a measure of the DNA
aneuploidy of the population. Cellfit provides five models to estimate the percentage of cells in each phase of the cycle. The model which best fits the data DNA profile is the SOBR (Sum of Broadened Rectangles).

2.4.1.5 The analysis of DNA profiles using SOBR.

Propidium iodide or 7-aminoactinomycin D stained DNA can be used. SOBR assumes that cells are distributed in all phases of the cell cycle, for this reason, SOBR should not be used for synchronised cell populations. A complex calculation fits the G0/G1 and G2/M populations with single Gaussian curves producing successive approximations to the actual histogram. A selected number of Gaussian convolved rectangles fit the S phase; the greater the number of fits the better the data. The specifications of the SOBR fit are that it estimates the initial % CV by determining the peak width at the inflection point (60% of peak height) and then generates the final reported % CV during the iterated fit. The reported % CV is determined by the final Gaussian curve that is fitted to the G0/G1 peaks.

2.4.2 The Hypoxic Probe, 2-nitroimidazole-theophylline (NITP)

The hypoxic probe is a 2-nitroimidazole linked to a theophylline side-chain. The 2-nitroimidazole is metabolised in a similar way to misonidazole by reduction in low oxygen concentrations resulting in binding to cellular macromolecules. The highest proportion of binding is nuclear and probably to the DNA. The theophylline side-chain, acting as a hapten, allows antibodies raised against theophylline to become attached to the bound compound and thus identify and quantify hypoxia indirectly using two step antibody staining.

2.4.2.1 Double staining procedure for hypoxia and DNA

The formulation of the hypoxic probe, 2-nitroimidazole-theophylline (NITP) is described in 2.3.3.2. For a 35g mouse, 0.38ml of the NITP mixture was injected intraperitoneally and this ratio adjusted appropriately depending on mouse weight. The tumour was excised after 2 hours,
minced finely and incubated with 10ml of 0.2% collagenase and 0.02% DNAase in MEM without serum for 30 minutes. The resulting tumour pieces were pipetted to break them up, filtered through a 35μm nylon mesh which was washed with 10ml of serum containing medium to neutralise the enzymes. The single cell suspension was centrifuged for 10 minutes at 1000rpm, the pellet resuspended in 1ml PBS, then 9ml of 70% ethanol was added and mixed. The sample was stored at 4°C until ready for analysis. The protocol for immunochemical staining for flow cytometry is listed in protocol 1.

Protocol 1  Flow cytometry immunological staining for hypoxia and DNA
1. The fixed cells were counted using a haemocytometer.
2. 2 x 10⁶ cells of each specimen were aliquoted into 10ml conical bottomed centrifuge tubes.
3. 5ml of PBS were added and centrifuged for 5 minutes at 2000rpm. The supernatant was decanted.
4. 0.25ml of neat rabbit antiserum to theophylline-8-KLH* was added, vortexed, then incubated for 1 hour at room temperature with occasional mixing.
*Theophylline antiserum was prepared by addition of 1ml of deionised water to a 100 test vial of lyophilized power, mixed gently until the powder dissolved, with subsequent addition of 9ml of PBS.
5. 5ml of PBS were added, the tubes centrifuged, and the supernatant discarded.
6. 0.25ml of PBS containing 0.5% Tween 20 and 0.1% normal goat serum with 25μl IgG fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit antiserum were added and the tubes vortexed and incubated for 1 hour at room temperature with occasional mixing.
7. 5ml of PBS were added, the tubes centrifuged, and the supernatant discarded.
8. The pellet was resuspended in 2ml of PBS containing 1mg/ml
RNAse and 10μg/ml propidium iodide.

9. The stained suspensions were analysed on the FACScan using LYSYS II software.

This original protocol was modified in the subsequent triple staining procedure to quantify hypoxia within the nucleus. After step 3 the cells were incubated with 2.5ml of 0.2mg/ml pepsin in 2M HCl for 20 minutes, followed by two washes in 5ml of PBS to eliminate the acid. The protocol was then followed from step 4 onwards except no RNAse was required in step 8.

This modified method was validated below for inter- and intra-assay variability and compared with the original double staining method as in protocol 1.

2.4.2.2 Inter-assay variability of NITP and DNA double stain

Four positive samples and a control were stained and run on five consecutive days to compare the statistical variability between samples run at different times (Table 2.3).

<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>17.7</td>
<td>14.2</td>
<td>20.5</td>
<td>7.1</td>
</tr>
<tr>
<td>Day 2</td>
<td>19.4</td>
<td>13.4</td>
<td>18.1</td>
<td>5.5</td>
</tr>
<tr>
<td>Day 3</td>
<td>15.9</td>
<td>14.1</td>
<td>18.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Day 4</td>
<td>16.3</td>
<td>13.5</td>
<td>19.8</td>
<td>3.7</td>
</tr>
<tr>
<td>Day 5</td>
<td>19.4</td>
<td>11.0</td>
<td>18.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Mean</td>
<td>17.7</td>
<td>13.2</td>
<td>19.2</td>
<td>5.2</td>
</tr>
<tr>
<td>SD</td>
<td>1.66</td>
<td>1.30</td>
<td>0.95</td>
<td>1.23</td>
</tr>
<tr>
<td>SEM</td>
<td>0.74</td>
<td>0.58</td>
<td>0.42</td>
<td>0.55</td>
</tr>
<tr>
<td>CV%</td>
<td>9.38</td>
<td>9.83</td>
<td>4.97</td>
<td>23.50</td>
</tr>
</tbody>
</table>

83
The data shows that there is relatively good agreement from day to day in the percentage of hypoxia found within the aneuploid population of cells, although the coefficient of variance is greater for samples with fewer positive cells.

2.4.2.3 Intra-assay variability of NITP and DNA double stain

Ten aliquots of a single sample were stained simultaneously for hypoxia and DNA content using modified protocol 1 and the results statistically compared for variability (Table 2.4).

Table 2.4 Intra-assay variability of NITP and DNA content staining

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aneuploid %</th>
<th>Aneuploid Hypoxia %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72.7</td>
<td>17.4</td>
</tr>
<tr>
<td>2</td>
<td>71.2</td>
<td>17.4</td>
</tr>
<tr>
<td>3</td>
<td>71.0</td>
<td>17.3</td>
</tr>
<tr>
<td>4</td>
<td>70.9</td>
<td>17.3</td>
</tr>
<tr>
<td>5</td>
<td>71.8</td>
<td>16.9</td>
</tr>
<tr>
<td>6</td>
<td>71.8</td>
<td>17.2</td>
</tr>
<tr>
<td>7</td>
<td>69.1</td>
<td>16.6</td>
</tr>
<tr>
<td>8</td>
<td>69.9</td>
<td>17.6</td>
</tr>
<tr>
<td>9</td>
<td>71.3</td>
<td>17.6</td>
</tr>
<tr>
<td>10</td>
<td>70.5</td>
<td>17.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aneuploid %</td>
<td>71.0</td>
<td>1.03</td>
<td>0.34</td>
<td>1.45</td>
</tr>
<tr>
<td>Hypoxia %</td>
<td>17.3</td>
<td>0.30</td>
<td>0.10</td>
<td>1.76</td>
</tr>
</tbody>
</table>

These results show good agreement with a low % C.V. so within the same sample run, with the same staining procedure and FACScan operation combined, there is little variation. This implies that samples are better run
on the same day for comparative purposes, although samples run on different days can be satisfactorily compared if a control is stained concurrently.

2.4.2.4 Analysis of NITP profiles using LYSYS II software

Figure 2.3 illustrates how regions were derived to calculate hypoxic parameters of aneuploid SaF tumours. Figure 2.3A is a control sample which had no NITP administered but nuclei were processed through immunostaining for NITP adducts. A region is drawn above these control nuclei, spanning the whole width of DNA content, containing <1% of the total number of events (region 1). Figure 2.3B is a positively stained NITP dot plot displaying hypoxic nuclei now present in region 1. The DNA histogram of Figure 2.3B is shown in Figure 2.3C. The histogram is divided into diploid (region 2) and aneuploid populations (region 3) to obtain the number of host cells present in the tumour. The aneuploid population is sub-divided into G1 (region 4), S (region 5) and G2/M (region 6) phases of the cell cycle, so that total numbers of nuclei in each region can be calculated. The hypoxic population is then highlighted by selecting region 1 only (Figure 2.3D). The hypoxic DNA profile (Figure 2.3E) can be used to quantify the proportion of hypoxic cells in each region of the cell cycle. These figures can then be used to quantify % hypoxia in each region i.e. cell cycle phase, and in turn the proportion of hypoxia within each phase can be calculated (Figure 2.3F).

2.4.2.5 Comparison of the original and modified NITP staining protocols

Ten samples were stained for NITP adducts by the original and modified protocol on the same day. The hypoxia profiles were then analysed for number of diploid and aneuploid cells and % aneuploid hypoxia. Each DNA profile was analysed by the SOBR model of Cellfit to ascertain the % of cells within each cell cycle phase. Table 2.5 shows the difference in % diploid population, cells in each phase and aneuploid hypoxia for whole cells and pepsin/HCl digested nuclei.
Figure 2.3  Analysis procedure for NITP samples
There is a decrease in the number of diploid cells when nuclei are used, mean of % diploid 56.3 and 40.8 respectively, probably due to the more fragile nature of diploid cells. The % of cells in each phase of the cell cycle in the aneuploid population also changes. The number of cells in G1 drops, this could either be from a decrease in the underlying peak of G2 diploid cells or the effect of the sharpening of the DNA profiles produced by analysis of nuclei (displayed in Figure 7.6). The better defined phases of the cell cycle with nuclei also probably account for the changes in S and G2/M phases, especially since the aneuploid hypoxia value changes very little for each sample.

**Table 2.5** Comparison of the original and modified NITP staining protocols

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Diploid Population</th>
<th>Difference in Original and modified protocols (Whole cells - Nuclei)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole</td>
<td>Nuclei</td>
</tr>
<tr>
<td>1</td>
<td>49.8</td>
<td>42.8</td>
</tr>
<tr>
<td>2</td>
<td>60.2</td>
<td>45.2</td>
</tr>
<tr>
<td>3</td>
<td>58.0</td>
<td>41.0</td>
</tr>
<tr>
<td>4</td>
<td>53.8</td>
<td>43.0</td>
</tr>
<tr>
<td>5</td>
<td>53.7</td>
<td>33.5</td>
</tr>
<tr>
<td>6</td>
<td>55.4</td>
<td>41.9</td>
</tr>
<tr>
<td>7</td>
<td>56.7</td>
<td>38.1</td>
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<tr>
<td>8</td>
<td>56.5</td>
<td>43.5</td>
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<tr>
<td>9</td>
<td>62.4</td>
<td>39.5</td>
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<tr>
<td>10</td>
<td>56.8</td>
<td>39.1</td>
</tr>
<tr>
<td>Mean</td>
<td>56.3</td>
<td>40.8</td>
</tr>
</tbody>
</table>
2.4.3 The Proliferation Probe, 5-Bromo-2'-deoxyuridine (BrdUrd)

5-Bromo-2'-deoxyuridine (BrdUrd) is a thymidine analogue. Administration of BrdUrd is described in 2.3.3.3. It is incorporated into DNA during synthesis and can be detected using monoclonal antibodies raised against it. As the antibody needs a single strand of DNA, the double helix is denatured by incubation in hydrochloric acid exposing the antigenic site. The protocols for immunochemical staining for BrdUrd in whole tumours and cell suspensions are listed in protocols 2 and 3 respectively.

Protocol 2 5-Bromo-2'-deoxyuridine staining of whole tumours
1. Solid tumours were excised from cervically dislocated mice 1 hour after administration of BrdUrd and fixed directly in 70% ethanol and stored for at least 12 hours prior to staining.
2. Approximately 3mm³ of the sample from the tumour was removed and minced finely with scissors.
3. 8ml of 0.4mg/ml pepsin in 0.1M HCl were added. The pepsin was dissolved in a small amount of PBS first, then the HCl added, the resulting pH should be 1.5. The suspension was incubated at 37°C for 30 minutes, with constant rotation. It was checked after 20 minutes, as the time spent in this solution was dependent on the consistency of the tumour.
4. The sample was further dissociated by pipetting. If the solution was still clear, containing lumps, it was incubated for a longer period of time and checked every 5 minutes.
5. The suspension was filtered through a 35μm nylon mesh, to exclude any remaining large lumps of tumour, into 10ml conical bottomed centrifuge tubes. The nuclei may have been counted at this stage in the procedure.
6. The sample was centrifuged at 2000rpm for 5 minutes and the pepsin solution decanted. The pellet resuspended in 2.5ml of 2M HCl and incubated at room temperature for 12 minutes with...
occasional mixing.

7. 5ml of PBS were added, the tubes centrifuged for 5 minutes at 2000rpm and the supernatant decanted. This was repeated.

8. The pellet was resuspended in 0.5ml PBS containing 0.5% normal goat serum and 0.5% Tween 20 (PNT). 25μl rat anti-BrdUrd monoclonal antibody was added, mixed, and incubated for at least 1 hour at room temperature with occasional mixing.

9. 5ml of PBS were added, the tubes centrifuged for 5 minutes at 2000rpm and the supernatant decanted.

10. The pellet was resuspended in 0.5ml PNT and 25μl anti-rat FITC conjugate added and mixed, followed by incubation for 30-60 minutes at room temperature with occasional mixing.

11. 5ml of PBS were added, the tubes centrifuged for 5 minutes at 2000rpm. The pellet was resuspended in 2ml PBS and 20μl of 1mg/ml propidium iodide added and mixed.

12. Stained suspensions were analysed using LYSYS II software.

Protocol 3  5-Bromo-2'-deoxyuridine staining of a cell suspension
There was a slight variation in the staining protocol for the cell suspensions as the samples did not need to be dissociated, they only required enucleation and denaturation of the DNA double helix.

1. 2 x 10^6 cells of each specimen were aliquoted into 10ml conical bottomed centrifuge tubes and 5ml of PBS added.

2. The sample was centrifuged for 5 minutes at 2000rpm. The supernatant decanted and the pellet resuspended in 2.5ml of 2M HCl, followed by incubation at room temperature for 25 minutes with occasional mixing.

3. 5ml of PBS were added, the tubes centrifuged for 5 minutes at 2000rpm and the supernatant decanted. This was repeated.

4. The pellet was resuspended in 0.5ml PBS containing 0.5% normal goat serum and 0.5% Tween 20 (PNT). 25μl rat anti-BrdUrd monoclonal antibody was added, mixed, and incubated for at least
1 hour at room temperature with occasional mixing.
5. 5ml of PBS were added, the tubes centrifuged for 5 minutes at 2000rpm and the supernatant decanted.
6. The pellet was resuspended in 0.5ml of PNT, 25μl anti-rat IgG FITC conjugate added and mixed, then incubated for 30-60 minutes at room temperature with occasional mixing.
7. 5ml of PBS were added, the tubes centrifuged for 5 minutes at 2000rpm and the supernatant decanted. The pellet was resuspended in 2ml PBS and 20μl of 1mg/ml propidium iodide added.
8. The stained suspensions were analysed on the FACScan using LYSYS II software.

The analysis of BrdUrd profiles using LYSYS II software is carried out in a similar manner to the NITP profiles except the positive and negative boundaries are better defined. Short time periods between BrdUrd injection and tumour excision lead to S phase positivity only, due to the nature of the probe.

2.5 IMMUNOHISTOCHEMISTRY

Initially, all tumour tissue for histological staining was frozen in liquid nitrogen, taking care not to crack large tumours, and stored at -70°C. A protocol for producing NITP staining in paraffin embedded sections was then developed, as paraffin embedded sections have better cellular definition than frozen sections. The frozen samples were then defrosted, fixed in 70% ethanol and processed as described below.

2.5.1 Paraffin Embedding of Sections

Whole tumours and large peritoneal spheroids fixed in 70% ethanol were sent to the histology department of Mount Vernon Hospital for routine paraffin embedding. In vitro spheroids and smaller peritoneal spheroids were fixed in 70% ethanol, embedded in 2% agar and wrapped in tissue paper to prevent loss of tumour tissue during the embedding procedure.
2.5.2 Immunohistochemical Staining of Ethanol Fixed Paraffin Sections

Protocol 4 NITP Staining

1. 5μm sections were cut, picked up on poly-l-lysine coated slides and dried for 48 hours at 37°C.

2. All the following procedures were run at room temperature. The sections were taken through xylene, 100%, 90% to 70% ethanol to deparaffinize and rehydrate.

3. Endogenous peroxidase was blocked with 0.1% H₂O₂ in methanol for 30 minutes.

4. The slides were wiped, sections encircled with resin pen, then washed 3 times in PBS.

5. Non-specific binding sites were blocked with normal goat serum diluted 1/5 with PBS for 10 minutes.

6. Primary antibody, neat rabbit anti-theophylline with 1% Human AB serum was added for 1½ hours.

7. Sections were washed 3 times in PBS, 1 minute for each wash.

8. Secondary antibody, biotinylated swine anti-rabbit IgG diluted 1/300 with 1% Human AB serum in PBS was added for 1 hour.
   (Step 10 solution was made up at this time, as it needed to stand for at least 30 minutes before use).

9. Sections were washed 3 times in PBS, 1 minute for each wash.

10. ABC (avidin biotin complex) reagent was added for 1 hour.

11. Sections were washed 3 times in PBS, 1 minute for each wash.

12. Sections were incubated from 4-10 minutes in DAB solution. (A 10mg 3,3′-diaminobenzidine tablet dissolved in 16.6mls of PBS, filtered through a 5μm filter and just prior to use activated with 83μl of 6% H₂O₂ and mixed well).

13. Sections were washed for a minute in tap water.

14. Sections were counterstained with Mayers Haematoxylin, and blued in running tap water for a minute.

15. The sections were cleared by immersing in graded ethanol (70%, 90%, 100%), then xylene for 1-2 minutes and mounted in DPX.
Protocol 5  BrdUrd Staining

1. 5μm sections were cut, picked up on poly-l-lysine coated slides and dried for 48 hours at 37°C.
2. The sections were taken through xylene, 100%, 90% to 70% ethanol to deparaffinize and rehydrate.
3. Endogenous peroxidase was blocked with 0.1% H₂O₂ in methanol for 30 minutes.
4. Slides were transferred to preheated 1M HCl at 60°C for 15 minutes, then washed well in tap water.
5. Slides were flooded with Tris buffered saline pH 7.6 (TBS) for 5 minutes, which was then tipped off and the section wiped around and encircled with resin pen.
6. Mouse anti-BrdUrd was added, diluted 1/30 in 1% Human AB Serum in TBS for 1 hour.
7. The section was washed 3 times in TBS, 1 minute for each wash.
8. The secondary antibody, biotinylated rabbit anti-mouse diluted 1/300 in 1% Human AB Serum in TBS was added for 1 hour.
9. The section was washed 3 times in TBS, 1 minute for each wash.
10. ABC reagent was added for 1 hour.
11. The section was washed 3 times in TBS and rinsed in Tris Buffer.
12. DAB solution was added for 4-10 minutes.
13. Sections were washed in tap water.
14. Sections were counterstained in Mayers Haematoxylin, and blued in running tap water for 1 minute.
15. Sections were then dehydrated, cleared and mounted.
Protocol 6  Haematoxylin and Eosin Staining

1. 5µm sections were cut, picked up on slides and dried for ½ hour at 60°C.

2. The sections were taken through xylene, 100%, 90% to 70% ethanol to deparaffinize and rehydrate.

3. Sections were placed in Mayers Haematoxylin for 10 minutes, then washed for 1 minute in tap water.

4. The sections were differentiated in 1% acid/alcohol (1% HCl in 70% ethanol) for 30 seconds, then blued for 5 minutes in tap water.

5. Sections were placed in 1% Eosin for 5 minutes, then washed for 1 minute in tap water.

6. Slides were then dehydrated, cleared and mounted.
CHAPTER 3 SPHEROID CHARACTERISATION AND IMPLANTATION METHODS

3.1 SPHEROID DEVELOPMENT

There are two standard methods of developing spheroids from cell lines. These are the Yuhas method of liquid-overlay culture (Yuhas et al. 1977) and the Sutherland spinner method (Sutherland et al. 1971). The Yuhas method is the simplest form of producing spheroids as it involves preventing the cells from attaching to flasks or dishes in normal culture conditions; this is achieved by using bacterial dishes or agar coating the surface of culture vessels. The Sutherland approach involves keeping the cells in constant motion in spinner culture so that they do not attach to any surface. Often spheroids are initiated in a liquid-overlay culture and transferred into spinner culture after a few days growth. These studies describe the attempts to produce spheroids from a number of tumour lines and subsequent characterisation of the spheroids by these two methods. Attempts to grow spheroids by microcarrier bead culture and alginate gels were described in sections 2.2.2 and 2.2.3 respectively.

One of the aims of producing spheroids was to implant the spheroids, grown in vitro, into syngeneic mice to act as a more complex tumour model where the effects of vascularisation could be evaluated. Initial experiments to standardise implantation are also detailed.

3.1.1 Tumour Selection

Tumours were selected from a range of Gray Laboratory tumours derived from two strains of mice including sarcomas and carcinomas both poorly and well differentiated tumour types with a wide variation in latency and doubling times. The specific characteristics of each tumour are listed in Table 3.1.

3.1.2 The Liquid-overlay Method

This method is described in 2.2.1.1. Initially, cells were plated in T-75 agar coated flasks with MEM or MEMS alone or at ratios of these (2:1, 1:1,
Table 3.1  Origin of Gray Laboratory tumours

* This is data from various sources within the Gray Laboratory

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Mouse Strain</th>
<th>Year Arose</th>
<th>Histological Description, Latency (L) and Doubling Time in vivo ($T_d$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaF</td>
<td>CBA</td>
<td>1957</td>
<td>Anaplastic sarcoma L= 6 days, $T_d$= 2.1 days</td>
</tr>
<tr>
<td>SaS</td>
<td>CBA</td>
<td>1962</td>
<td>Poorly differentiated, infiltrative round-celled sarcoma L= 6 weeks, $T_d$= 9.7 days</td>
</tr>
<tr>
<td>CaRH</td>
<td>WHT</td>
<td>1966</td>
<td>Moderately differentiated adenocarcinoma L= 2 months, $T_d$= 6.1 days</td>
</tr>
<tr>
<td>CaNT</td>
<td>CBA</td>
<td>1968</td>
<td>Poorly differentiated, rather vascular adenocarcinoma L= 2 weeks, $T_d$= 2.6 days</td>
</tr>
<tr>
<td>SaFB</td>
<td>WHT</td>
<td>1979</td>
<td>Fibrosarcoma, vascular L= 6-8 weeks, $T_d$= 11.1 days</td>
</tr>
<tr>
<td>CaX</td>
<td>CBA</td>
<td>1979</td>
<td>Poorly differentiated adenocarcinoma L= 8 months, $T_d$= 6.5 days</td>
</tr>
<tr>
<td>SaHM</td>
<td>CBA</td>
<td>1980</td>
<td>Anaplastic fibrous sarcoma L= 3-4 weeks, $T_d$= 6.2 days</td>
</tr>
<tr>
<td>CaWW</td>
<td>CBA</td>
<td>1980</td>
<td>Moderately differentiated adenocarcinoma L= 1-2 months, $T_d$= 5.2 days</td>
</tr>
<tr>
<td>CaRD</td>
<td>WHT</td>
<td>1980</td>
<td>Moderately differentiated carcinoma, predominantly squamous differentiation and some glandular differentiation, adenosquamous carcinoma L= 1 month, $T_d$= 4.0 days</td>
</tr>
<tr>
<td>SaNeo</td>
<td>WHT</td>
<td>1981</td>
<td>Pleomorphic soft-tissue sarcoma with marked plasma cell infiltration in some areas L= 3-4 weeks, $T_d$= 3.9 days</td>
</tr>
</tbody>
</table>
1:2) with cell densities of 6, 4 and 2 x 10^4 cells/ml. Subsequently, it was decided that the addition of a buffer may be beneficial to prevent fluctuations in pH. 10, 20, 30, 40 and 50mM HEPES buffer were tested with V79-379 spheroids in their optimum medium (2:1 MEMS:MEM). Spheroids grown in 40mM HEPES increased most in size over 14 days. The success of this method with the range of tumour types is listed in Table 3.2.

Table 3.2  Tumour cell lines grown by the Yuhas method

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Appearance of cells after 2 weeks growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaF</td>
<td>Spheroids, large aggregates.</td>
</tr>
<tr>
<td>SaFB</td>
<td>Large loose aggregates formed, none spheroidal.</td>
</tr>
<tr>
<td>SaHM</td>
<td>No aggregates, many dead cells and cell debris.</td>
</tr>
<tr>
<td>SaNeo</td>
<td>Spheroids, large aggregates formed.</td>
</tr>
<tr>
<td>CaNT</td>
<td>Spheroids, large aggregates formed.</td>
</tr>
<tr>
<td>CaRD</td>
<td>Spheroids, but very slow growing.</td>
</tr>
<tr>
<td>CaRH</td>
<td>Many dead cells and cell debris.</td>
</tr>
<tr>
<td>SaS</td>
<td>Many single cells, no spheroids.</td>
</tr>
<tr>
<td>CaWW</td>
<td>Aggregates formed, none spheroidal.</td>
</tr>
<tr>
<td>CaX</td>
<td>Small approximately 8 cell aggregates formed.</td>
</tr>
</tbody>
</table>

Spheroids were generated from SaF, SaNeo, CaNT and CaRD cell lines; discussed in 3.1.4. SaHM and CaRH produced no indications of aggregating in the media with cells dying in culture and disintegrating to produce debris. SaS cells remained as a single cell suspension but were viable and capable of growing as a monolayer on tissue culture flasks. SaFB, CaWW and CaX formed small cellular aggregates, but either increasing the initial cell density or individual selection of aggregates into fresh media did not result in the formation of larger or more spheroidal aggregates.

3.1.3 The Spinner Method

Excess cells from the above experiments were used to initiate spinner cultures, as described in 2.2.1.2, initially with 40ml of 2:1
MEMS:MEM and 40mM HEPES buffer in a 100ml Bellco hanging bar spinner vessel at a density in excess of $4 \times 10^4$ cells/ml. Spheroids proved difficult to grow by this culture method. There may have been several reasons why the Sutherland method proved unsuccessful including control of the pH. The most likely reason was not achieving optimal stirring conditions. This may have either caused sheering forces which were too great, such that the cells did not remain as aggregates or that there was not enough agitation for the cells to aggregate in the first instance. Transfer of spheroids from liquid-overlay culture to spinner vessels at days 3 to 7 caused the spheroids to disintegrate, even at slow spin speeds, indicating that the spheroids were loosely bound at this stage in their growth.

3.1.4 Spheroid Selection and Growth Optimisation

Figure 3.1 shows the growth curves of spheroids derived from Chinese hamster V79-379A cells and two of the three murine cell lines which grew successfully with the Yuhas method; SaF and SaNeo. The perpendicular diameters ($a$ and $b$) of at least 24 spheroids in individual wells of a 24 well plate containing 2.5ml of medium were measured using a 90° graduated graticule. The mean diameter and volumes were then calculated as below.

\[
\text{Mean Diameter} = \sqrt{ab} \\
\text{Mean Volume} = \frac{4}{3} \pi \left( \frac{\sqrt{ab}}{2} \right)^3
\]

The volume doubling times for V79-379A, SaF and SaNeo spheroids were 0.60, 1.05 and 4.35 days respectively. The three growth curves had different characteristics; the V79-379A spheroids grew rapidly up to about 7 days, then slowed down. After an initial spurt of growth, the SaF spheroids had linear growth rate, slowing down when approaching 900μm diameter (0.38mm$^3$). Both V79-379A and SaF spheroids reached a mean diameter of approximately 1000μm (0.52mm$^3$) by 20 days growth. The SaNeo was very slow growing, reaching only 400μm diameter (0.03mm$^3$) after 20 days.
Figure 3.1  Growth curves of spheroids derived from three different cell lines.

Figure 3.2  Growth curves of SaF spheroids grown in different mixtures of media.
growth, and was found to contain inherent mycoplasma even when the earliest passage was removed from frozen stocks and transplanted into mice. CaRD spheroids also grew very slowly and in conjunction with low yield and a large variation in growth rates between spheroids were deemed unsuitable for large scale experimentation. The CaNT cell line eventually proved to grow as spheroids but at an advanced stage in this study when it was not possible to utilise them in this thesis. With these considerations the SaF was chosen as the cell line to investigate further, and supplementary studies were performed to optimise the growth conditions.

SaF spheroids formed well in three mixtures of media thus growth curve experiments were performed to obtain the optimum growing conditions. Comparison showed (Figure 3.2) that although MEMS with 40mM HEPES buffer produced slightly more rapid growth of SaF spheroids, qualitatively MEM 2:1 MEMS with 40mM HEPES buffer produced the more regular spheroids. The measurement of spheroids grown in MEMS with HEPES could have been complicated by uneven growth at the spheroid circumference creating a ragged appearance.

The routine method of SaF spheroid culture once these parameters had been optimised are detailed in section 2.2.4. 1 x 10⁶ cells were used to initiate cultures because a larger number generated strings of aggregates and less regular spheroids, a lower cell density generated fewer spheroids. A higher (80mM) and lower (20mM) concentration of HEPES buffer was compared to the V79-379A optimised 40mM HEPES and there was no visible difference in spheroid structure or spheroid yield, so 40mM HEPES buffer was retained.

3.2 SAF SPHEROID CHARACTERISATION

3.2.1 Spheroid cell number

Spheroids were grown by the optimised method in section 2.2.4. During growth spheroids were individually selected at diameters of 100, 200, 250, 300, 400, 500, 600, 700, 800, 900 and 1000μm (within limits of ± 10μm) using a 90° graticule and placed in a conical bottomed vial of PBS.
The number of spheroids per diameter were counted, rinsed twice in PBS then 4ml of 0.05% trypsin/0.02% EDTA added. The spheroids were incubated at 37°C for 10 minutes, mixing on a vortex before incubation, and at 5 and 10 minutes, when the suspension was checked for the presence of large cellular masses. Once a single cell suspension was obtained, 4ml of MEM was added and the suspension centrifuged at 1000rpm for 10 minutes. The pellet was resuspended in 200μl of PBS and the number of cells counted on a haemocytometer. The total number of cells in PBS was calculated and adjusted for the initial number of spheroids trypsinized to determine the number of cells per spheroid.

Figure 3.3 shows the increase in cell number as the spheroid increases to a maximum diameter of approximately 1mm.
3.2.2 Flow Cytometry

Spheroids were selected as above at diameters of 250, 400 and 600µm and placed in flasks of warm media. The spheroids were left for at least two hours to equilibrate in the incubator and then either 20µM BrdUrd or 100µM NITP, or both, were added to the media for 1 or 2 hours respectively. Spheroids were rinsed in PBS then disaggregated as above. After resuspension in 200µl of PBS, 5ml of 70% ethanol was added to fix the cells which were stored at -4°C. The samples were stained by protocols 3 and 1 (sections 2.4.3 and 2.4.2) for BrdUrd incorporation and hypoxia respectively.

Table 3.3 shows the proportions of cells in each phase of the cell cycle, total hypoxia and the labelling index of the three different diameter spheroids. This shows that as the spheroid increases in size the number of cells in G1/G0 increases and cells in S and G2/M phases of the cell cycle decrease. The labelling index also decreases as the spheroid increases in size. Hypoxia increases as the spheroid grows, with measureable hypoxia present even at 250µm diameter.

<table>
<thead>
<tr>
<th>Spheroid diameter (µm)</th>
<th>% of cells in each phase</th>
<th>% Hypoxia</th>
<th>BrdUrd labelling index %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>S</td>
<td>G2/M</td>
</tr>
<tr>
<td>250</td>
<td>62.4</td>
<td>22.8</td>
<td>14.9</td>
</tr>
<tr>
<td>400</td>
<td>75.8</td>
<td>15.3</td>
<td>8.9</td>
</tr>
<tr>
<td>600</td>
<td>83.9</td>
<td>10.6</td>
<td>5.5</td>
</tr>
</tbody>
</table>

3.2.3 Histology

Spheroids were treated as above and, after incubation with both BrdUrd and NITP, were fixed in 70% ethanol. Spheroids were stained with 1% Eosin, for ease of identification, and embedded in 2% agar which was then processed and immunohistochemically stained as in sections 2.5.1 and 2.5.2. Figure 3.4 shows staining of SaF spheroids.
Figure 3.4  Histological staining of 250μm diameter SaF in vitro spheroids. Magnification x200.
(A) Haematoxylin and Eosin staining
(B) NITP staining
(C) BrdUrd staining
3.3 SPHEROID IMPLANTATION

Lord first implanted EMT6 spheroids into the peritoneum of a mouse to investigate which cells would infiltrate and destroy the foreign tumour masses (Lord et al. 1979). Later, Zwi implanted them to investigate the vasculature of spheroids which would attach to the surrounding organs compared to the free floating avascular spheroids (Zwi et al. 1989). We were interested to use this system to investigate the influence of neovascularisation on proliferative and hypoxic characteristics of spheroids. The preliminary experiment was carried out using SaNeo spheroids but subsequent experiments used SaF spheroids.

3.3.1 Pilot SaNeo Implantation

Ten SaNeo spheroids, between 150 and 350μm in diameter, were aseptically selected in 1ml of medium and injected using a 19g needle into the peritoneum of anaesthetised WHT female mice. Three mice were randomly selected every week for 6 weeks, sacrificed and the peritoneum examined macroscopically for spheroids or any tumour cell growth. Recovered spheroids were placed immediately in neutral buffered formalin.

SaNeO spheroids grew successfully in the peritoneum, although the yield of spheroids recovered was small, some had become attached and others were found free floating with a maximum diameter of 1mm. By 21 days more spheroids were being recovered than the initial 10 implanted which suggested that metastases were developing in the peritoneum. Histological sections of the spheroids and tissues obtained were stained with Haematoxylin and Eosin showing that some of the attached spheroids had become vascularised.

Figures 3.5 and 3.6 show the diversity of peritoneal spheroids, all of which were recovered three weeks after implantation. Figure 3.5A shows a free floating spheroid with a maximum diameter of 1050μm. It has an outer viable rim of proliferating cells with a central region of necrosis. Figure 3.5B demonstrates a spheroid surrounded by fatty tissue which was embedded in the inguinal fat pad. This 800μm diameter spheroid had a
Figure 3.5  Haematoxylin and Eosin staining of SaNeo peritoneal spheroids.
(A) Free floating spheroid. x100
(B) Spheroid embedded in the inguinal fat pad. x100
Figure 3.6 Haematoxylin and Eosin staining of SaNeo peritoneal spheroids.
(A) Ingrowing spheroid attached to the liver. x200
(B) Vascularised spheroid attached to the spleen. x100
100-150μm viable rim of cells with central necrosis similar to *in vitro* spheroids. Figure 3.6A shows a spheroid attached to the liver with tumour cells beginning to invade the normal tissue and evidence of a blood vessel among the tumour cells. Figure 3.6B demonstrates a spheroid attached by a small margin (200μm) to the spleen. The presence of blood vessels throughout the spheroid was indicated by compact groups of red blood cells. Figure 3.7 displays the variation of a single spheroid when histological sections are prepared at different depths. The spheroid was attached to the gastro-intestinal tract. Figure 3.7A shows the spheroid invading the muscle layer with a central area of muscle cells. Figure 3.7B shows a section through the centre of the spheroid and Figure 3.7C a section on the outer edge of the spheroid where tumour cells are invading intestinal villi. Further use of SaNeo spheroids was prohibited due to positive mycoplasma testing.

### 3.3.2 Pilot SaF Implantation

Ten SaF spheroids between 200 and 300μm in diameter were selected and injected using a 19g needle into the peritoneum of CBA female mice. The SaF is a more rapid growing tumour than the SaNeo so 3 mice were examined daily from day 3 to day 10 after implantation.

No spheroids were located until day 5 and these tended to be attached to the peritoneal wall at the injection site. The numbers recovered were low and none were found unattached. After day 8 the tumours tended to be large, due to the number of spheroids injected the percentage tumour mass in relation to the body mass of the mouse could have become great if the technique was improved. It was decided that day 7 would be the optimum day of examination with SaF implanted spheroids.

Haematoxylin and Eosin sections of peritoneal spheroids were indistinguishable from subcutaneous tumours. Examples of SaF peritoneal samples are shown in Chapter 5.
Figure 3.7 Variation in structure of a single attached SaNeo spheroid stained with Haematoxylin and Eosin. x100
3.3.3 Modifications of Implantation

Delivery of spheroids into the peritoneum was improved by a combination of tapping the syringe to detach spheroids on the syringe wall and washing out the syringe and needle with 0.5ml of saline solution and injecting this into the peritoneum. The injection technique was also improved by primarily using the left hand side then the right hand side of the abdomen and making sure the needle tip was not in contact with any internal organs on injection. Recovery of spheroids improved on familiarisation with the technique.

3.4 DISCUSSION

Spheroid development

Cell lines from Gray Laboratory sources proved difficult to develop into a reliable spheroid model. SaS, CaRh and SaHM cell lines would not even aggregate. Other cell lines, CaX, CaWW and SaFB, produced aggregates of cells but these could not be encouraged to become spheroidal or increase in size. Of the four cell lines which did produce spheroids, SaNeo was infected with mycoplasma, CaRD was very slow growing with irregular sized spheroids and CaNT spheroids were only produced very late in the development studies. SaF spheroids were the best for modelling purposes. Growth was fairly rapid and the spheroids produced were regular in shape and size.

A major problem was encountered when histological studies were required. SaF spheroids, although tightly bound enough not to disintegrate when picked up by pipette, had a large degree of central necrosis. This was first encountered when trying to grow SaF spheroids in spinner culture to increase yield and create a more uniform oxygen distribution in the spheroid. Spheroids initiated in liquid-overlay culture and transferred when the spheroids were approximately 200-300μm in diameter would fall apart once spinning was initiated, even at low speeds. Large SaF spheroids also fell apart when processed for paraffin embedding. H & E staining of frozen sections demonstrated the central necrotic core present in large
spheroids, however did not produce high definition staining so was not utilised for BrdUrd and NITP immunohistochemistry. Sections of 250μm diameter spheroids still demonstrated the expected distribution of BrdUrd incorporation and hypoxia (Figure 3.4). Hypoxic staining was confined to the necrotic core and surrounding layer of cells, whilst BrdUrd incorporation was only in the outer most cell layers.

Once the histological structure of the spheroids was established flow cytometry was a more satisfactory method for quantitation of hypoxia and proliferation. Flow cytometry showed that distribution of cells throughout the cell cycle changed as the diameter increased. A decrease in proliferating cells due to an increase in size is a well known feature of spheroid growth and there have even been reports of changes in ploidy in some cell lines (Durand 1990a). Flow cytometry data is also probably a more accurate method of quantifying hypoxia when oxygen is unevenly distributed in the spheroid due to the static nature of liquid-overlay culture. This is especially relevant when quantitation is required to assess the action of bioreductive drugs in this model.

**Spheroid Implantation**

The injection of spheroids into the peritoneum was relatively easy. The most difficult action was ascertaining whether all the spheroids were deposited in the peritoneal cavity or were still present in the needle or syringe. Introduction of the second injection of a saline washout increased recovery of spheroids when expelled into a petri dish and peritoneal spheroid yield did increase slightly. Recovery of spheroids was a far more difficult task. Excision had to be methodical and precise so that all internal organs were examined and any free spheroids detected, although the SaF tumour did not produce any free spheroids.

The SaNeo spheroid model would have been preferable to the SaF model if vascular effects were being studied because it produced free and attached spheroids and the slow growth of the tumour would have enabled finer vascular studies to be performed. Evidence of blood vessels was
obtained from H & E staining of groups of red blood cells in SaNeo sections. This could have been explored further with specific endothelial cell histological markers such as Factor VIII, to ascertain the quantity and depth of penetration of these vessels. The SaF tumour model has a very rapid growth rate, and as such tumours would outgrow their host within a week providing less scope for examination, but it also leads to rapid turnover of experiments. The lack of free spheroids is probably due to the aggressive nature of the SaF tumour. The SaNeo spheroids also appeared to retain their shape to a higher degree than SaF implanted spheroids which could have been due to their more compact, resilient structure. This led to SaNeo implanted spheroids being more distinct in shape and structure from subcutaneous tumours unlike their SaF counterparts.

The diversity of recovered SaNeo spheroids was shown in Figures 3.5, 3.6 and 3.7. Free and attached spheroids were recovered. Spheroids were found attached to the liver, spleen, intestines and peritoneal fat pads as well as the peritoneal wall. By week 6 the mice were becoming sick due to the size of tumour and many more spheroids were recovered than the initial implanted number. This implies that the tumour has metastasized. This tumour model appears to be better suited to a slow growing tumour in order for the investigation of vascular development to be defined.
CHAPTER 4  CELL LINE STUDIES : HYPOXIA

Tumour model systems vary in complexity, the cell line represents the simplest model in which the cellular environment can be controlled by external manipulation. It has been recognised for many years that cells irradiated under hypoxic conditions are more resistant than their aerobic counterparts (Gray et al. 1953) usually by a factor of three. Many different modalities including oxygen and radiosensitizers have been used to exploit the hypoxic nature of cells to increase damage to the tumour (Dische 1985; Overgaard 1989; Kjellen et al. 1991). Bioreductives are a class of drugs with selective activity to hypoxic cells and are currently under investigation in combination with radiotherapy (Rockwell & Kennedy 1979; von der Maase 1986; Weissberg et al. 1989; Grau & Overgaard 1991). In this Chapter the two selected bioreductive drugs, mitomycin C and porfiromycin, were investigated with respect to cell survival under hypoxic and aerobic conditions to establish their differential response for later comparison with more complex model systems. Subsequently, this led to investigation of the precise relationship between cell survival and oxygen concentration at a fixed dose of mitomycin C or porfiromycin. In addition, the oxygen dependent binding of the hypoxic probe, NITP, and radiation were also evaluated in this model system to establish whether they share similar characteristics.

4.1  DOSE RESPONSE OF SAF CELLS TO BIOREDUCTIVE DRUGS

In the following experiments cells from at least the second passage of SaF tumours were used; this eliminated the diploid component, as they fail to divide in culture. Inclusion of these would, in theory, artificially reduce the plating efficiency calculations required to measure surviving fractions. However, analysis of DNA profiles, from control in vivo tumours, by the flow cytometer indicated that a higher percentage of aneuploid cells within the tumour did not necessarily mean a concomitant rise in plating efficiency.
4.1.1 Method

SaF cells, in exponential growth, were trypsinised with 0.05% trypsin/0.02% EDTA (4ml for T-75 flasks, 8ml for a roller bottle) for 4 minutes at 37°C. The trypsin/EDTA was quenched with double the volume of MEM supplemented with 10% fetal calf serum and the vessel rinsed out with a further volume of MEM. The resulting cell suspension was centrifuged for 10 minutes at 1000rpm. Cells were resuspended in 5ml of medium using a 21 gauge needle and 5ml syringe, all the separate flask contents were pooled, the suspension diluted to 50ml and the resulting cell suspension counted on a Coulter counter. Dreschel gas washing bottles modified with a side arm, containing 30ml of medium with SaF cells at a density of $2 \times 10^5$ cells/ml were placed in a 37°C warm room and kept in suspension with a magnetic stirrer. The appropriate gas concentration was obtained by connecting the bottles via butyl rubber tubing and a copper sulphate containing bubble trap to gas cylinders of either 95% N₂, 5% CO₂ or 95% air, 5% CO₂. Gas flow rate was checked using a flowmeter on the exhaust gas. Cells were gassed for at least 1 hour prior to drug administration at a flow rate of at least 0.4L/min.

The drug was made up as described previously (section 2.3.3.1) to provide final concentrations of 0.5, 1, 2, 3 and 4μM which were added as a 1ml aliquot to the pregassed 30ml of cells. The control vessel was treated as the drug vessels but with 1ml of sterile water being added instead of the drug.

After 1 hour the suspension was centrifuged at 1000rpm for 10 minutes. The cells were resuspended in 5ml of medium and then made up to 25ml (during the whole process cells were kept at 4°C). Cells were then processed for a colony forming assay described in section 2.3.2.1. The experiment was repeated at least three times to provide means and errors.

4.1.2 Results

Plating efficiencies of SaF cells were in the range of 0.17-0.35 with a mean of 0.27±0.03 when a feeder layer of lethally irradiated Chinese
hamster V79-379A cells was used. Plating efficiencies without the feeder layer were reduced with a mean of 0.15±0.02.

The survival curves for mitomycin C and porfiromycin are shown in Figure 4.1. It can be seen that mitomycin C caused significant aerobic toxicity reducing the surviving fraction by almost 2 decades at 4μM. In contrast, porfiromycin showed very little aerobic toxicity with only a modest reduction in surviving fraction (0.3) at the same 4μM dose. However, the hypoxic toxicity of the two drugs was similar, the surviving fractions at 4μM were 0.0002 and 0.0004 respectively for mitomycin C and porfiromycin.

The differential toxicities between hypoxic and aerobic conditions of the drugs were 1.7 and 3.5 for mitomycin C and porfiromycin respectively at a survival level of 0.1. To enable calculation of the differential for porfiromycin the survival curve was extrapolated to extend below a 10% reduction of cell survival. Figure 4.2A shows the difference in survival between hypoxic and aerobic cells in decades of cell kill at each dose. This demonstrates that as dose rises the difference in hypoxic and aerobic cytotoxicity increases. Porfiromycin has a larger differential at each dose than mitomycin C.

The two drugs were directly compared in Figure 4.2B by calculating the difference in decades of cell kill under aerobic or hypoxic conditions. This histogram clearly shows the oxygen dependent toxicity of porfiromycin compared to mitomycin C. Under hypoxic conditions, porfiromycin matches mitomycin C toxicity with little difference in cell kill. However, in aerobic cultures mitomycin C showed increasing toxicity, while porfiromycin toxicity changed very little, thus a difference of a decade in cell kill at 4μM was achieved.
Figure 4.1  Dose response of SaF cells to mitomycin C (upper panel) and porfiromycin (lower panel) after 1 hour exposure to the drug. Solid symbols (●, ■) in air and open symbols in nitrogen (○, □) are means ± SEM of at least 3 experiments. Absence of error bars indicates they are within the symbols.
Figure 4.2  

(A) Comparison of the difference in hypoxic and aerobic SaF cell survival in decades of cell kill after 1 hour exposure to mitomycin C or porfiromycin at different doses.

(B) Comparison of the difference between porfiromycin and mitomycin C in decades of SaF cell kill under hypoxic or aerobic conditions after 1 hour exposure at different doses.
4.2 CYTOTOXICITY AND TIME-DEPENDENCE OF EXPOSURE TO BIOREDUCTIVE DRUGS

4.2.1 Method

The experiments were carried out as above with drug concentrations of 1 and 2µM but aliquots of cells were aseptically removed at 1, 2, 3, 4, 5 and 6 hours. The experiments were repeated at least three times to provide means and errors for each time and dose point.

4.2.2 Results

The survival curves for mitomycin C and porfiromycin following different times of exposure are shown in Figure 4.3. Mitomycin C showed increasing toxicity up to 6 hours with a surviving fraction of 0.03 and 0.0002 at a doses of 1 and 2µM respectively for SaF cells under aerobic conditions. Under hypoxic conditions toxicity was greater, 1µM mitomycin C showing a similar toxicity to the 2µM concentration under aerobic conditions, and 2µM mitomycin C reaching 5 decades of cell kill in 6 hours.

As indicated from the dose response curves in Figure 4.1, porfiromycin maintained a modest toxicity under aerobic conditions up to 6 hours attaining 2 decades of cell kill with the higher 2µM dose; 1µM achieving half the toxicity. Again, under hypoxic conditions, porfiromycin exhibited a much greater toxicity, with four and five decades of cell kill at 1 and 2µM respectively, compared to aerobic survival at 6 hours.

The difference in decades of cell kill between mitomycin C and porfiromycin toxicity under hypoxic and aerobic conditions at 2µM is shown in Figure 4.4A. The difference in cell kill of porfiromycin increases up to 6 hours to nearly 3 decades due to the increase in hypoxic toxicity, whilst the difference in mitomycin C toxicity remains at about a decade as both aerobic and hypoxic toxicity increases. Direct comparison of the two drugs under aerobic and hypoxic conditions by calculating the difference in decades of cell kill at 2µM is shown in Figure 4.4B. Corresponding to the dose response ratios, this histogram shows the oxygen-dependent toxicity of porfiromycin. Under hypoxic conditions, the difference increases to a
Figure 4.3 Influence of incubation time on the response of SaF cells to 1 (■□) and 2µM (●○) mitomycin C (upper panel) or 1 (■□) and 2µM (●○) porfiromycin (lower panel). Solid symbols in air (■, ●) and open symbols in nitrogen (□, ○) are means ± SEM of at least 3 experiments. Absence of error bars indicates they are within the symbols.
Figure 4.4  (A) Comparison of the difference in hypoxic and aerobic SaF cell survival in decades of cell kill for 2μM mitomycin C or porfiromycin after different exposure times.

(B) Comparison of the difference between 2μM porfiromycin and mitomycin C in decades of SaF cell kill under hypoxic or aerobic conditions after different exposure times.
decade at 2 hours, then decreases up to 6 hours such that there is very little difference between hypoxic cytotoxicity of the two drugs. This reflects the underlying shapes of the two curves as the toxicity of mitomycin C tends to be linear under hypoxia whereas the porfiromycin curve has a larger shoulder. The aerobic toxicity of mitomycin C increases over time, whereas the aerobic toxicity of porfiromycin increases only slightly, thus the difference in decades of cell kill gradually increases reaching over 1.5 decades at 6 hours.

4.3 OXYGEN DEPENDENCY OF MITOMYCIN C AND PORFIROMYCIN

4.3.1 Experimental Design

The previous results showed the response of SaF cells to mitomycin C and porfiromycin at the two extremes of oxygen concentration. Further experiments were used to show the precise response of cells to the two bioreductives under different oxygen concentrations. An optimum drug concentration of 3μM was chosen as both drugs displayed large differentials in toxicity at this dose (Figure 4.1). "K" curves were originally designed for radiosensitivity assessment at different oxygen concentrations but similar principles can be applied to drugs whose cytotoxicity is oxygen dependent.

4.3.2 Method

Large numbers of SaF cells were grown on roller bottles until they reached near confluence. The cells were then trypsinised using 8ml of 0.05% trypsin/0.02% EDTA solution for 4 minutes at 37°C. The trypsin was quenched with 20ml of MEM with 10% fetal calf serum and the mixture centrifuged for 10 minutes at 1000rpm. The cells were then resuspended, pooled and counted. Spinner vessels, 55mm in diameter containing side arms for gas inlet and outlet and a maximum 30ml capacity, were prepared with 20ml of MEM at a cell density of 5 x 10^5 cells/ml. The vessels were then placed in a 37°C waterbath in a warm room and stirred continuously
for the duration of the experiment. Gas cylinders were attached via butyl rubber tubing and a copper sulphate containing bubble trap to the spinner vessels. The cells were then gassed for at least 1 hour with the appropriate concentration of oxygen, 5% CO₂ and a balance of N₂. For each individual run a control of 95% N₂, 5% CO₂ was used.

Mitomycin C and porfiromycin were prepared as previously described to achieve a final concentration of 3μM in the spinner vessel.

After 1 hour exposure to mitomycin C or porfiromycin, the cells were removed and immediately centrifuged at 1000rpm for 10 minutes. Cells were kept at 4°C, resuspended in 5ml of medium, then made up to 25ml and processed for a colony forming assay as described in section 2.3.2.1. Control dishes were plated consisting of cells which had not been exposed to drugs to obtain a surviving fraction.

The oxygen concentration of the nitrogen sample was measured using a Thermox probe (Thermo-Lab Instruments Inc.), and a value of 20ppm oxygen was recorded. To establish whether this small amount of oxygen had any effect on the cytotoxicity of mitomycin C or porfiromycin a "scrubbed" sample was prepared, where the last traces of oxygen from the N₂/CO₂ gas mixture were removed. This was achieved by putting the gas through a wash bottle of Fieser's Solution (20g KOH in 100ml H₂O and 2g sodium anthraquinone-β-sulfonate (C₁₁H₇O₅) with 15g sodium hydrosulfite (Na₂S₂O₄ - sodium dithionate) added after the rest of the solution had been deoxygenated by bubbling with nitrogen). A blood-red solution was obtained, which remains effective until it turns brown or a precipitate forms. Any sulphurous products were removed by passage through saturated sodium bicarbonate solution.

4.3.3 Results

There was no significant difference between the "scrubbed" and normal nitrogen toxicity for either mitomycin C or porfiromycin as shown in Figure 4.5.

Mitomycin C displayed a plateau up to 200ppm oxygen followed by
Figure 4.5 Surviving fraction of SaF cells \textit{in vitro} under different oxygen concentrations following exposure to 3\(\mu\)M mitomycin C (upper panel) or 3\(\mu\)M porfiromycin (lower panel). All values are means \(\pm\) SEM of at least 3 experiments. Absence of error bars indicates they are within the symbols.
a steep increase between 600 and 6000 ppm, and then a gradual rise up to 200,000 ppm oxygen; the air sample. The "K" value, oxygen concentration for half maximum effect, was calculated by using the surviving fractions (SF) of the low plateau and the air sample and finding the midpoint.

\[
\log "K" = \frac{\log SF (Nitrogen) - \log SF (Air)}{2} - \log SF (Air)
\]

Half theoxic: hypoxic differential was 2500 ppm or 0.25% oxygen, although this value is of oxygen passed over the surface of medium not the actual oxygen concentration within the cell suspension.

Porfiromycin showed two plateaux with a steep slope between 200 and 3000 ppm oxygen. Calculation of the "K" value proved easier as surviving fractions for both plateaux were used in the equation above. The "K" value for porfiromycin was 600 ppm or 0.06% oxygen, lower than that of mitomycin C.

4.4 OXYGEN DEPENDENCY OF THE HYPOXIC MARKER; NITP

4.4.1 Method

Cells to be incubated with NITP were treated as previously described in section 4.3.2, keeping cell densities and volumes constant, except that the NITP was weighed, dissolved in a small amount of DMSO (for a final concentration of <1% DMSO in the 20 ml of medium) and the final concentration achieved by addition of medium. The cells were exposed to 100 µM NITP for 2 hours at 37°C. Samples were centrifuged, the supernatant was discarded, cells resuspended in 1 ml of PBS, then 19 ml of 70% ethanol was added and the suspension mixed. The ethanol fixed cells were counted on a haemocytometer, followed by immunofluorescent NITP staining and analysis on the flow cytometer as described in protocol 1 in section 2.4.2.1. The control sample consisted of fixed SaF cells not incubated with NITP but taken through the staining procedure.

Analysis of the flow cytometric data was carried out to determine the mean green fluorescence values, median green fluorescence values and the percentage of cells with positive fluorescent staining compared to that
of control cells not exposed to NITP.

Samples were also analysed to assess whether the NITP bound to the same extent in all phases of the cell cycle. Each flow cytometry profile was subdivided into the constituent (G1, S and G2/M) parts of the cell cycle and individual mean fluorescence values calculated for each phase corrected for the baseline slope.

4.4.2 Results

Figure 4.6A and B show the two extremes of fluorescence obtained using the NITP hypoxic probe measured with the same photomultiplier gains on the flow cytometer. The ten overlaid histograms in Figure 4.6C indicate the amount of cellular fluorescence for each oxygen concentration. The peak becomes both displaced to the right as overall fluorescence increases and broader due to a range of bound NITP adducts incorporated into the cells, which therefore take up different amounts of the fluorescent antibody.

The mean cellular fluorescence is displayed graphically in Figure 4.7. Each symbol represents a single experimental vessel, with data pooled from two groups of six vessels run on the same day with the same batch of SaF cells. All the samples were stained on the same day. Duplicate samples at 20% and 1% show the difference obtained on one day between runs. Median green fluorescence and percentage of cells with greater fluorescent staining than controls provided similar shaped curves but with less differentiation between nitrogen and air samples.

"K" values were calculated, as below, by marking the midpoint of mean green fluorescence, and locating the corresponding oxygen concentration.

\[
\frac{\text{Highest fluorescence} - \text{Lowest fluorescence} + \text{Lowest fluorescence}}{2}
\]

By this means a "K" value of 3000ppm or 0.3% oxygen was calculated.

Table 4.1 shows the ratio of mean green fluorescence of G1, S and G2/M phases to total mean green fluorescence in ten samples incubated under different oxygen concentrations. There was no order of NITP
Figure 4.6  Bivariate dot plots of fluorescence versus DNA content of 100μM NITP incubated with SaF cells under (A) 20% oxygen and (B) nitrogen. (C) Fluorescence histograms of SaF cells incubated under different oxygen concentrations.
Figure 4.7  The mean green fluorescence of SaF cells incubated with 100µM NITP for 2 hours under different oxygen concentrations.
binding with the three values randomly distributed about the mean. Paired t tests to establish whether there was any difference between the three values for each sample showed no significant difference. Although there were slight discrepancies between each cell cycle phase there was no consistent trend of NITP binding specificity.

Table 4.1 NITP binding in each cell cycle phase.

<table>
<thead>
<tr>
<th>Gas</th>
<th>Ratio of Phase to Total Mean Green Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>Nitrogen 0.002% O₂</td>
<td>1.05</td>
</tr>
<tr>
<td>0.02% O₂</td>
<td>1.14</td>
</tr>
<tr>
<td>0.06% O₂</td>
<td>1.00</td>
</tr>
<tr>
<td>0.10% O₂</td>
<td>0.86</td>
</tr>
<tr>
<td>0.10% O₂</td>
<td>0.83</td>
</tr>
<tr>
<td>0.30% O₂</td>
<td>0.94</td>
</tr>
<tr>
<td>0.60% O₂</td>
<td>0.91</td>
</tr>
<tr>
<td>1.00% O₂</td>
<td>1.06</td>
</tr>
<tr>
<td>2.50% O₂</td>
<td>1.03</td>
</tr>
<tr>
<td>Air 20% O₂</td>
<td>1.01</td>
</tr>
<tr>
<td>Mean</td>
<td>0.98</td>
</tr>
<tr>
<td>SEM</td>
<td>0.03</td>
</tr>
</tbody>
</table>
4.5 OXYGEN DEPENDENCY OF RADIATION CYTOTOXICITY

4.5.1 Method

Large numbers of near confluent cells were obtained as previously described from roller bottles and 20ml aliquots containing $5 \times 10^5$ cells/ml were placed in spinner vessels, as the previous experiments for mitomycin C, porfiromycin and NITP. Two vessels could be irradiated simultaneously on the Pantak X-ray set as described in section 2.1.3.5. The spinner vessels were gassed with the appropriate concentration of oxygen for 30 minutes prior to irradiation, when a control sample was taken. Five samples of cell suspension were taken per oxygen concentration at the increasing doses of radiation listed in Table 4.2. X-ray doses were estimated from previous work with Chinese hamster V79-379A cells (Begg et al. 1985b).

<table>
<thead>
<tr>
<th>Gas</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.002% $O_2$</td>
<td>18.00</td>
<td>21.50</td>
<td>25.00</td>
<td>29.00</td>
<td>32.20</td>
</tr>
<tr>
<td>0.06% $O_2$</td>
<td>13.63</td>
<td>16.29</td>
<td>18.93</td>
<td>21.97</td>
<td>24.39</td>
</tr>
<tr>
<td>0.10% $O_2$</td>
<td>12.41</td>
<td>14.82</td>
<td>17.24</td>
<td>20.00</td>
<td>22.20</td>
</tr>
<tr>
<td>0.30% $O_2$</td>
<td>9.42</td>
<td>11.25</td>
<td>13.09</td>
<td>15.18</td>
<td>16.85</td>
</tr>
<tr>
<td>0.60% $O_2$</td>
<td>8.00</td>
<td>9.55</td>
<td>11.11</td>
<td>12.88</td>
<td>14.31</td>
</tr>
<tr>
<td>1.00% $O_2$</td>
<td>7.41</td>
<td>8.85</td>
<td>10.29</td>
<td>11.93</td>
<td>13.25</td>
</tr>
<tr>
<td>2.00% $O_2$</td>
<td>6.82</td>
<td>8.15</td>
<td>9.47</td>
<td>10.98</td>
<td>12.19</td>
</tr>
<tr>
<td>Air</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20% $O_2$</td>
<td>6.00</td>
<td>7.00</td>
<td>8.00</td>
<td>9.00</td>
<td>10.00</td>
</tr>
</tbody>
</table>

Each sample was centrifuged at 1000rpm for 10 minutes. The cells were resuspended in 5ml of medium and then made up to 25ml; during the whole process the cells were kept at 4°C. Cells were counted on a Coulter counter and processed as before for a colony forming assay (section 2.3.2.1). The resulting colonies were counted and the surviving fractions calculated from the irradiated and control plating efficiencies.
4.5.2 Results

The surviving fractions for each sample were plotted against the dose of radiation received. The data were fitted by computer to the equation:

$$\ln \text{SF} = \ln (N) - \frac{X}{D_0}$$

for each oxygen concentration, where SF is the surviving fraction, N is the extrapolation number, X is the dose and $D_0$ is the slope. The mean extrapolation number was calculated for the 8 curves and curves refitted for each oxygen concentration with this common value (Figure 4.8). The $D_0$ values for each of these curves were then plotted against oxygen concentration to create the radiation "K" curve (Figure 4.9).

The "K" value was calculated from the oxygen concentration at the midpoint of the $D_0$ curve, as for the NITP value. This produced a "K" value of 2500ppm or 0.25% oxygen, which is very close to the "K" value for NITP.

4.6 DISCUSSION

*Mitomycin C and porfiromycin in vitro cytotoxicity*

The oxic:hypoxic differential, at a surviving fraction of 0.1, for mitomycin C and porfiromycin in SaF cells was established as 1.7 and 3.5 respectively (Figure 4.1). The differential for mitomycin C was small with only a slight increase in toxicity under hypoxic conditions, however this is also true for mitomycin C in cell lines tested by other investigators (Rockwell *et al.* 1979; Bremner *et al.* 1990). Porfiromycin demonstrated a larger differential than mitomycin C in SaF cells, however there are reports of other bioreductive drugs with much greater differential cytotoxicity (Adams 1992). The most promising aspect of the cytotoxicity of porfiromycin in respect to clinical application is that it has very low aerobic toxicity which means that it would not harm normal well oxygenated cells in the body as much as mitomycin C at equivalent concentrations. This could be an important consideration as it is often the normal tissue toxicity which is the limiting factor in chemotherapy regimes.
Figure 4.8  Radiation survival curves of SaF cells under different oxygen concentrations and their respective curves fitted to the equation \( \ln SF = \ln 17.95 - \frac{X}{D_g} \).

<table>
<thead>
<tr>
<th>Key Symbol</th>
<th>Gas</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>Nitrogen</td>
<td>( \ln SF = \ln 17.95 - \frac{X}{3.730} )</td>
</tr>
<tr>
<td>■</td>
<td>0.06% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>( \ln SF = \ln 17.95 - \frac{X}{3.595} )</td>
</tr>
<tr>
<td>◆</td>
<td>0.1% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>( \ln SF = \ln 17.95 - \frac{X}{2.892} )</td>
</tr>
<tr>
<td>●</td>
<td>0.3% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>( \ln SF = \ln 17.95 - \frac{X}{2.349} )</td>
</tr>
<tr>
<td>▼</td>
<td>0.6% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>( \ln SF = \ln 17.95 - \frac{X}{1.602} )</td>
</tr>
<tr>
<td>○</td>
<td>1.0% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>( \ln SF = \ln 17.95 - \frac{X}{1.502} )</td>
</tr>
<tr>
<td>■</td>
<td>5.0% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>( \ln SF = \ln 17.95 - \frac{X}{1.307} )</td>
</tr>
<tr>
<td>▲</td>
<td>20% O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>( \ln SF = \ln 17.95 - \frac{X}{1.063} )</td>
</tr>
</tbody>
</table>
Figure 4.9  Radiation "K" curve based on $D_0$ values derived from Figure 4.8 for SaF cells.
Oxygen dependency of mitomycin C, porfiromycin, radiation and NITP binding

The oxygen dependence of the two bioreductive drugs showed slightly different features. Both "K" curves reached a plateau at 200ppm oxygen below which there was no significant increase in cytotoxicity. The porfiromycin curve then proceeded via a steep slope to reach an upper plateau, whereas the mitomycin C curve had a short steep slope which then decreased to a gradual incline which never reached a plateau. This is probably characteristic of the mechanism of action of the two drugs, as porfiromycin displays little aerobic toxicity and there is hardly any change in toxicity once the higher oxygen concentrations have been attained so that a plateau is formed. However, mitomycin C forms cytotoxic species in both hypoxic and aerobic environments. Thus mitomycin C in better oxygenated cells is still capable of undergoing metabolism to cytotoxic species, albeit at a lower rate as oxygen increases, so a plateau of toxicity is never attained.

The oxygen dependence of radiation acted as a standard to compare to NITP binding and the bioreductive drugs. The "K" values of radiation and NITP binding were very similar at 2500 and 3000ppm oxygen respectively. If binding of NITP is used to reflect the oxygen status of tissues, for radiotherapy regimes in the future, then the two parameters need to be in good agreement to be relevant. Thus, according to this "K" curve data, metabolism of NITP should be a good predictor of radiosensitivity.

In order to compare the oxygen dependency of radiation, NITP, mitomycin C and porfiromycin, the data was converted into a form suitable for plotting on a linear scale between 0 and 1 (Figure 4.10). The linear plots of radiation and NITP binding were easily transposed by expressing highest $D_0$ or green fluorescence as 1 and lowest $D_0$ or green fluorescence as 0. The surviving fractions for mitomycin C and porfiromycin were made linear by taking logarithms to base 10 and expressing the lowest value (the lowest surviving fraction) as 1 and the highest value (the highest surviving fraction) as 0. Radiation, NITP binding and mitomycin C all have
similar "K" values whereas the "K" value for porfiromycin is 1900 ppm oxygen lower.

![Graph showing comparison of K curves for mitomycin C, porfiromycin, NITP, MMC, POR, and radiation.](image)

Figure 4.10 Comparison of the "K" curves of mitomycin C, porfiromycin, NITP and radiation. Calculation is described in the text.

The larger differential of porfiromycin compared to mitomycin C, at the same concentration, while still producing the same hypoxic cell kill (Figures 4.1 & 4.2) would seem to be an advantage when considering the two extremes of aerobic and hypoxic environments. However, the low "K" value of porfiromycin indicates that it is only effective at very low oxygen concentrations. For example, using radiation and either of the bioreductives in combination on a tumour with a cluster of cells with an oxygen concentration of 1000 ppm. At this oxygen concentration radiation has only 30% of its maximal oxygen effect, so one would expect to use the bioreductive with the greatest differential, porfiromycin, to attack the surviving cells. However, Figure 4.10 shows at 1000 ppm oxygen mitomycin C is much more effective than porfiromycin with corresponding surviving...
fractions of 0.03 and 0.1 respectively. This suggests that cells with intermediate oxygen values may be missed as cells may be relatively resistant to radiation but not sufficiently hypoxic to be affected by porfiromycin.

When discussing differentials for bioreductive drugs it may be important to look at the intermediate oxygen levels as drugs with a steep slope between aerobic and hypoxic cytotoxicity may be unsuitable for targeting certain oxygen levels in a tumour. There are other factors which need to be taken into account when assessing bioreductive drugs, such as systemic toxicity and tissue penetration, but as oxic:hypoxic differential is the main criterion for bioreductive drug development, the direct relationship between oxygen and cytotoxicity needs to be investigated.

_Uptake and fluorescence of NITP throughout the cell cycle_

Flow cytometry analysis of NITP profiles showed that there was no difference in the uptake and fluorescence of NITP throughout the cell cycle. This is an important fact to verify due to subsequent experiments included in this thesis. The baseline of the profile is likely to rise as DNA content increases due to increased background fluorescence (see Figure 4.6A and B). This slight incline is taken into consideration when a control sample is used for quantitative analysis so that further correction is unnecessary.

_Single dose radiation "K" curve_

To maintain consistency of methodology for calculation of the "K" value radiation was assessed at a single dose. The equations in Figure 4.8 were used, where X=10Gy, to calculate a surviving fraction. Adjustment of these surviving fractions to a linear plot, as the bioreductive drugs, led to the radiation "K" curve being shifted slightly to the right of the multi-dose radiation "K" curve with an increase in K value from 3000ppm to 6000ppm oxygen. This shift means that NITP still indicates the presence of the most radioresistant population of cells. At 50% of the maximal radiation cell kill there is still some NITP binding occurring, thus estimations of oxygen status and radiosensitivity are still highly relevant.
CHAPTER 5  **IN VITRO AND IN VIVO SPHEROIDS**

Spheroids are an intermediate model of tumour complexity between cells and subcutaneous tumours. They are ideal to study different subpopulations of cells in an *in vitro* system because as they grow a central core of necrosis develops where nutrients are unable to reach and regions of cellular heterogeneity evolve. An hypoxic zone has been demonstrated in larger spheroids at the limit of oxygen penetration prior to the onset of necrosis and also proliferation characteristics change as depth into the spheroid increases. All the following experiments have used SaF spheroids characterised in Chapter 3. These spheroids, grown as described previously, develop necrosis early in their development between 200 and 300μm in diameter and have an increasing degree of hypoxia as their diameter increases. SaF spheroids were assessed *in vitro* and *in vivo* with respect to their response to mitomycin C and porfiromycin and their suitability as model systems.

5.1 **DOSE RESPONSE OF IN VITRO SPHEROIDS TO BIOREDUCTIVE DRUGS**

5.1.1 Experimental Design

Spheroids were grown from a tumour cell suspension derived from freshly excised SaF tumours. The cells were seeded at $1 \times 10^6$ cells in a solution of 25ml of 2:1 MEM:MEMS + 40mM HEPES buffer in 2% agar coated T-75 flasks. On day 3, the spheroids were fed and the large aggregates and single cells removed by density settling. The large aggregates settled first within 1 minute, the supernatant was removed and allowed to settle for a further 5 minutes. At this time the spheroids had settled but the majority of single cells were still suspended. A fresh 25ml of medium was added, the spheroids resuspended, the resulting suspension divided between two agar coated T-75 flasks and a further 12.5ml of medium added per flask. The spheroids were then fed three times a week and the number of spheroids per flask reduced as the spheroids grew. The spheroids were routinely used for experimentation the
day after feeding, as spheroids were selected for size before being fed.

Spheroids were selected at 250, 400 and 600μm diameter to demonstrate the effect of different percentages of hypoxia on the action of mitomycin C and porfiromycin.

5.1.2 Method

Modified Dreschel gas washing bottles with a spinner bar had approximately 100 SaF spheroids of either 250μm, 400μm or 600μm diameter in 30ml of medium. The spheroids were gassed with 95% air, 5% CO₂ for at least 1 hour prior to treatment in a 37°C warm room. A 2mg vial of mitomycin C powder was dissolved in 5ml of sterile water and then further diluted to produce final concentrations of 1, 2, 4, 8, and 16μM in 30ml of medium with the addition of 1ml of drug. Porfiromycin was weighed, sterile water added and the solution sonicated and warmed to 37°C to aid dissolution. This solution was also further diluted to produce final concentrations of 1, 2, 4, 8 and 16μM in 30ml of medium with the addition of 1ml of drug. The control vessel had 1ml of sterile water added instead of the drug. The drug was then stirred with the spheroids for 1 hour. The suspension was removed and poured into a universal and the spheroids allowed to settle. Immediately the spheroids had settled the supernatant was removed. The spheroids were put on ice, 10ml of PBS added, the spheroids allowed to settle and the PBS removed by pipette. A further 10ml of PBS was added and the spheroids allowed to stand for 3 minutes to permit the drug to permeate from the central regions of the spheroid. The supernatant was removed. The spheroids were then divided into 2 groups for growth delay assessment or disaggregation for colony forming assays.

5.1.2.1 Disaggregation of spheroids for dose response

10ml of 0.05% trypsin/0.02% EDTA was added to the spheroids for disaggregation, the spheroids vortexed and put in a 37°C waterbath. After 5 minutes the spheroids were vortexed again and replaced in the waterbath
until 10 minutes had elapsed. The spheroids were then revortexed and checked to see if they were single cells. The trypsin was then quenched with serum containing medium and centrifuged for 10 minutes at 1000rpm. The single cells were resuspended in medium, counted and plated for a colony forming assay (section 2.3.2.1). Plating efficiencies and surviving fractions were calculated for each drug concentration. The experiment was repeated three times to obtain mean surviving fractions.

5.1.2.2 Growth delay of spheroids for dose response

10ml of medium was added to the spheroids for growth delay assessment. Each spheroid was then placed individually with a pasteur pipette into 2.5ml of medium in a well of a 24 well plate. The 2 diameters of the spheroid were measured using a 90° graduated graticule and the mean diameter calculated as in 3.1.4. The spheroids were then monitored every 2 days until they reached approximately 1mm in diameter. The spheroids were kept in a 5% O₂, 5% CO₂, 37°C incubator and were fed 3 times a week until the end of the experiment.

5.1.3 Results

The plating efficiency of SaF cells from disaggregated spheroids was 0.44±0.03. Figure 5.1 shows the dose response of 250, 400 and 600µm diameter spheroids for mitomycin C and porfiromycin. The difference between the three sizes of spheroids is very small which is surprising due to the difference in the percentage of hypoxic cells within each spheroid. The differential at a surviving fraction of 0.1 between mitomycin C and porfiromycin decreases (4.77, 4.52, 3.87µM), as the spheroid diameter increases (250, 400 and 600µm) and hypoxia within the spheroid increases. This is due to the greater cytotoxicity of porfiromycin in hypoxia. The cytotoxicity of both mitomycin C and porfiromycin was linear up to 8µM where the curves flattened towards 16µM as the range of the assay reached a limit.

Growth delay data demonstrates the difference in aerobic and
Figure 5.1 Dose response of 250μm (upper panel), 400μm (central panel) and 600μm (lower panel) diameter spheroids after exposure to mitomycin C (■) or porfiromycin (●) for 1 hour. Symbols represent means ± SEM of three experiments. Absence of error bars indicates they are within the symbol.
hypoxic toxicity between mitomycin C and porfiromycin to a greater
degree than survival data. Figure 5.2 shows how 250μm diameter spheroids
exposed to increasing doses of mitomycin C have an increase in growth
delay. The majority of spheroids at 16μM and about 50% at 8μM
mitomycin C lost their spheroidal integrity and disintegrated into single
cells, a few of which regrew into separate spheroids and were thus counted
as regrowth of the original spheroid. In Figure 5.3 the 250μm spheroids
treated with porfiromycin hardly showed any growth delay but this would
be consistent with them having little or no hypoxia such that the aerobic
toxicity would be insufficient to cause disruption to normal growth. These
two figures also show that, at larger diameters, toxicity has a much lesser
impact on the size of the spheroid even though surviving fractions are
maintained in the excision assays. Porfiromycin shows greater growth
delay as size and hypoxia of the spheroid increases, whereas mitomycin C
maintains a consistent growth delay. Porfiromycin appeared to cause
asymmetrical toxicity in the liquid-overlay culture probably due to uneven
distribution of oxygen throughout the spheroid. In this case a mean
diameter between the narrowest diameter and its perpendicular diameter
was obtained. There was no apparent asymmetry to the toxicity of
mitomycin C probably due to the smaller oxic:hypoxic differential.

Figure 5.4 shows the comparison between the growth delay of each
spheroid treatment size and dose measured as the time to reach the
diameter of control spheroids at day 5. This value takes into account any
variations in growth patterns, media and incubation conditions between
experiments. Increases in spheroid diameter or volumes could not be
normalised as growth is no longer exponential after about 700μm diameter,
thus the 600μm spheroids would have taken a much greater time to reach
multiples of initial diameter or volume. Mitomycin C growth delay
decreases slightly as diameter increased and porfiromycin growth delay
increased proportionally as hypoxia within the spheroid increased,
however mitomycin C maintained a higher growth delay than
porfiromycin.
Figure 5.2  Growth delay of 250μm (upper panel), 400μm (central panel) and 600μm (lower panel) diameter spheroids after exposure to 0 (●), 1 (○), 2 (∆), 4 (□), 8 (◇) and 16μM (■) mitomycin C for 1 hour. Symbols represent means ± SEM of at least 24 spheroids. Absence of error bars indicates they are within the symbol.
Figure 5.3 Growth delay of 250μm (upper panel), 400μm (central panel) and 600μm (lower panel) diameter spheroids after exposure to 0 (●), 1 (○), 2 (△), 4 (□), 8 (○) and 16μM (■) porfiromycin for 1 hour. Symbols represent means ± SEM of at least 24 spheroids. Absence of error bars indicates they are within the symbol.
Figure 5.4 Comparison of the growth delay of 250µm (---), 400µm (---) and 600µm (---) diameter spheroids to mitomycin C (■) or porfiromycin (○).
Specific growth delay is measured as the time to reach the same diameter as the control on day 5 after treatment.
5.2 DOSE RESPONSE OF *IN VIVO* SPHEROIDS TO BIOREDUCTIVE DRUGS

5.2.1 Experimental Design

Intermediate doses of drugs were required to compare subcutaneous tumours described in Chapter 6 and *in vitro* spheroids described previously. Optimal implantation size, duration and conditions were characterised in Chapter 3. Spheroid implants were performed on three consecutive days so that dissection could be comfortably achieved, and dose groups divided into three to avoid daily fluctuations in conditions affecting results.

5.2.2 Method

Six male CBA mice per dose group were injected with 20 spheroids of approximately 250-350μm diameter with a 19g needle as described fully in section 2.3.4. Seven days after implantation, NITP was administered i.p. followed 1 hour later by BrdUrd and mitomycin C or porfiromycin at doses of 0, 3, 6, 9 and 12mg/kg. 1 hour later the animal was sacrificed and peritoneal spheroids excised as described in section 2.3.4. On excision, the spheroid was immediately rinsed in PBS and placed in a dish of fresh PBS. Excess normal tissue was removed from around the spheroid which was then cut in half, perpendicular to the plane of the normal tissue to which it was attached. One half was immediately frozen in liquid nitrogen. At a later date this was placed in 70% ethanol and processed for histological staining. The other half was washed twice in PBS, then chopped finely with scissors and a single cell suspension prepared for a colony forming assay (described in section 2.3.2.1). Plating efficiencies and surviving fractions were calculated from the resulting stained colonies. Remaining cells were centrifuged, resuspended in 200μl of PBS and fixed in 70% ethanol for flow cytometry analysis. There were insufficient cells from the majority of samples for full correlation of degree of hypoxia and survival therefore only a limited number of samples were processed for hypoxic staining.
5.2.3 Results

Figure 5.5 shows the dose response of SaF in vivo spheroids to mitomycin C and porfiromycin. The upper panel shows individual spheroids excised from different locations in the peritoneum and the variation in surviving fraction. The lower panel shows the mean±SEM surviving fraction from this data. There was a large spread of both mitomycin C and porfiromycin surviving fractions. The location of spheroids did not unduly influence the cytotoxicity of either drug. The number and size of tumour recovered from each mouse differed and thus each tumour potentially had a different dose of bioreductive drug. A number of disaggregated spheroids produced no colonies although many cells were produced and plated and were thus not included in the graphs. When counting cells on a Coulter counter diploid and aneuploid populations could not be distinguished. It could be that those samples which did not produce colonies contained a high proportion of diploid cells.

Flow cytometry analysis of samples showed that some were wholly aneuploid whilst others had a high proportion of diploid cells but there were not enough remaining cells for each sample to adjust the surviving fractions. Figure 5.6 shows two samples with a diploid component (A and C) and two consisting of aneuploid cells only (B and D), deduced from the variation in the red gain on the FACScan. From 14 samples analysed on the flow cytometer for hypoxia the range of aneuploid hypoxia was from 4.6 to 22.0% with a mean of 13.2±1.6%. In the 7 populations of spheroids with a diploid component the percentage of diploid cells ranged from 22 to 58%, each containing some hypoxic cells, this wide range could easily have influenced the surviving fraction.

Figure 5.7 shows sequential sections of a peritoneal spheroid which have been stained for hypoxia (NITP) and proliferation (BrdUrd). This showed that for this peritoneal spheroid, which was attached to the peritoneal wall and reached a maximum diameter of 2350μm, included large areas of hypoxia. The region marked N was a circular area of
Figure 5.5  Dose response of individual (upper panel) and mean±SEM (lower panel) \textit{in vivo} spheroids after exposure to mitomycin C (solid symbols) or porfiromycin (open symbols) for 1 hour.
Hypoxic profile

(A) 15.1%

DNA profile

(B) 19.0%

Green Fluorescence

(C) 22.2%

(D) 5.9%

DNA Content

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</tr>
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<tr>
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<td>D</td>
<td>78.4</td>
<td>3.3</td>
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</tr>
</tbody>
</table>

Figure 5.6 Flow cytometry hypoxia (%) and DNA profiles from SaF peritoneal spheroids.
The accompanying table lists the % of cells in each cell cycle phase of the aneuploid population.
Figure 5.7  Sequential sections of a peritoneal SaF spheroid. x100
(A) NITP staining
(B) BrdUrd staining
necrosis, which was surrounded by hypoxic cells with no BrdUrd staining present. This illustrated that regions of hypoxia and proliferation tend to be segregated although there are a few overlapping areas.

The background staining of the spheroid was due to the biotinylated secondary antibody used on this section, although the hypoxic areas can be easily identified even when this was present. Later staining for NITP adducts with a different antibody (listed in section 2.5.2) produced better definition (see Figures 7.16, 7.17 and 7.18).

5.3 DISCUSSION

*In Vitro Spheroids*

The *in vitro* model worked well displaying an effect of differential toxicity to subpopulations of cells. Growth delay of spheroids exhibited a more clinically relevant method of analysing tumours in situ, whereas surviving fraction provided a quantitative endpoint. Increasing the size of the spheroid increased the percentage of hypoxic cells (Table 3.3). This had little effect on mitomycin C toxicity or growth delay, but a greater effect was demonstrated by porfiromycin with this increase. One possibility could be that mitomycin C was metabolised by aerobic cells so that the drug may not penetrate to the inner more hypoxic cells, whereas porfiromycin is metabolised to a lesser extent by the aerobic cells so a higher concentration of porfiromycin reaches the hypoxic cells. This may also be an explanation for the reduction in growth delay produced by mitomycin C as spheroids increase in size. Larger spheroids have a greater number of cells and thus the limited amount of mitomycin C available could have been utilised prior to reaching the hypoxic cells.

Another feature which highlighted the specificity of porfiromycin cell kill to the hypoxic cells was the asymmetric growth delay of spheroids. When spheroids are grown in liquid-overlay culture the distribution of oxygen is biased, being greater nearer the surface of the media and reduced on the lower portion of the spheroid. Porfiromycin selectively
killed these hypoxic cells creating irregular indentations in the initially round spheroid. Mitomycin C displayed no selectivity reducing the whole spheroid diameter or producing total disintegration of spheroidal integrity.

The loss of spheroid integrity also emphasised that spheroids are probably derived from a subpopulation of SaF cells. On reduction to single cells, all cells which did not disintegrate grew as spheroids unless they became embedded in the agar coating. This probably means that certain SaF cells are better adapted for spheroidal growth and thus when not allowed to attach will grow as spheroids, whereas other cells would die.

**In Vivo Spheroids**

Disadvantages with the peritoneal model of *in vivo* spheroids were that the status of the spheroids could not be judged until after excision so that some animals were treated and no tumours were recovered. Excision of the tumour alone is difficult as normal tissue is also excised and these cells could dilute the resultant cell suspension for colony forming assays and decrease surviving fraction. The spheroids in the peritoneum will be exposed to drug for a longer period of time and at a higher concentration than subcutaneous tumours which have to wait for the drug to be absorbed and transported in the vasculature. The extent of vascularisation in each spheroid is different so this may affect drug metabolism. A problem with the SaF spheroid in particular was that the spheroids appeared to be quite fragile and a low yield of attached spheroids was recovered, all the recovered spheroids were also attached there were no avascular free floating spheroids for comparative analysis.

For the majority of samples insufficient cells remained after colony forming assays were plated to allow flow cytometry analysis. With hindsight, it may have been advisable to keep a number of cells to quantify DNA content with propidium iodide so that adjustments to surviving fraction could have been made with regard to the proportions of diploid and aneuploid cells present.

Figure 3.4 demonstrated that spheroids as small as 250μm contained
hypoxia which increased as the spheroid diameter increased (Table 3.3). Peritoneal spheroids contained a relatively high degree of hypoxia (13.2%) despite becoming vascularised. Histological staining showed that hypoxia was distributed throughout the peritoneal spheroid, although hypoxia tended to be concentrated in large patches. This may have been evidence of blood vessels not reaching these areas of the peritoneal spheroid in the 7 days between implantation and excision. Regions of BrdUrd and NITP staining tended to be mutually exclusive, however there were regions where an overlap could be found.

A double staining procedure for hypoxia and/or proliferation and blood vessels would be a useful technique to explore these findings further. Flow cytometry data is quick and informative, however histology data showing the relationship of these two parameters to tumour structure is a powerful tool. Visual confirmation of a hypothesis is often more convincing than a plethora of numerical data.
CHAPTER 6 SUBCUTANEOUS TUMOURS: HYPOXIA

The most complex tumour model is the *in vivo* tumour where it is very difficult to control internal conditions and individual hosts may display varying reactions to treatments. Subcutaneous tumours are one of the easiest *in vivo* tumours to use as they can be measured easily and any macroscopic effects brought about by treatments may be visualised. The response of cells to the two selected bioreductive drugs, mitomycin C and porfiromycin, has been studied in conjunction with the amount of hypoxia within the tumour, measured by metabolic binding of the hypoxic probe, NITP. The toxicity of mitomycin C and porfiromycin to the whole body and the tumour have been assessed. The paired survival curve method for measurement of hypoxic fraction has been utilised for comparison with flow cytometry data. Flow cytometric techniques have been employed for further analysis of tumour hypoxia relative to tumour size and the distribution of hypoxia throughout the cell cycle, in both an anaplastic and well differentiated tumour.

6.1 EFFECT OF HYPOXIA ON BIOREDUCTIVE CYTOTOXICITY

6.1.1 Time-dependent Response of SaF Tumours to Mitomycin C by Excision Assay

6.1.1.1 Method

Subcutaneous SaF tumours on CBA females were selected at a geometric mean diameter of 9-11mm. A dose of 6mg/kg mitomycin C, 0.3ml at 0.6mg/ml for a 30g mouse, was injected intraperitoneally using a 25g needle. Four mice per time point were sacrificed by cervical dislocation at 0.25, 0.5, 0.75, 1, 1.5, 2 and 24 hours after injection. Control mice were injected with sterile water and sacrificed immediately. The tumour was excised as described in section 2.3.2, and the single cell suspension used in a colony forming assay (section 2.3.2.1) to provide surviving fractions from the number of colonies on the control and treated dishes.
6.1.1.2 Results

A plating efficiency of 0.38±0.02 was obtained for the control animals. The survival curve of SaF tumours exposed to mitomycin C is shown in Figure 6.1. The maximum cell kill at 6mg/kg occurred at 1 hour reaching a surviving fraction of 0.18. The cytotoxic effect of mitomycin C was rapid producing 70% of the maximal cell kill at 15 minutes and varied only slightly between 1 and 2 hours. At 24 hours the mean surviving fraction had risen from 0.22 at 2 hours to 0.36, possibly due to repair of potentially lethal damage, proliferation of survivors diluting dead cells or scavenging of dead cells by macrophages.

6.1.2 Dose Response of SaF Tumours to Mitomycin C by Excision Assay

6.1.2.1 Method

From the previous experiment it was decided that 1 hour would be the optimum time of sacrifice to see the maximal effect of mitomycin C. Doses up to 15mg/kg were selected based on data reported by Rockwell and Kennedy (1979). The experiment was carried out as above with each dose in equivalent volumes of vehicle. Doses of 3, 6, 9, 12 and 15mg/kg of mitomycin C were given intraperitoneally to CBA female mice bearing SaF tumours with a geometric mean diameter of 9-11mm. Each dose point consisted of 6 mice to account for inter-animal variation.

6.1.2.2 Results

At 1 hour the mice treated with 15mg/kg were showing adverse affects from the drug, by hunching their bodies and having ruffled fur and death would have been expected within 24 hours (section 6.1.5). At 15mg/kg there was up to 2 decades of cell kill as shown in Figure 6.2. There was no difference between surviving fraction at a dose of 3mg/kg of mitomycin C and control values.
Figure 6.1  Time-dependent response of SaF tumours to 6mg/kg mitomycin C.
Symbols represent means ± SEM of 4 mice. 
Absence of error bars indicates they are within the symbols.
Figure 6.2  Dose response of SaF tumours to mitomycin C. Symbols represent means ± SEM of 6 mice without NITP administration (□) and 4 mice with NITP administration (■). Absence of error bars indicates they are within the symbols.
6.1.3 Effect of Hypoxic Fraction on Mitomycin C Cytotoxicity to SaF Tumours

6.1.3.1 Method

Groups of 4 mice were selected and treated with mitomycin C as described in section 6.1.2.1. NITP was administered intraperitoneally as described in section 2.3.2 two hours prior to sacrifice and 1 hour before mitomycin C administration. The survival curve in section 6.1.2 acted as a control to establish whether administration of NITP interfered with the action of mitomycin C. After plating cells for the survival curve, the remainder of the cell suspension was centrifuged at 1000rpm for 10 minutes, the pellet resuspended in 1ml of PBS and 19ml of 70% ethanol added to fix the cells. The samples were then stored at 4°C, counted on a haemocytometer and stained for hypoxia by modified protocol 1 in section 2.4.2.1.

6.1.3.2 Results

Figure 6.2 shows that prior administration of NITP had little effect on the toxicity of mitomycin C except for the 12mg/kg dose where the second experiment obtained a higher surviving fraction. Flow cytometric analysis of the tumour cell suspensions, as described in section 2.4.2.4, provided hypoxic fractions for each survival measurement. The four hypoxic fraction values for each dose group were plotted against the corresponding surviving fractions to show any relationship between hypoxic fraction and surviving fraction. Figure 6.3 shows, for each dose of mitomycin C, there is no direct relationship between hypoxic fraction and cytotoxicity. Correlation coefficients for the 4 points were not consistently positive or negative and low, except for a high r value of 0.98 at 15mg/kg which shows a correlation in reverse of that expected.
Figure 6.3  Effect of hypoxia on the cytotoxicity of mitomycin C in vivo at 0, 3, 6, 9, 12 and 15mg/ kg (■,□,●,○,▲,◆).
6.1.4 Effect of Hypoxic Fraction on Porfiromycin Cytotoxicity to SaF Tumours

6.1.4.1 Method

Six CBA female mice were selected with SaF tumours of a geometric mean diameter of 9-11 mm for each dose. Dose groups of 3, 6, 9, 12 and 15 mg/kg were selected so that direct comparisons between mitomycin C and porfiromycin could be made. Porfiromycin was prepared as in section 2.3.3.1 so that equivalent volumes of drug were administered to each mouse (i.e. 9 mg/kg = 0.3 ml of 0.9 mg/ml porfiromycin to a 30 g mouse). Mice were injected intraperitoneally with NITP 2 hours and porfiromycin 1 hour before sacrifice by cervical dislocation. A control group of mice were given 6 mg/kg porfiromycin without prior administration of NITP. Tumours were excised as described previously and a single cell suspension prepared for a 7 day colony forming assay (section 2.3.2.1) with the remaining cells fixed for flow cytometry analysis (section 2.3.2). The samples were then stored at 4°C, counted and stained for hypoxia by modified protocol 1 as described in section 2.4.2.1.

6.1.4.2 Results

The survival of cells from SaF tumours after administration of porfiromycin is shown in Figure 6.4. There was nearly 2 decades of cell kill at the highest dose of 15 mg/kg with a gradual increase in cytotoxicity as dose increased. There was no difference in surviving fraction at 6 mg/kg when NITP was not administered thus NITP did not affect porfiromycin cytotoxicity. Figure 6.4 shows that mitomycin C and porfiromycin were similarly toxic at each dose level.

Figure 6.5 shows the relationship between hypoxic fraction and the cytotoxicity of porfiromycin. There was a direct relationship, unlike mitomycin C, as hypoxia within the tumour increases so does the cytotoxicity of porfiromycin. Correlation coefficients of -0.76, -0.91, -0.87, -0.88, -0.76 were obtained for 3, 6, 9, 12 and 15 mg/kg respectively indicating correlation occurred at all doses of porfiromycin.
Figure 6.4 Comparison of dose response of SaF tumours to mitomycin C (■) and porfiromycin (●).
Symbols represent means ± SEM of 10 and 6 mice respectively.
Mean ± SEM of 6 mice given porfiromycin without prior administration of NITP (○).
Figure 6.5  Effect of hypoxia on the cytotoxicity of porfiromycin in vivo at 0, 3, 6, 9, 12 and 15mg/ kg (■, □, ●, ○, △, ◆).
6.1.5 In Vivo Toxicity of Mitomycin C and Porfiromycin

6.1.5.1 Method

SaF tumour bearing and non-tumour bearing female CBA mice were injected intraperitoneally with a single dose of mitomycin C or porfiromycin. Non-tumour bearing mice were given 3, 6 or 9mg/kg mitomycin C and SaF tumour bearing mice were given 3, 6, 9 or 12mg/kg mitomycin C when the tumour was 4-6mm in diameter. Porfiromycin was given up to 12mg/kg in SaF tumour bearing mice and growth delay data obtained as described in the next section. Non-tumour bearing females were only given a 12mg/kg dose of porfiromycin to ascertain whether a comparable dose to the highest dose of mitomycin C administered had any toxic effect. There were 4 mice per dose point. The mice were then observed for up to 90 days for non-tumour bearing mice or until the tumours reached a geometric mean diameter of approximately 11mm.

6.1.5.2 Results

Table 6.1 shows the toxicity of mitomycin C and porfiromycin to CBA females. If mice appeared, by eye, to be having no adverse effects they were classed as healthy. Mice classed as sick were cool to the touch with ruffled fur and tended to stay in a huddled posture rather than move around the cage. Non-tumour bearing mice were subject to greater

<table>
<thead>
<tr>
<th>Dose mg/kg</th>
<th>Mitomycin C</th>
<th>Porfiromycin</th>
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<tbody>
<tr>
<td></td>
<td>Non-tumour bearing</td>
<td>SaF tumour bearing</td>
</tr>
<tr>
<td>3</td>
<td>Healthy</td>
<td>Healthy</td>
</tr>
<tr>
<td>6</td>
<td>Sick at day 5</td>
<td>Healthy</td>
</tr>
<tr>
<td>9</td>
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<td>Sick at day 8</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>Death by 24hrs</td>
</tr>
</tbody>
</table>
systemic toxicity than tumour bearing mice after mitomycin C administration. Sick mice were humanely killed when symptoms of toxicity became severe.

6.1.6 Growth Delay of SaF Tumours by Mitomycin C and Porfiromycin

6.1.6.1 Method

CBA female mice bearing SaF tumours were injected intraperitoneally with a single dose of mitomycin C or porfiromycin when the tumours reached a geometric mean diameter of approximately 6mm. Doses of 1, 2, 3, 4, 5, 6 and 9mg/kg mitomycin C and 1.5, 3, 6, 9 and 12mg/kg porfiromycin were administered. Four or five mice were selected per dose point and measurements of tumour diameters were recorded at least every second day. Growth curves were calculated for each dose group. The growth curves were normalised by taking the mean of all curves at day zero and adjusting subsequent values by the difference in real and calculated starting point.

6.1.6.2 Results

Figures 6.6 and 6.7 show the normalised growth delay curves of mitomycin C and porfiromycin respectively. For mitomycin C, there was no significant difference between the control and doses up to 6mg/kg. The 9mg/kg data consisted of two mice whose tumours grew comparative to the control tumours and two mice whose tumours had delayed growth. The two tumours with delayed growth shrunk slightly before starting to regrow on day 6 after mitomycin C administration, however the mice began to suffer from toxicity by day 8 exhibited in a lowering of body temperature and a reluctance to move. There was a significant (p<0.05) difference between the control and 9mg/kg mitomycin C using an unpaired t test. For porfiromycin, there was evidence of slight but not significant delay at 9 and 12mg/kg, but porfiromycin did not cause any shrinkage of tumours. It was also less toxic than mitomycin C as there were no manifestations of systemic toxicity in the porfiromycin dose groups.
Figure 6.6  Growth delay of SaF tumours after a single dose of 0, 3, 6 and 9mg/kg (■,□,●,○) of mitomycin C. Other lines represent doses of 1, 2, 4 and 5mg/kg of mitomycin C. Curves were normalised to a common value at day 0. Each point represents the mean ± SEM of at least 4 mice.
Figure 6.7  Growth delay of SaF tumours after a single dose of 0, 1.5, 3, 6, 9 and 12mg/ kg (■,□,●,○,◇,▲) of porfiromycin. Curves were normalised to a common value at day 0. Each point represents the mean ± SEM of at least 4 mice.
6.2 MEASUREMENT OF HYPOXIC FRACTION BY IRRADIATION

Hypoxic fraction can be measured *in vivo* by a number of methods described in the introduction. The method of paired survival curves used here compared cell survival in tumours from air-breathing mice with that in fully hypoxic tumours using an excision assay endpoint.

6.2.1 Method

Six SaF tumour bearing mice with diameters of 8-10mm were used per dose point of radiation. Doses of irradiation were 5, 10, 15, 20, 25 and 30Gy for air-breathing mice and 10, 15, 20, 25 and 30Gy for hypoxic mice. Two hours prior to irradiation the air breathing groups were given NITP intraperitoneally as described in section 2.3.3.2. A control group of un-irradiated mice, not treated with NITP, were included to verify that NITP had no adverse effects on the plating efficiency of SaF cells as well as a control group of un-irradiated mice to which NITP was administered. The mice were irradiated on a Pantak X-ray set (240kVp, 15mA) in a box connected to an air pump to provide them with a supply of free flowing air. Hypoxic groups were sacrificed by cervical dislocation and immediately irradiated in a nitrogen atmosphere. After irradiation tumours were excised and processed to produce a single cell suspension, plated in a colony forming assay with the remaining cells fixed for flow cytometry (section 2.3.2). The fixed cells were then stained for NITP adducts by modified protocol 1 (section 2.4.2.1) and analysed on the flow cytometer.

6.2.2 Hypoxic Fraction of SaF Tumours by Survival Assay.

Figure 6.8 demonstrates the difference in survival of totally hypoxic tumours and tumours from air-breathing mice. A curve can be fitted to the hypoxic data using the equation

\[ \ln(SF) = -\alpha D - \beta D^2 \]  

(1)

where SF = surviving fraction, D = radiation dose and \( \alpha, \beta \) are constants.
Fitting of this curve by computer to all the survival data for individual hypoxic tumours provides values for $\alpha$ and $\beta$ and the unbroken line in Figure 6.8.

A curve fitted to the air-breathing data needs to take into account both the hypoxic and better oxygenated populations of the cells. An equation to express this data is below.

$$\ln(SF) = \ln[p \exp(-\alpha_1 D - \beta_1 D^2) + (1-p) \exp(-\alpha_2 D - \beta_2 D^2)] \quad (2)$$

where $SF = \text{surviving fraction}$, $p = \text{hypoxic fraction}$, $D = \text{radiation dose}$ and $\alpha_1, \beta_1, \alpha_2, \beta_2$ are constants.

Use of this equation requires a number of assumptions. For calculation purposes $SF$ and $D$ are variables and $\alpha_1$ and $\beta_1$ are determined from the data for the hypoxic tumours (Eqn 1). The aerobic population can be related to the hypoxic population by a dose modification factor, $m$, which can be expressed as a function of $\alpha_1, \alpha_2$ and $\beta_1, \beta_2$ assuming strict dose modification at all levels of survival and also assuming there are two discrete populations.

$$m = \frac{\alpha_2}{\alpha_1} \quad (3) \quad \text{and} \quad m = \frac{\sqrt{\beta_2}}{\sqrt{\beta_1}} \quad (4)$$

If a dose modification factor of 3 is used then $\alpha_2 = 3\alpha_1$ and $\beta_2 = 3^2\beta_1$. The curve fitted using equation 2 is shown as the dashed line in Figure 6.8 with the value of $p$, the hypoxic fraction, calculated as 44% ± 5.2.

Alternatively, ratios of the five paired dose points can be taken and a value of hypoxic fraction calculated from

$$\text{Hypoxic fraction} = \exp(\ln \text{aerobic} - \ln \text{hypoxic})$$

which provides a mean result of 49% ± 12.9.
Figure 6.8  Comparison of survival of SaF tumour cells from air-breathing (●) and hypoxic mice (○) to determine hypoxic fraction.

Hypoxic curve  \[ (SF) = -0.0866 \, D - 0.0037 \, D^2 \]

Air-breathing curve  \[ (SF) = \ln (0.4453 \, \exp(-0.0866 \, D - 0.0037 \, D^2)) + (1-0.4453) \, \exp(-0.2597 \, D - 0.0334 \, D^2)) \]
6.2.3 Hypoxic Fraction of SaF Tumours by Flow Cytometry

Flow cytometric profiles of hypoxia were measured against a control tumour and the percentage of hypoxic cells in total and in the aneuploid population were calculated, as described in section 2.4.2.4. Figure 6.9 shows that there was not a positive correlation between surviving fraction and hypoxic fraction in the aneuploid population, except at 10Gy where \( r = 0.91 \) relying on a single outlying point, other doses had \( r \) values less than 0.2. From all the SaF tumours analysed a mean value for aneuploid hypoxia of 9.9% ± 1.6 was obtained for the hypoxic fraction calculated from flow cytometry profiles. These values are much lower than the calculated hypoxic fraction from radiation curves.

6.3 EFFECT OF TUMOUR SIZE ON THE DEGREE OF HYPOXIA

6.3.1 Effect of Size on the Degree of Hypoxia in SaF Tumours

6.3.1.1 Experimental design

CBA mice bearing subcutaneous SaF tumours were measured in three dimensions with callipers and tumours selected between 4 and 11mm in geometric diameter. Each mouse was injected with NITP to distinguish the hypoxic cells and the tumour processed as described in section 2.4.2.1. Flow cytometry profiles were then analysed to estimate the degree of hypoxia within the tumour and this was related to dimensional size and to weight. The hypoxic profiles were then dissected further and hypoxia for and within each phase in the cell cycle calculated, as described in section 2.4.2.4.

6.3.1.2 Results

The amount of hypoxia in the SaF tumour ranges from less than 1 to 29% with a mean from 31 tumours of 9.9%. The degree of hypoxia in the aneuploid cells ranges from less than 1 to 40%, with a mean value of 11.6%. The values for the whole tumour are lower because these values include the diploid cells consisting of endothelial cells, infiltrating macrophages and monocytes which are either in more oxic areas of the
Figure 6.9 Effect of hypoxia on the surviving fraction of irradiated SaF tumours at 0, 10, 20, 25 and 30Gy.
tumour or are less efficient at binding NITP than the aneuploid cells. The amount of hypoxia within the diploid population ranges from less than 1 to 15% with a mean of 5.5%. The percentage of hypoxia in the aneuploid population of individual, different sized tumours is shown in Figure 6.10. This demonstrates that there is no relationship between percentage of hypoxia and size, either geometric mean diameter or weight, in this anaplastic murine tumour.

The constituent parts of the cell cycle were then investigated by defining regions around the G1, S and G2/M phases of the cell cycle and the cells positive for hypoxia. The percentage of cells within each phase was calculated using the Cellfit program on the flow cytometer as described in section 2.4.1.4. The number of hypoxic cells in each phase of the cell cycle is shown in Figure 6.11. It shows that the highest number of hypoxic cells were in the G1 phase as would be expected as this phase contains the greatest number of cells. S and G2/M phases have lower numbers of hypoxic cells. When each phase is examined individually, calculating the percentage of hypoxic cells within that phase (Figure 6.12), it can be seen that the G2/M phase contains the highest proportion of hypoxic cells, approaching 60% in some cases, although these cells constitute a tiny fraction of the total aneuploid component of the tumour.

6.3.2 Effect of Size on the Degree of Hypoxia in Rh Tumours

6.3.2.1 Experimental design

A tumour with totally different characteristics, the Rh, was chosen for comparison of the distribution of hypoxia within the cells with that observed in the SaF tumour. The Rh is a moderately well differentiated carcinoma grown in WHT mice with an \textit{in vivo} doubling time of 6.1 days whereas the SaF is an anaplastic sarcoma grown in CBA mice with an \textit{in vivo} doubling time of 2.1 days.

The experiment was carried out as before (section 6.3.1) and analysed by the same methods for total, aneuploid and diploid hypoxia as well as phase specific hypoxia.
Figure 6.10  Effect of size, in geometric mean diameter and weight, on the total and aneuploid hypoxia of SaF tumours.
Figure 6.11 The percentage of hypoxic cells in G1(○), S(□) and G2/M(♦) phases of the cell cycle in SaF tumours.
Figure 6.12 The proportion of hypoxia within G1(●), S(□) and G2/ M(♦) phases of the cell cycle in SaF tumours.
6.3.2.2 Results

In contrast to the SaF tumour, the Rh had a range of total hypoxic fractions from 12 to 67% with a mean of 32.3%. The aneuploid population had a mean of 35.6% with a range from 11 to 71% and the diploid had lower values overall with a mean of 27.8%, ranging from 10 to 52%. Figure 6.13 shows that like the SaF, the Rh tumour demonstrated no relationship between the size of the tumour, either in geometric mean diameter or weight, and the degree of hypoxia.

The distribution of hypoxia within each phase of the cell cycle was similar to that observed in the SaF, as the greatest number of hypoxic cells were in the G1 population (Figure 6.14A). Analysis of the distribution of hypoxia within each cell cycle phase (Figure 6.14B) showed that, there was a higher proportion of G2 cells which were hypoxic compared with other phases, although the difference was less pronounced than that of the SaF tumour.

6.4 DISCUSSION

Bioreductive Cytotoxicity

Mitomycin C caused rapid cytotoxicity to the SaF tumour after a single 6mg/kg ip. injection (Figure 6.1) This is in agreement with pharmacokinetic data in SaF tumour bearing mice showing a peak plasma concentration of mitomycin C (10.1μM) and porfiromycin (8.2μM) at 10 minutes after a single 6mg/kg ip. injection (M.R.L Stratford, personal communication). However, by 24 hours, tumours had begun to recover from mitomycin C toxicity evidenced by a higher surviving fraction. There are several possible reasons for this reduction in toxicity. Firstly, the SaF tumour is very fast growing with an in vivo volume doubling time of only 2.1 days at 10mm diameter (see Chapter 7), thus the tumour could quickly repopulate with new cells produced by surviving tumour cells. Secondly, Dulhanty et al. (1989) showed that repair deficient cells displayed greater toxicity than their repair proficient parental line towards mitomycin C suggesting that
Figure 6.13 Effect of size, in geometric mean diameter and weight, on the total and aneuploid hypoxia of Rh tumours.
Figure 6.14  
(A) The percentage of hypoxic cells in G1(●), S(□) and G2/M(♦) phases of the cell cycle in Rh tumours.  
(B) The proportion of hypoxia within G1(●), S(□) and G2/M(♦) phases of the cell cycle in Rh tumours.
some of the damage caused by mitomycin C is repairable. Thirdly, flow cytometry DNA profiles demonstrate that 40-50% of cells derived from the SaF tumour are diploid, of which some are probably infiltrating macrophages which could phagocytose dead cells and decrease the number of non-colony forming cells; this would be in addition to the phagocytic cells which would invade the tumour after treatment.

The dose response curve to mitomycin C was basically exponential, although there was a some variation between experiments (Figure 6.2). Porfiromycin also had an exponential dose response curve, but a surprising feature was that it had equivalent in vivo toxicity to mitomycin C (Figure 6.4). From the cell studies in Chapter 4, it would be reasonable to assume that, at a particular dose, the greatest cell kill would be achieved by mitomycin C due to a combination of both aerobic and hypoxic cell kill whereas porfiromycin cell kill is predominantly hypoxic. When this feature is considered with the reduced systemic toxicity of porfiromycin, compared to mitomycin C, then porfiromycin is by far the more efficient bioreductive. Mitomycin C had systemic toxicity at 6mg/kg whereas 12mg/kg porfiromycin did not provide obvious systemic toxicity. Therefore, increasing the dose by this amount would increase tumour toxicity by a decade of cell kill. Rockwell and colleagues showed the LD$_{50}$ of mitomycin C and porfiromycin in EMT6 tumour bearing mice was 8.1 and 43mg/kg respectively, thus if this increase in therapeutic ratio could be transferred to man, porfiromycin would be much more effective as a bioreductive agent than mitomycin C (Rockwell 1992).

A further measure of the effectiveness of treatment is growth delay of murine tumours. Mitomycin C displayed no growth delay up to 6mg/kg and mice given 9mg/kg, where tumour growth was delayed, were affected by systemic toxicity. Likewise, porfiromycin also displayed no growth delay up to the highest dose used of 12mg/kg. The SaF is not a good tumour model for growth delay as an endpoint because it has such a rapid doubling time. Shrinkage of tumours involves death of cells and their removal from the tumour mass to reduce volume. Despite the fact that
both drugs may have killed a significant number of cells, cell loss by phagocytosis, dissolution or apoptosis, and stromal resorption, would take hours to days, thus with an in vivo volume doubling time of 1.9 days at a diameter of 6mm (Wilson et al. 1993) birth of new cells would be sufficient to prevent loss of tumour volume.

Effect of hypoxia on bioreductive cytotoxicity

NITP acts via a bioreductive mechanism, so it had to be ascertained if metabolism of NITP affected the reductive environment of the tumour cells in combination with mitomycin C or porfiromycin. Addition of NITP had no effect on cytotoxicity when combined with either mitomycin C or porfiromycin.

Comparison of surviving fraction with the degree of hypoxia estimated by flow cytometry shows there is no relationship between these two parameters for mitomycin C. There was a large variation in the degree of aneuploid hypoxia as a whole from 2 to 70%. Each dose group contained different distributions of hypoxia. At 3mg/kg mitomycin C, all four tumours had less than 20% hypoxia so in combination with the low dose it was perhaps not surprising there was little correlation. At 9 and 15mg/kg mitomycin C doses there was only a range of 20% difference between the lowest and highest degree of hypoxia so this could have contributed to there being no correlation between cell survival and hypoxia. At 6mg/kg there was a wide spread of hypoxic values so it would have been expected that a tumour with 70% hypoxic cells would have produced a much greater cell kill than one with only 10% hypoxia. One reason for this lack of correlation could be due to the smalloxic:hypoxic differential of mitomycin C producing less distinction between hypoxic and aerobic cell populations.

There was a positive correlation between decreased surviving fraction and increased hypoxia for porfiromycin. The degree of hypoxia in this set of tumours ranged from 1 to 40% so that overall hypoxia in comparison with the mitomycin C set of tumours was reduced. Even the
lowest dose group of six tumours at 3mg/kg, with a 20% range in hypoxia, produced significant correlation between surviving fractions and hypoxia. The toxicity of porfiromycin predominantly depends on the presence of an hypoxic environment; as the aerobic toxicity is only small it will have a limited influence on toxicity, and thus greater hypoxia will be associated with a higher toxicity.

Rockwell and colleagues found that porfiromycin uptake was greater in hypoxic than aerobic cells, so porfiromycin may be retained in the cells over the hour of exposure whilst mitomycin has a more even distribution. Changes in oxygenation due to opening and closing of blood vessels could lead to efflux of porfiromycin from cells and subsequent toxicity to other newly hypoxic cells. Mitomycin C may have a more blanket effect having a short period of greater toxicity as it is metabolised by both aerobic and hypoxic cells whilst porfiromycin has a more specific effect as it is primarily metabolised by the smaller hypoxic population.

**Hypoxic measurement by paired survival curves**

The conventional means of measuring the hypoxic fraction of tumours, as described in the introduction, is by radiation. A pair of air-breathing and dead mice survival curves were used to estimate the percentage hypoxia as a standard for measurement of hypoxia using NITP and flow cytometry. Calculation of the hypoxic fraction gives a value of 44%. One of the major assumptions made when using this method is that all the tumours have the same hypoxic population. Tumours becoming fully hypoxic following death would quickly use any pockets of oxygen by respiration of cells, so the hypoxic curve is only affected by the efficiency with which cells are recovered from tumours in the excision process. However, as individual implanted SaF tumours will have developed a vascular network independently, although histologically classified as the same tumour, morphology will differ from tumour to tumour and thus so should the degree of hypoxia. Vaupel has shown in human tumours that although tumour $pO_2$ measurements may have similar mean values,
variation within tumours is large (Vaupel et al. 1991).

In comparison using flow cytometry analysis, the maximum hypoxia achieved in the aneuploid population of the SaF tumour was 35% (mean 9.9±1.6%). Unfortunately there was no correlation between hypoxia and surviving fraction after irradiation. Even at the highest dose of radiation, 30Gy, with the widest spread of hypoxia from less than 1 to 35% there was no positive correlation between cytotoxicity and hypoxia. As the OER for the effect of radiation on cells is usually around 3, a marked contrast would have been expected, especially at higher doses, between tumours with high and low proportions of hypoxic cells in the same dose group. This is especially true when porfiromycin which has an oxic:hypoxic differential at 10% survival of 3.5 yet shows strong correlation. This expectation is supported by the data in Chapter 4 showing that NITP binding to cells and radiation had very similar "K" values for their oxygen dependency. NITP would be expected to reflect hypoxia to the same extent as toxicity by irradiation. A probable cause of the discrepancy are the intermediate oxygen concentrations within the tumours, which may modulate the radiosensitivity of the tumour while not being marked with NITP. NITP injected 1 hour before irradiation may not be reflecting the oxygen status of the cells at the time of irradiation. Chaplin et al. showed that there may be changes in acute hypoxia over as little as 20 minute intervals because of changes in blood vessel perfusion (1986). Thus, significant change in oxygenation may have occurred in the 1 hour gap between irradiation and NITP binding. Mismatch of two dyes disclosing whether vessels are open in the SaF tumour demonstrated an average of 6.6% mismatch over 20 minutes (S.A. Hill, personal communication). This would be sufficient over an hour to reduce the correlation between radiation toxicity and NITP binding to hypoxic cells.
**Tumour size**

Hypoxic fraction consistently increases with tumour size as size increases from microscopic to a macroscopic lesion, where cells are further from a blood capillary than the diffusion distance of oxygen through tissues. Within the macroscopic range the relationship of tumour size to hypoxic fraction is less clear. There have been conflicting reports on correlation between tumour size and hypoxic fraction. A review by Moulder & Rockwell (1984) showed that in general the size of a tumour did not dictate a high or low hypoxic fraction. However, when specific tumours were compared at different sizes there was a trend for the hypoxic fraction to increase as size increased although this was not true of all tumour types (Fu et al. 1990; Okunieff et al. 1986; Siemann 1980; Stanley et al. 1977; Wallen et al. 1980). Over the size range of 4 to 11mm diameter, both SaF and Rh tumours showed no correlation between size and hypoxic fraction as measured by flow cytometric analysis of bioreductively bound NITP adducts. The range in hypoxic fraction was large, even for tumours of similar diameter or weight. The spread in hypoxic fractions was not related to poor NITP distribution as immunostaining of sections of large tumours (10mm diameter) show hypoxia distributed throughout the tumour.

**Hypoxia and cell cycle**

Profiles of hypoxic cells produced by NITP and flow cytometry showed that most hypoxic cells were in the G1 population, but there were significant numbers of hypoxic cells in the other phases of the cell cycle. The predominance of hypoxic G1 cells in the cell cycle would conform to the theory that cells become hypoxic as they move away from the blood vessels by cell migration. In this state they are thought to arrest in G1 and become quiescent entering a "G0" phase (Tannock 1968; Hirst & Denekamp 1979). However, it is often overlooked that most cells in tumours tend to be in a G1/G0 phase thus one might expect to see greater numbers of hypoxic cells with G1 DNA content. Spheroid work has also shown that as...
the spheroid diameter increases, and a greater proportion of cells become nutritionally deprived, the percentage of G1 cells increases while that in S and G2 decreases (Sutherland et al. 1986). However, other investigators have also found that although the greatest proportion of hypoxic cells are within G1, there are significant numbers of S, G2 and M cells which are hypoxic (Pallavicini et al. 1979; Siemann & Keng 1988).

A more significant point is that when each cell cycle phase (defined by DNA content) was investigated individually, in both SaF and Rh tumours, the highest proportion of hypoxic cells were located within G2. Most in vitro cell cycle studies have shown that induction of hypoxia produces either total arrest of the cell cycle or cell cycle specific arrest. There is usually a block of entry of cells into S resulting in an accumulation at the G1/S border. Cells can either be totally arrested in S, such that no further DNA synthesis occurs or cells may continue to synthesise DNA uninterrupted but have a prolonged duration in S phase. Cells in G2/M tend to be unaffected and continue through to G1 where they may become arrested. In the in vivo studies in this thesis, a high proportion of hypoxic cells in G2 may be due to hypoxic cells in S completing this phase but being unable to continue through mitosis. There is also the possibility that some cells with G2 DNA content are no longer viable but represent cells about to die due to hypoxia regulated events in S or G2. Survival studies on different populations of the cell cycle after the introduction of hypoxic conditions have shown that S phase is the most sensitive, with G2 greatly reduced and G1 slightly reduced. This could mean that hypoxic cells in G2 are cells which have completed S phase but are not re-entering the cell cycle. Wilson et al. (1994) have studied the SaF and Rh tumours in radiation experiments, but showed that 20 to 25% of unirradiated cells labelled with BrdUrd arrested in G2 in the first cycle. This arrest could be prior to cell death, possibly by apoptosis, or it could be an indication that the cells are hypoxic so can not progress through mitosis.
**Comparison of SaF tumour models**

Examination of the percentage of cells in each phase of the cell cycle of the three solid tumour models shows a pattern. Table 6.2 overleaf summarises the mean values of the cell cycle phase distribution of all the subcutaneous and peritoneal SaF tumour data analysed by flow cytometry and the spheroid data from Table 3.3.

**Table 6.2  Cell Cycle Phases and Aneuploid Hypoxia**

<table>
<thead>
<tr>
<th>Model</th>
<th>G1</th>
<th>S</th>
<th>G2/ M</th>
<th>Aneuploid Hypoxia %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous Tumour</td>
<td>57.8</td>
<td>35.5</td>
<td>6.7</td>
<td>9.2</td>
</tr>
<tr>
<td>Peritoneal Spheroid</td>
<td>65.5</td>
<td>24.9</td>
<td>9.6</td>
<td>13.2</td>
</tr>
<tr>
<td>250µm Spheroid</td>
<td>62.4</td>
<td>22.8</td>
<td>14.9</td>
<td>5.0</td>
</tr>
<tr>
<td>400µm Spheroid</td>
<td>75.8</td>
<td>15.3</td>
<td>8.9</td>
<td>15.8</td>
</tr>
<tr>
<td>600µm Spheroid</td>
<td>83.9</td>
<td>10.6</td>
<td>5.5</td>
<td>36.0</td>
</tr>
</tbody>
</table>

The better organised vascularised subcutaneous tumour had the greatest proportion of cells actively synthesising DNA and dividing; in S and G2/M phases. The lesser organised peritoneal spheroids, on average, had fewer cells in S and G2/M, however this tumour model displayed great variation in distribution of cells throughout the cell cycle (Figure 5.6). The distribution of cells in S and G2/M phases of the in vitro spheroid model decreased as size increased indicating a greater proportion of cells entering the dormant G0 phase. These tumour models demonstrate the hypothesis that tumours require vasculature to enable growth and rapid proliferation.

The percentage of hypoxia, which is related to vascular density (Siracka *et al.* 1988; Revesz *et al.* 1989) increases as unvascularised spheroid size increases (5.0 → 15.8 → 36.0), however it decreases sharply as spheroids are vascularised (13.2) and become better organised (9.2) as tumours.
CHAPTER 7 SIMULTANEOUS HYPOXIA AND PROLIFERATION STUDIES

The distribution of hypoxia throughout the cell cycle and in particular the observation that cells in the G2/M phase showed the greatest proportion of hypoxia needed to be explored further. The questions to be answered were (a) Did these cells become hypoxic in G2? (b) As cells become hypoxic in S phase do they arrest in G2? (c) Do hypoxic cells divide? To start to answer these questions the base line cell cycle parameters for the SaF tumour needed to be established.

7.1 CELL CYCLE PARAMETERS IN THE SAF TUMOUR

7.1.1 Experimental Design

CBA mice bearing subcutaneous SaF tumours with a mean diameter of 10mm were injected intraperitoneally with 100mg/kg BrdUrd prepared as described in section 2.3.3.3. Mice were sacrificed every 2 hours, by cervical dislocation, up to 30 hours after injection, the tumour excised and placed immediately in 70% ethanol. Tumours were stained for flow cytometric analysis using modified protocol 2 in section 2.4.3.

7.1.2 Analysis for Cell Cycle Parameters

The following regions were delineated using Lysis II software: labelled and unlabelled BrdUrd cells on a bivariate BrdUrd (FL1) versus DNA content (FL3) dot plot, and diploid G1, total aneuploid cells, aneuploid G1, S, G2 cells and a narrow mid S window on a DNA (FL3) histogram (Figure 7.1). These regions were then used to calculate cell cycle phase durations. Examples of the change in BrdUrd distribution over the cell cycle time are shown in Figure 7.2.

The estimation of duration of the total cell cycle, S phase and G2/M phases are shown in Figures 7.3, 7.4 and 7.5 respectively.

Further cell cycle parameters were calculated from these initial data values combined with the volume doubling time determined from growth curves of the SaF tumour in vivo (measurement of three orthogonal
diameters by callipers every day until the tumour approached 12mm in diameter, followed by calculation of time to increase volume twofold).

**Bivariate Dot Plot**

**DNA Histogram**

**DNA Content**

**DNA Content**

**Regions**

**Bivariate dot plot**

R1  Labelled BrdUrd cells
R2  Unlabelled BrdUrd cells

**DNA histogram**

R3  Diploid G1 cells
R4  Total aneuploid cells
R5  Aneuploid G1 cells
R6  Aneuploid S cells
R7  Aneuploid G2/M cells
R8  Mid-S window

**Figure 7.1** The regions used to calculate cell cycle phase durations on a bivariate BrdUrd versus DNA dot plot and its corresponding DNA histogram for the aneuploid SaF tumour.
Figure 7.2  Distribution of BrdUrd labelled cells throughout the cell cycle, at 1, 2, 4, 6, 12 and 22 hours after intraperitoneal administration of 100mg/ kg BrdUrd in PBS.
Figure 7.3  Estimation of cell cycle time, $T_c$

Cell cycle time ($T_c$) was calculated using the total number and the number of BrdUrd labelled cells in the narrow mid-S window. A ratio of mid-S phase BrdUrd labelled to mid-S total cells over time produces the above graph. The cell cycle time was calculated as the mid-point of the second peak of BrdUrd labelled cells in mid-S phase. This value was estimated as 22 hours.
The duration of S phase ($T_s$) was calculated from the relative movement technique. This expresses the mean DNA content of BrdUrd labelled cells in S phase as a function of the difference in DNA content between G1 and G2/M. This ratio versus time produces the above graph. The length of S phase was calculated as the time when the ratio attained a value of 1 using linear regression. The equation for the computer fitted line was

$$\text{Relative Movement} = 0.047 \times \text{Time} + 0.519$$

which produced a value of 10.2 hours for the duration of S phase.

Figure 7.4  Estimation of duration of S phase, $T_s$

The duration of S phase ($T_s$) was calculated from the relative movement technique. This expresses the mean DNA content of BrdUrd labelled cells in S phase as a function of the difference in DNA content between G1 and G2/M. This ratio versus time produces the above graph. The length of S phase was calculated as the time when the ratio attained a value of 1 using linear regression. The equation for the computer fitted line was

$$\text{Relative Movement} = 0.047 \times \text{Time} + 0.519$$

which produced a value of 10.2 hours for the duration of S phase.
Figure 7.5 Estimation of duration of G2/M phase, $T_{G2/M}$
The duration of G2/M ($T_{G2/M}$) was calculated by plotting the entry of BrdUrd labelled cells into G1. Initially, there should be no BrdUrd labelled cells in G1 but there is an overlap of positive cells from the diploid population. A regression line is thus fitted to the data points between 2 and 8 hours after BrdUrd administration. Extrapolation of this line to zero BrdUrd labelled cells in G1 marks the time in G2/M. The equation for the computer fitted line was

$$\% \text{ BrdUrd labelled cells in G1} = 5.5 \times \text{Time} - 6.3$$

which produced a value of 1.2 hours for the duration of the G2/M phase.
7.1.3 Results

From the three graphs in Figures 7.3, 7.4, and 7.5, $T_C$, $T_S$ and $T_{G2/M}$ were calculated, therefore the duration of the G1 phase is the result of subtraction of the sum of time in S and G2/M phases from the total cell cycle time; $T_{G1} = 10.6$ hours. A volume doubling time ($T_d$) was calculated from the growth of SaF tumours in control animals in the experiment described in 6.1.6. Cell kinetic characteristics were calculated using the following assumptions:- the tumours were growing exponentially, some non-proliferating cells were present and cell loss was random with respect to the cell cycle. In Table 7.1 the cell kinetic characteristics determined in this experiment, with the required equations for calculation overleaf, were compared to those calculated previously for the SaF tumour at the Gray Laboratory (Wilson et al. 1992).

Table 7.1  Cell kinetic parameters of the SaF tumour.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Aneuploid SaF</th>
<th>Aneuploid SaF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI%</td>
<td>23.1</td>
<td>30.6</td>
</tr>
<tr>
<td>$T_C$ (hours)</td>
<td>22.0</td>
<td>22.0</td>
</tr>
<tr>
<td>$T_{G1}$ (hours)</td>
<td>10.6</td>
<td>12.3</td>
</tr>
<tr>
<td>$T_S$ (hours)</td>
<td>10.2</td>
<td>8.1</td>
</tr>
<tr>
<td>$T_{G2/M}$ (hours)</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>$T_d$ (hours)</td>
<td>62.1</td>
<td>45.6</td>
</tr>
<tr>
<td>$T_{FOT}$ (hours)</td>
<td>34.9</td>
<td>21.4</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>0.79</td>
<td>0.81</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>1.54</td>
<td>2.04</td>
</tr>
<tr>
<td>$\beta$</td>
<td>0.020</td>
<td>0.032</td>
</tr>
<tr>
<td>$\theta$</td>
<td>0.44</td>
<td>0.53</td>
</tr>
<tr>
<td>GF</td>
<td>0.55</td>
<td>1.03</td>
</tr>
</tbody>
</table>

*data from Wilson et al. (1992).
Cell Cycle Equations

LI = labelling index, percentage of BrdUrd labelled cells

\[
\frac{\text{BrdUrd Labelled Aneuploid Cells}}{\text{Total Aneuploid Cells}} \times 100
\]

\[T_{\text{POT}}, \text{ the potential doubling time } = \lambda \cdot \frac{T_s}{LI}\]

\[\lambda, \text{ the age distribution of the population calculated by iteration of } \frac{T_{\text{POT}}}{T_s} \left(\exp(\beta(T_s+T_{g2}) - \exp(\beta \cdot T_{g2})\right) , \text{ where } T_{\text{POT}} = \frac{T_s}{LI}\]

\[\alpha, \text{ the average number of daughter cells at each division that stay in cycle } \exp(\beta \cdot T_c)\]

\[\beta, \text{ the growth constant } = \frac{\log_2 2}{T_{\text{POT}}}\]

\[\theta, \text{ the cell loss factor } = 1 - \frac{T_{\text{POT}}}{T_d}\]

\[\text{GF, the growth fraction } = \frac{T_d}{T_c} = \frac{\log_2 2}{(1-\theta) \log_2 (\text{GF}+1)}\]

The variation in data can be mainly attributed to the difference in size of the tumours on excision. This experiment used 10mm diameter SaF tumours whereas the tumours in the former experiment were 6mm in diameter. The larger tumours had a lower labelling index which would be expected due to growth constraints on the tumour mass. The volume doubling time for the 10mm tumour was also greater, as tumour growth tends to decrease as tumour size increases, so this affected the calculations for the cell loss factor and growth fraction. The potential doubling time was increased by calculation of both a longer duration of S phase and a lower labelling index for the tumour.
7.2 DEVELOPMENT OF A TRIPLE STAINING METHOD FOR HYPOXIA, PROLIFERATION AND DNA CONTENT

7.2.1 Experimental Design

The double staining protocols for NITP or BrdUrd and DNA content are listed in sections 2.4.2 and 2.4.3 respectively. Both protocols used propidium iodide for measuring relative DNA content and FITC as the fluorochrome to attach to the particular probe. However, in order to be able to analyse three parameters using the FACScan, fluorochromes of different emission wavelengths were required. As seen in Figure 2.1 the emission spectra of the DNA binding dye, 7-aminoactinomycin D, has less spectral overlap than propidium iodide with the other two fluorochromes which could be used, FITC and R-phycoerythrin. Even so, all of the fluorochromes overlap to some extent in their emission spectra. A successful staining procedure depends critically on sample preparation, antibody dilutions and the choice of fluorochromes for the strongest and weakest signals such that electronic compensation can be minimised.

There were some initial problems with achieving triple staining which could not be attributed to any one step, therefore the techniques for cell and nuclei preparation, DNA denaturation and antibody type and sequence were used in different combinations to achieve success.

7.2.2 Cell and Nuclei Preparation

Mice bearing SaF tumours were injected with NITP 2 hours before and BrdUrd 1 hour before cervical dislocation (as in section 2.4.2). The tumour was excised ready for a variety of cell or nuclei preparations or fixed, intact, in 70% ethanol.

Cell suspensions were made by either mechanical disaggregation or by enzymatic digestion. For mechanical disaggregation, tumours were finely minced, with scissors, then suspended in 5ml of MEM in a 30ml universal. To detach cells the tumour fragments were pipetted with successively smaller bore pipette tips, trying to prevent introduction of air.
bubbles which might disrupt the cell membrane. The resulting suspension was filtered through 35µm nylon mesh and centrifuged at 1000rpm for 10 minutes. The pellet was resuspended in 200µl of PBS and fixed in 10ml of 70% ethanol. This procedure causes the least amount of disturbance to cells and preserves both surface, cytoplasmic and nuclear proteins.

For enzyme digestion, tumours were cut as small as possible then suspended in an enzyme cocktail consisting of either 0.2% collagenase, 0.02% DNAase and 0.02% pronase or just 0.2% collagenase and 0.02% DNAase in 10ml MEM without fetal calf serum. The suspension was rotated on a wheel at 37°C for half an hour, after which MEM containing 10% serum was added to prevent further enzymatic dissociation. The suspension was then filtered through a 35µm nylon mesh and centrifuged at 1000rpm for 10 minutes. The pellet was resuspended in 200µl of PBS and fixed in 10ml of 70% ethanol. This provided a higher yield of cells than just mechanical disaggregation (1.5 fold increase). This procedure has been reported to remove some cell surface antigens but was found to have no effect on either NITP or BrdUrd detection.

Due to the initial lack of success with intact cells, a further method studied was enucleation of tumour cells, to determine whether it would improve the access of the relatively large R-phycoerythrin molecule to the BrdUrd/antibody complex in DNA. In this procedure mechanically disaggregated single cells were put in a lysing buffer (0.25M sucrose, 0.33mM CaCl₂, 0.25% non-ident P40) on ice for 10 minutes, washed and resuspended in 200µl PBS. 7ml of ice cold methanol was added and the suspension kept in the -20°C freezer for 10 minutes. 3ml of distilled water were added to provide a final concentration of 70% methanol. The fixed nuclei were then denatured either with 2.5ml 2M HCl for 12 minutes or with the Endo/Exo III procedure below. This method did not produce distinguishable profiles and was abandoned as a viable alternative.

Intact tumours were disaggregated and enucleated as BrdUrd protocol 2. This method produced DNA histograms containing high proportions of debris and little positive staining so was not used further.
7.2.3 DNA Denaturation

Single whole cell suspensions processed as in protocol 3 produced well defined DNA profiles and double parameter staining but the third parameter with R-phycoerythrin (BrdUrd) did not provide any positive staining.

Addition of pepsin to the HCl solution as in protocol 2, but on cell suspensions rather than intact tumours, led to a good DNA profile being produced but there was little evidence of positive staining with the antibody combination used.

To overcome this an alternative method of denaturation of DNA in fixed single cell suspensions was tried using restriction endonucleases and Exonuclease III to expose halogenated pyrimidines to the antibodies (Dolbeare & Gray 1988) using a less traumatic procedure. The protocol is listed in protocol 7.

**Protocol 7: Denaturation of fixed single cells with Exo/ Endonucleases**

1. Approximately 2 million cells were washed in 5ml of PBS.
2. The pellet was resuspended in 1ml of 1mg/ml RNAse for 10 minutes at 37°C to remove double stranded RNA, which the DNA marker could subsequently bind to, followed by centrifugation at 2000rpm for 5 minutes and the supernatant discarded.
3. Cells were resuspended in 1ml of cold 0.1M citric acid and 0.5% Triton X-100, and put on ice for 10 minutes; to remove histones and other nuclear proteins which could effect enzyme activity. Then centrifuged and the supernatant discarded.
4. The cells were washed in 3ml of 0.1M Tris-HCl, pH 7.5 with 50mM NaCl and 10mM MgCl₂.
5. The pellet was resuspended in 100μl Bam H1 incubation buffer (10mM Tris-HCl, 100mM NaCl₂, 5mM MgCl₂, 1mM 2-mercaptoethanol in distilled water adjusted to pH 8.0) with 60 units of Bam H1 for 30 minutes at 37°C.
6. 3ml of PBS diluted ten times with distilled water were added followed by centrifugation.
7. The pellet was then resuspended in 100μl Exo III incubation buffer (66mM Tris-HCl, 0.66mM MgCl₂, 1mM 2-mercaptoethanol in distilled water adjusted to pH 7.6) and 100 units of Exo III for 30 minutes at 37°C.

8. 3ml of PBS diluted ten times with distilled water were added, then centrifuged. The pellet was then ready for suspension in the first antibody.

DNA profiles prepared by this method also contained a large proportion of debris and showed very little positive staining. The lack of positive staining with different denaturation procedures suggested that there may be deficiencies in the antibody procedures. Thus the protocol producing the best defined DNA profile was selected for further optimisation.

This protocol utilised fixed enzyme digested single cell suspensions and the cells were denatured with pepsin and HCl to expose the antigenic sites (Figure 7.6).

7.2.4 Antibody Incubation

Primary antibodies available for attachment to the proliferative and hypoxic probes consisted of rat or mouse anti-BrdUrd and rabbit antitheophylline. Secondary antibodies consisted of FITC conjugates of rat, mouse and rabbit immunoglobulins or biotin conjugates of rat, mouse and rabbit immunoglobulins which could be biochemically linked to streptavidin-R-phycoerythrin or directly to R-phycoerythrin as the second antibody fluorochrome.

The first combination tried was simultaneous incubation with the standard primary antibodies 25μl rat anti-BrdUrd (1 in 10 dilution) and 250μl rabbit anti-theophylline (neat) for 1 hour as they were derived from different species. The weakest primary antibody (rabbit anti-theophylline) was combined with the strongest fluorochrome (goat anti-rabbit FITC) and biotin conjugated anti-rat immunoglobulin at 1 in 10 dilutions for 1 hour.
Figure 7.6 Comparison of DNA profiles from different DNA denaturation procedures using SaF tumours.
in PNT. Then 1:10 diluted R-phycoerythrin-conjugated streptavidin was incubated with the nuclei for 30 minutes and 7-AAD used as the DNA marker. There was no positive staining for either of the markers with pepsin digested nuclei direct from tumour pieces but positive NITP adduct staining when denatured single cell suspensions were used with the same antibody combination. In all subsequent staining attempts the antibodies were kept at the same dilutions as above, unless otherwise stated, and the primary and secondary antibodies incubated for 1 hour at room temperature.

Further manipulations were carried out on the BrdUrd portion of the staining procedure. Double staining of BrdUrd with single cell preparations using the standard rat anti-BrdUrd and FITC conjugated anti-rat antibodies showed evidence of positive staining but the same samples utilising anti-rat biotin with R-phycoerythrin conjugated streptavidin did not produce positive results when either diluted in PNT or theophylline antiserum. The secondary antibodies were then switched so that anti-rat FITC conjugate and anti-rabbit biotin conjugate were used in conjunction with R-phycoerythrin conjugated streptavidin. This produced little positive staining for either investigative probe. The biotin-streptavidin complex may not have produced good results because the large R-phycoerythrin molecule could not penetrate the DNA double helix to attach to the biotin.

The binding of R-phycoerythrin to BrdUrd appeared to be the main problem. Therefore in an attempt to simplify this procedure, a direct conjugate of R-phycoerythrin to a mouse IgG was tried. It should be noted that this was not used originally because the signal intensity can be improved using the biotin/streptavidin system, and that the standard BrdUrd antibody was developed in rat and no anti-rat IgG R-phycoerythrin conjugates were available. Therefore the monoclonal was changed to a mouse derived antibody from Dako. Initially this protocol demonstrated no positive staining for BrdUrd so a Caltag mouse monoclonal (BR3), which recognised bromodeoxyuridine specifically, was used in combination with the anti-mouse R-phycoerythrin.
All the procedures tried above failed to provide positive triple staining so it was thought that the two primary antibodies could be blocking each other and thus the two staining processes were tried in succession.

2M HCl denaturation of fixed single cell suspensions for 25 minutes was followed by different combinations of primary and secondary antibodies. NITP/anti-rabbit biotin conjugate and BrdUrd/anti-rat FITC conjugate failed to provide good NITP staining. NITP/anti-rabbit FITC conjugate and BrdUrd/anti-rat biotin conjugate produced good hypoxic and BrdUrd staining profiles, as did the combination NITP/anti-rabbit FITC conjugate and the direct anti-mouse R-phycoerythrin conjugate for BrdUrd. However, the second positive combination with the direct anti-mouse R-phycoerythrin conjugate had greater green fluorescence and the calculated values for NITP and BrdUrd staining were more comparable with those of double parameter staining protocols (Figure 7.7).

7.2.5 Optimisation of Triple Staining Technique

Once a preliminary staining procedure was found, it could be refined. Addition of pepsin to the HCl denaturation was tested to improve the access of the large R-phycoerythrin molecule to the DNA attached mouse monoclonal. Different pepsin concentrations were added to aliquots of the same cell sample for 20 minutes. DNA profiles were then examined, as demonstrated in Figure 7.6, for extent of damage and definition and the BrdUrd profiles analysed for greatest fluorescence (Table 7.2).

### Table 7.2 Effect of different pepsin concentrations on BrdUrd staining

<table>
<thead>
<tr>
<th>Pepsin mg/ml</th>
<th>Total BrdUrd (%)</th>
<th>Aneuploid BrdUrd</th>
<th>Aneuploid BrdUrd (%)</th>
<th>Mean Y Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>13.2</td>
<td>1138/6856</td>
<td>16.6</td>
<td>250</td>
</tr>
<tr>
<td>0.15</td>
<td>14.5</td>
<td>1254/6687</td>
<td>18.8</td>
<td>237</td>
</tr>
<tr>
<td>0.2</td>
<td>12.4</td>
<td>1145/6149</td>
<td>18.6</td>
<td>241</td>
</tr>
<tr>
<td>0.3</td>
<td>13.8</td>
<td>1184/6660</td>
<td>17.8</td>
<td>219</td>
</tr>
<tr>
<td>0.4</td>
<td>12.2</td>
<td>1025/6933</td>
<td>14.8</td>
<td>203</td>
</tr>
</tbody>
</table>
Figure 7.7  A single triple stained sample displaying the variation in binding produced by different secondary antibody combinations.
There was little difference between digestion with 0.15 and 0.2mg/ml pepsin in 2M HCl, both yielding similar high percentages of aneuploid BrdUrd labelled cells, but 0.2mg/ml pepsin had slightly higher mean \( y \) (BrdUrd related) fluorescence with a better defined BrdUrd profile and was chosen as the optimum concentration of pepsin.

Varying lengths of time in 0.2mg/ml pepsin/2M HCl solution were then tested and compared to the standard preparation of 25 minutes in 2M HCl. The DNA profiles showed that at 10 minutes the DNA was not sufficiently denatured and at 30 minutes was being disrupted. At 25 minutes the BrdUrd staining was decreasing. The optimum time of 20 minutes was chosen as the percentage aneuploid figure was closest to that obtained with the standard technique (25 minutes in 2M HCl) used already in the laboratory (Table 7.3).

**Table 7.3** Effect of different times in 0.2mg/ml pepsin/2M HCl on BrdUrd staining

<table>
<thead>
<tr>
<th>Time minutes</th>
<th>Total BrdUrd</th>
<th>Aneuploid BrdUrd</th>
<th>Aneuploid BrdUrd (%)</th>
<th>% Aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>542/2852</td>
<td>441/2232</td>
<td>19.8</td>
<td>78.3</td>
</tr>
<tr>
<td>15</td>
<td>563/3744</td>
<td>399/2137</td>
<td>18.7</td>
<td>57.1</td>
</tr>
<tr>
<td>20</td>
<td>589/3786</td>
<td>432/2329</td>
<td>18.6</td>
<td>61.5</td>
</tr>
<tr>
<td>25</td>
<td>481/3677</td>
<td>377/2364</td>
<td>15.9</td>
<td>64.3</td>
</tr>
<tr>
<td>30</td>
<td>521/3762</td>
<td>409/2549</td>
<td>16.1</td>
<td>67.8</td>
</tr>
<tr>
<td>25 HCl only</td>
<td>------</td>
<td>------</td>
<td>--</td>
<td>60.6</td>
</tr>
</tbody>
</table>

The hypoxia staining procedure was then analysed with the addition of pepsin to the denaturation step for varying amounts of time. The DNA profiles were analysed using the SOBR model on Cellfit as described in 2.4.1.5. Hypoxia was defined by setting a region around the positive cells, by using a tumour not treated with NITP but taken through the staining procedure as a control sample. All the DNA profiles of the pepsin/HCl digests were better defined than digestion with HCl alone for 25 minutes as demonstrated previously with the BrdUrd staining (Figure 7.6).
There was very little difference between samples regarding the percentages of cells in each cell cycle phase and the percentage aneuploid hypoxia had only a slight variation (Table 7.4). It was therefore concluded that the optimum time and concentration for the pepsin/HCl digest was 20 minutes in 0.2mg/ml pepsin in 2M HCl.

Table 7.4 Effect of pepsin/2M HCl digest on hypoxic staining

<table>
<thead>
<tr>
<th>Pepsin mg/ml</th>
<th>Time mins</th>
<th>G1 %</th>
<th>S %</th>
<th>G2 %</th>
<th>Total Hypoxia</th>
<th>% An. Hypoxia</th>
<th>Mean Y Fluores.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl</td>
<td>25</td>
<td>73.7</td>
<td>14.1</td>
<td>12.2</td>
<td>18.2</td>
<td>14.7</td>
<td>130</td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>60.2</td>
<td>33.9</td>
<td>5.9</td>
<td>22.2</td>
<td>19.7</td>
<td>112</td>
</tr>
<tr>
<td>0.1</td>
<td>15</td>
<td>62.2</td>
<td>31.0</td>
<td>6.8</td>
<td>17.4</td>
<td>15.5</td>
<td>140</td>
</tr>
<tr>
<td>0.1</td>
<td>20</td>
<td>61.1</td>
<td>33.4</td>
<td>5.5</td>
<td>19.0</td>
<td>16.3</td>
<td>135</td>
</tr>
<tr>
<td>0.15</td>
<td>10</td>
<td>63.2</td>
<td>31.0</td>
<td>5.9</td>
<td>22.0</td>
<td>16.6</td>
<td>118</td>
</tr>
<tr>
<td>0.15</td>
<td>15</td>
<td>59.0</td>
<td>34.9</td>
<td>6.1</td>
<td>17.5</td>
<td>14.2</td>
<td>141</td>
</tr>
<tr>
<td>0.15</td>
<td>20</td>
<td>62.5</td>
<td>32.9</td>
<td>4.6</td>
<td>21.3</td>
<td>17.5</td>
<td>154</td>
</tr>
<tr>
<td>0.2</td>
<td>10</td>
<td>61.0</td>
<td>32.8</td>
<td>6.2</td>
<td>20.1</td>
<td>16.5</td>
<td>122</td>
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<tr>
<td>0.2</td>
<td>15</td>
<td>62.1</td>
<td>29.9</td>
<td>8.0</td>
<td>22.0</td>
<td>18.4</td>
<td>128</td>
</tr>
<tr>
<td>0.2</td>
<td>20</td>
<td>60.5</td>
<td>31.9</td>
<td>7.6</td>
<td>19.0</td>
<td>15.1</td>
<td>139</td>
</tr>
</tbody>
</table>
Once this was verified the triple staining protocol was subjected to further optimisation. Pepsin/HCl digested samples were double and triple stained and compared to the same sample stained using the standard double staining procedures for NITP and BrdUrd probes. DNA profiles were analysed using the SOBR model on Cellfit as described in section 2.4.1.5 and the results are shown in Table 7.5. Pepsin/HCl digested samples (3-7) had much better defined DNA profiles so the cut off points for G1, S and G2 regions were much easier to delineate. The G2 peak was noticeably much easier to define.

This experiment demonstrated that BrdUrd staining needed to be prior to hypoxic staining (6 and 7) to obtain accurate results for hypoxia. The triple staining with BrdUrd before hypoxia showed comparative results to the double stained samples.

<table>
<thead>
<tr>
<th></th>
<th>G1 %</th>
<th>S %</th>
<th>G2 %</th>
<th>% Total BrdUrd</th>
<th>% Aneu. BrdUrd</th>
<th>% Total Hypoxia</th>
<th>% Aneu. Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74.8</td>
<td>14.6</td>
<td>10.6</td>
<td>19.3</td>
<td>21.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>77.0</td>
<td>13.9</td>
<td>9.1</td>
<td>18.9</td>
<td>21.3</td>
<td>26.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>64.3</td>
<td>29.8</td>
<td>5.9</td>
<td>18.5</td>
<td>21.1</td>
<td>24.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>64.1</td>
<td>30.7</td>
<td>5.2</td>
<td>18.5</td>
<td>23.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>62.4</td>
<td>31.9</td>
<td>5.6</td>
<td>20.7</td>
<td>25.8</td>
<td>10.0</td>
<td>10.7</td>
</tr>
<tr>
<td>6</td>
<td>64.0</td>
<td>29.9</td>
<td>6.1</td>
<td>20.7</td>
<td>25.8</td>
<td>10.0</td>
<td>10.7</td>
</tr>
<tr>
<td>7</td>
<td>63.4</td>
<td>30.2</td>
<td>6.4</td>
<td>19.4</td>
<td>23.8</td>
<td>23.6</td>
<td>26.9</td>
</tr>
</tbody>
</table>

2. Standard NITP double staining preparation with rabbit anti-theophylline and anti-rabbit FITC conjugate.
3. Pepsin/HCl digest with theophylline antiserum and anti-rabbit FITC conjugate.
5. Pepsin/HCl digest control sample.
6. Pepsin/HCl digest with NITP adduct followed by BrdUrd staining.
7. Pepsin/HCl digest with BrdUrd followed by NITP adduct staining.
Optimisation of primary and secondary antibodies, mouse monoclonal to human BrdUrd and anti-mouse R-phycoerythrin conjugate, for the BrdUrd portion of the staining protocol is shown below (Table 7.6). Dilutions of 1/20 primary antibody and 1/5 secondary antibody produced the highest degree of staining with a well proportioned BrdUrd profile so were chosen as the optimum concentrations. The amount of PNT the cells were resuspended in was reduced to 100µl to increase cost efficiency of the triple staining method. To facilitate staining in a reduced volume the initial number of aliquoted cells was decreased to one million. After addition of R-phycoerythrin conjugate, which tends to be light sensitive, samples were kept in the dark for the remainder of the experiment.

Table 7.6 Antibody dilution for BrdUrd staining

<table>
<thead>
<tr>
<th>1° Antibody dilution</th>
<th>2° Antibody dilution</th>
<th>% Aneuploid</th>
<th>% Total BrdUrd</th>
<th>% Aneuploid BrdUrd</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/20*</td>
<td>1/20</td>
<td>72.2</td>
<td>22.5</td>
<td>28.9</td>
</tr>
<tr>
<td>1/20</td>
<td>1/5</td>
<td>74.2</td>
<td>27.4</td>
<td>33.4</td>
</tr>
<tr>
<td>1/20</td>
<td>1/10</td>
<td>73.1</td>
<td>19.8</td>
<td>24.6</td>
</tr>
<tr>
<td>1/20</td>
<td>1/12.5</td>
<td>75.1</td>
<td>20.0</td>
<td>24.2</td>
</tr>
<tr>
<td>1/20</td>
<td>1/20</td>
<td>73.9</td>
<td>19.0</td>
<td>23.8</td>
</tr>
<tr>
<td>1/20</td>
<td>1/50</td>
<td>74.9</td>
<td>12.6</td>
<td>15.9</td>
</tr>
<tr>
<td>1/5</td>
<td>1/10</td>
<td>73.2</td>
<td>22.7</td>
<td>27.8</td>
</tr>
<tr>
<td>1/10</td>
<td>1/10</td>
<td>73.3</td>
<td>15.8</td>
<td>21.3</td>
</tr>
<tr>
<td>1/50</td>
<td>1/10</td>
<td>73.9</td>
<td>12.5</td>
<td>15.2</td>
</tr>
</tbody>
</table>

* Standard 2M HCl digestion

The final staining procedure is listed in section 7.2.6 with examples of BrdUrd, NITP and DNA profiles for a positive sample and control in Figure 7.8.
7.2.6 Final Triple Staining Procedure

1. 1 million cells were washed in 5ml of PBS.

2. The pellet was resuspended in 2.5ml 0.2 mg/ml pepsin in 2M HCl for 20 minutes.

3. Twice, 5ml of PBS was added, the tubes centrifuged at 2000rpm for 5 minutes and the supernatant decanted.

4. The pellet was resuspended in 0.1ml of PBS with 0.1% normal goat serum and 0.5% Tween 20 (PNT) adding 5μl of Dako mouse anti-human antibody to BrdUrd and incubated at room temperature (RT) for 1 hour.

5. 5ml of PBS was added, the tubes centrifuged at 2000rpm for 5 minutes and the supernatant decanted.

6. The pellet was resuspended in 0.1ml of PNT adding 25μl of goat R-phycoerythrin conjugated anti-mouse antibody and incubated in the dark at RT for 1 hour. The samples were kept in the dark for the rest of the protocol.

7. 5ml of PBS was added, the tubes centrifuged at 2000rpm for 5 minutes and the supernatant decanted.

8. The pellet was resuspended in 250μl of rabbit anti-theophylline antibody for 1 hour.

9. 5ml of PBS was added, the tubes centrifuged at 2000rpm for 5 minutes and the supernatant decanted.

10. The pellet was resuspended in 0.25ml PNT with 25μl goat FITC conjugated anti-rabbit antibody for 1 hour.

11. The pellet was resuspended with 2ml of PBS and 20μl of 1mg/ml 7-aminoactinomycin D added.

12. The stained suspensions were analysed on the FACScan using LYSIS II software.
Figure 7.8  A sample and control specimen demonstrating triple staining for BrdUrd, NITP and DNA content in a SaF tumour.
7.3 CELL CYCLE PROGRESSION OF HYPOXIC CELLS

Two approaches were used to study cell cycle progression and oxygenation status. Firstly tumours were pulse-labelled with BrdUrd and then injected with NITP 2 hours before each sacrifice time. Secondly both probes were injected simultaneously and followed at different times as the labelled cells progressed through the cell cycle.

7.3.1 Experimental Design

Two different tumours were studied the SaF and CaNT. Tumour bearing mice were injected with 100mg/kg of BrdUrd intraperitoneally when the tumour approached 10mm in diameter. Two hours prior to sacrifice by cervical dislocation the mice were injected with NITP as described in section 2.3.3.2. Two mice were sacrificed every two hours up to 8 hours, then two mice every 4 hours, so that a complete cell cycle traverse by BrdUrd incorporated cells could be studied. Tumours were excised, a single cell suspension prepared, fixed in 70% ethanol and stored at 4°C as described in section 2.3.2. The fixed tumour cell suspensions were then triple stained as the protocol in section 7.2.6, with green fluorescence distinguishing hypoxic cells, orange/red fluorescence distinguishing cells in the S phase of the cell cycle and 7-aminoactinomycin D (far red) acting as the DNA marker.

7.3.2 Analysis of Flow Cytometric Profiles

Bivariate dot plots of hypoxia (FL1) or proliferation (FL2) versus DNA content (FL3) were produced from data collected on all tumours and regions of positive fluorescence defined from control, non-injected, tumours. DNA histograms were dissected into total aneuploid and constituent aneuploid G1, S and G2/M regions. The number of hypoxic cells, BrdUrd incorporated cells and cells containing both fluorochromes were analysed.

Figure 7.9 shows region 1 as hypoxic cells, region 2 as BrdUrd labelled cells, region 3 as total aneuploid cells, and regions 4, 5 and 6 as
Figure 7.9  Analysis of triple stained BrdUrd and NITP profiles.
G1, S and G2/M phases of the cell cycle. The top panel shows the total cells in each of these 6 regions and where the regions were defined. The second panel shows only those cells which were hypoxic in region 1. The third panel shows the total number of cells with BrdUrd incorporated and the forth panel shows only those cells which incorporated both BrdUrd and NITP (cells in region 1 and region 2). From each of these panels separate calculations for the numbers of cells in each region were made so that each subset of cells could be identified.

These calculations have been visualised to aid understanding (Figure 7.10). The data can be analysed from two approaches. The hypoxic and oxic cells can be defined and the cells from each of these regions displayed on a bivariate BrdUrd versus DNA dot plot, to establish whether hypoxic cells had previously incorporated BrdUrd. The alternative method is to define the positive BrdUrd incorporated cells and display whether they have become hypoxic with time.

7.3.3 Results

Figure 7.9 shows the regions defined and illustrated in Figures 7.11 and 7.12. BrdUrd positive cells were selected and this region was divided into total aneuploid, G1, S and G2/M phases by DNA histogram regions to obtain total and phase specific BrdUrd labelled cells. An hypoxic region was delineated on the NITP profile and the number of cells within both this and the region of BrdUrd labelled cells calculated in total and for each cell cycle phase.

Figures 7.11 and 7.12 show the distribution of hypoxia in each phase of the cell cycle, in cells from SaF and CaNT respectively, that have incorporated both BrdUrd and hypoxia labels. The dashed line represents total BrdUrd labelling distributed throughout the three cell cycle phases. For instance, the dashed line in Figure 7.11 (middle panel) demonstrates that the amount of BrdUrd labelled cells in S phase starts high, as would be expected, gradually decreases as BrdUrd labelled cells leave the S phase, and a second peak follows as cells that have been through G2, divided and
Figure 7.10  Various possible displays of triple stained data.
Figure 7.11 The percentage of hypoxia within cells incorporating BrdUrd from G1 (upper panel), S (middle panel) and G2/M (lower panel) cell cycle phases of the SaF tumour. The dashed line represents total BrdUrd labelled cell distribution among the three cell cycle phases.
Figure 7.12  The percentage of hypoxia within cells incorporating BrdUrd from G1 (upper panel), S (middle panel) and G2/M (lower panel) cell cycle phases of the CaNT tumour. The dashed line represents total BrdUrd labelled cell distribution among the three cell cycle phases.
proceeded through G1, re-enter S phase for the second time. The number of BrdUrd labelled cells in G2/M starts high because by the first time point of two hours, late S phase cells that have incorporated BrdUrd have traversed G2/M. The value remains stable as BrdUrd labelled cells continue to leave S phase. The number of G2/M cells with BrdUrd then decreases as all the labelled cells have progressed through S and G2/M and only rises again as cells on their second cell cycle pass through this phase. The number of BrdUrd labelled cells in G1 starts low and increases as the number of cells that have progressed through S and G2/M increases and grows until cells labelled in early S phase have traversed G1, when the numbers finally decrease. SaF cells have a cell cycle time of 22 hours and CaNT cells a cell cycle of 15 hours so the rate of progression of the BrdUrd cells represented by the dashed line in each panel differs between the two tumours but the pattern is similar. The well-defined pattern of BrdUrd labelled cells entry and exit into the cell cycle phases tends to become less distinguishable in the second cycle. This is a well known phenomena due to the decreased BrdUrd levels in each daughter cell, progression of cells at slightly different rates and the possible factor of cells leaving the active cell cycle and entering a resting state.

The symbols in Figures 7.11 and 7.12 represent the hypoxic BrdUrd labelled cells and how they are distributed around the cell cycle (Panel D-Figure 7.9) as a percentage of the total BrdUrd and NITP labelled population. In the SaF tumour (Figure 7.11), as BrdUrd labelled cells exit G2 the percentage of hypoxic cells in this phase rises, from about 20% at 2 hours up to 60% at 16 hours. In G1 the percentage of hypoxia in BrdUrd labelled cells appears to decrease up to 12 hours, as the total number of BrdUrd labelled cells in G1 reaches its peak, implying that hypoxic cells have not entered G1. In the S phase there is no particular pattern, and the remaining cells may be arrested. After 16 hours there is little variation in the percentage of hypoxia in the three cell cycle phases.

In the CaNT tumour (Figure 7.12), in G1 (upper panel) there appears to be two distinct falls in BrdUrd incorporated hypoxic cells which coincide
with two complete cell cycles. As cells enter G1 in the normal BrdUrd labelled manner there are fewer which are also hypoxic. In S phase (middle panel), in the duration of a single cell cycle, there is a sharp rise in hypoxia in S phase, whilst BrdUrd labelled cells leave S, followed with a fall in hypoxia as cells re-enter S phase for a second time; implying that hypoxic cells do not enter S phase. In G2 (lower panel), there is a rise in the percentage of hypoxic cells up to 8 hours, then a slight fall as the majority of BrdUrd labelled cells leave G2, however the initial percentage of hypoxia in G2 is not regained but a higher baseline for the second complete cell cycle.

7.4 SIMULTANEOUS ADMINISTRATION OF NITP AND BrdUrd

7.4.1 Method

In these experiments NITP and BrdUrd were administered together at time zero to monitor the progress of hypoxic cells throughout the cell cycle. NITP was administered and BrdUrd given within 1 minute by i.p. injection to SaF tumour bearing CBA males when the tumour approached 10mm in diameter. Tumours were removed at 2 and 4 hours and then at 4 hour intervals up to 28 hours. Tumours were excised (Section 2.3.2), stored as fixed single cell suspensions at 4°C, then stained as in section 7.2.6.

7.4.2 Analysis of Triple Stained Profiles

The regions were defined to analyse the BrdUrd labelled nuclei on the BrdUrd profile (FL2 vs FL3A), NITP labelled nuclei on the hypoxic profile (FL1 vs FL3A) and the DNA histogram (FL3A), each was divided into total aneuploid, G1, S and G2/M phases of the cell cycle (Figure 7.9). The number of events in total aneuploid, G1, S and G2/M phases were then calculated in total and for BrdUrd labelled cells only, NITP labelled cells only and for both BrdUrd and NITP labelled cells. Any combination of data could then be visualised, examples of which are shown in Figure 7.10.
7.4.3 Results

It has been shown previously in this chapter that BrdUrd staining could still be identified 28 hours after injection but NITP binding in cells had only been followed up to a maximum of 4 hours. Figure 7.13 shows the scatter of hypoxia over the time period of 28 hours. NITP binding could still be identified at 28 hours but due to the large range of hypoxia \textit{in vivo} in the SaF tumour at similar sizes and treated by the same conditions it is not possible to quantify changes of \textit{in vivo} hypoxia over time intervals without analysing substantially larger numbers of tumours.

Figure 7.14 shows how hypoxia is distributed throughout the cell cycle defined by the DNA content, the patterns are broadly similar to that seen in the previous section using staggered administration of the two probes. In G1, the percentage of the total hypoxia decreases from 60% at 2 hours to 45% at 4 hours, increases to a peak of 70% at 12 hours then evens out to about 60% at 16 to 28 hours. In the S phase the distribution of hypoxia increases from 25% at 2 to 35% at 4 hours, decreases to a trough at 12 hours of 20% and then plateaus at around 25% from 16 to 28 hours. The percentage of hypoxia in G2/M increases from 15% at 2 hours to 25% at 8 hours, decreases to 10% at 12 hours then there is little variation from 16 to 28 hours.

Figure 7.15 shows the proportions of BrdUrd labelled cells within each phase of the cell cycle. The solid symbols and unbroken line represent BrdUrd labelled cells which did not incorporate NITP (oxic) and the open symbols and dashed line represent cells that incorporated both BrdUrd and NITP (hypoxic). Each cell cycle phase is expressed as a percentage of the total cells which either incorporated only BrdUrd or both BrdUrd and NITP so that comparison is easier. All three phases of the cell cycle display similar shaped curves for hypoxic and oxic cells. The curves follow the expected pattern, BrdUrd labelling in G1 increases up to 12 hours as cells have progressed through S and G2/M, S phase BrdUrd labelling decreases as cells exit the S phase and there is a steady rate of BrdUrd labelled cells through G2/M up to 8 hours which decreases as all the cells have left S.
Figure 7.13  The scatter of aneuploid hypoxia in SaF tumours injected simultaneously with BrdUrd and NITP at time zero.
Figure 7.14 The percentage of hypoxia in G1 (●), S (□) and G2/ M (◆) phases of the cell cycle of the SaF tumour over a 28 hour period. Symbols represent individual tumours and the lines are mean values of the two tumours.
Figure 7.15 The distribution of BrdUrd labelled cells throughout the cell cycle. Solid symbols (unbroken line) represent cells incorporating BrdUrd only and open symbols (dashed line) represent cells incorporating both BrdUrd and NITP labels.
phase. There is a constant 10% of BrdUrd labelled cells that appear to remain in G2 and do not progress into mitosis.

The average of all the samples used in this experiment, of the aneuploid population (which was 68% of the whole tumour), showed that 23.7±4.7% were hypoxic, 28.6±1.5% were BrdUrd labelled and 7.8±1.5% were both hypoxic and BrdUrd labelled. The low proportion of cells which incorporated both probes in the SaF tumour, on average 5% of the total tumour population, means that some calculations relied on very few cells in each phase of the cell cycle, but numbers were made comparable by creating percentages.

7.5 IMMUNOHISTOCHEMICAL STAINING OF TUMOUR SECTIONS

7.5.1 Method

Sequential sections from SaF, CaNT and Rh tumours were stained for BrdUrd incorporation and NITP adducts as described in section 2.5.2. All tumours presented had NITP administered 2 hours and BrdUrd 1 hour prior to excision. Regions with distinctive, identifiable structures were then compared for positive areas of staining.

7.5.2 Results

The CaNT tumour has a corded structure which was evident in the hypoxic staining pattern of Figure 7.16A. Hypoxia was generally concentric surrounding blood vessels but several cell layers removed. Figure 7.16B shows the wide distribution of BrdUrd incorporation. The boxed region is detailed in Figure 7.16C and D. Vessel 1 had the classic pattern of chronic hypoxia and vessel 2 is suggestive of acute hypoxia where the central vessel in the cord has shut down. The adjacent section of BrdUrd staining confirms this (Figure 7.16D), as for both vessels proliferation was occurring close to the vessel where nutrients are most abundant. The central region between these two blood vessels also contains regions of both hypoxic and proliferating cells, as does the region around vessel 3. Figure 7.16E shows that chronic hypoxia, defined by NITP adduct staining, began 6 to 7 cell
Figure 7.16  Sequential staining of CaNT tumours
(A)  NITP staining, x125
(B)  BrdUrd staining, x100
Figure 7.16  (C)  NITP staining. x200  
(D)  BrdUrd staining. x200
Figure 7.16  (E) NITP staining displaying chronic hypoxia. x400  
(F) NITP staining displaying acute hypoxia. x400
layers from the blood vessel. The surmised closure of vessel 2 produced acute hypoxia adjacent and up to 8 to 9 layers from the vessel (Figure 7.16F). These sections confirm the flow cytometry findings that hypoxia and proliferation can be found within the same cells.

The anaplastic SaF tumour exhibited patchy hypoxia, with regions of high density surrounding necrotic areas (Figure 7.17A). The necrotic regions were easier to identify on BrdUrd stained sections as the dying cells stained a diffuse light brown colour (Figure 7.17B). These two sections showed that the majority of NITP and BrdUrd staining was mutually exclusive. NITP stained areas were lacking in proliferative activity. Areas of possible overlap with proliferative cells in hypoxic areas are labelled with arrows on Figure 7.17B. Figure 7.17C and D showed a more detailed sector of the SaF tumour. Although the areas of high proliferation and high hypoxia were well delineated with common boundaries there were a few pockets where the two stains may have coincided. (Marked by arrowheads in Figure 7.17D). In the corresponding region on Figure 7.17D to that marked 4 on Figure 7.17C there are proliferating cells within the hypoxic area. The hypoxic staining in this area although positive is less dense than that in other areas of the section. It may be the case that this area has a slightly higher oxygen concentration than surrounding hypoxic regions but NITP can still be bioreductively metabolised as demonstrated by NITP binding in Figure 4.7. Proliferation may be able to occur at relatively low oxygen concentrations but at a slower rate and this region may be an example of such in the SaF tumour. Proliferation studies of the SaF tumour under different oxygen concentrations would be required to verify these findings.

The Rh is a well differentiated tumour with highly organised structure. NITP adducts and BrdUrd were widely distributed throughout the tumour as shown in Figure 7.18A and B. There was no particular pattern to hypoxic staining, although proliferation was concentrated along the edges of the blood vessels. In this tumour hypoxic areas with proliferating cells were difficult to locate due to lack of distinctive characteristics. Areas of hypoxia tended to be devoid of proliferating cells but there may have been a few corresponding regions of positive staining.
Figure 7.17 Sequential staining of SaF tumours
(A)  NITP staining. x40
(B)  BrdUrd staining. x40
Figure 7.17  (C)  NITP staining. x100
(D)  BrdUrd staining. x100
Figure 7.18  Sequential staining of Rh tumours
(A)  NITP staining. x40
(B)  BrdUrd staining. x40
Figure 7.18  (C)  NITP staining. x100  
(D)  BrdUrd staining. x100
7.6 DISCUSSION

Cell Cycle Parameters

The SaF cell cycle showed similar cell kinetics to those produced by Wilson et al. (1992). The main difference could be attributed to the difference in growth kinetics between tumours of different sizes. It is well known that the larger a tumour becomes, the lower its labelling index and growth fraction, both of which affect calculation of further parameters. As tumours grow they also tend to slow down their rate of growth so that larger tumours have a longer volume doubling time.

Development of a triple staining technique

There were many problems associated with development of this technique. The main problem was the number of variables which had to be taken into account in each procedure. The final protocol was only arrived at after many different permutations of cell preparations, DNA denaturation and antibody combinations. Information on the diploid cell population was also lost as the pepsin/HCl treatment tended to reduce their numbers by about 40%. The binding of NITP to macromolecules other than DNA in the cell was also lost although this did not appear to influence the percentage of aneuploid hypoxia measured by flow cytometry (Table 7.5). All triple staining techniques have to accommodate some form of compensation when running the sample on the FACScan due to overlap of the three fluorochromes but efficient sample preparation should keep this to a minimum.

Simultaneous measurement of proliferation and hypoxia

The presence of hypoxia in actively cycling cells was approached by two methods. The first followed BrdUrd labelled cells through the cell cycle and assessed their hypoxia at intervals thereafter. The second followed the progress of both hypoxic and BrdUrd labelled cells through the cell cycle. The first procedure showed that cells that were actively progressing through the cell cycle did become hypoxic. This may support the concept
that cells become acutely hypoxic through shutdown of blood vessels, although a major drawback of this technique is that acutely and chronically hypoxic cells can not be distinguished.

The amount of hypoxia in BrdUrd labelled cells decreased in G1 but this was probably due to events occurring in other phases of the cell cycle. The percentage of hypoxia in BrdUrd cells increased in both S and G2 phases. The rise in S could have been due to arrest of cells in S once they had become hypoxic, or slower progression through S. The slow rise in G2 hypoxic BrdUrd labelled cells would suggest that cells become blocked in G2 and are unable to pass through mitosis. There could also be an indication that it is only cells labelled in late S that progress into G2, those cells labelled in early S may be the population that remains blocked in S. Once hypoxic cells have blocked in G2, they may be programmed for cell death, if not reoxygenated. There is always the possibility that cells only become hypoxic for a short period of time such that they may become reoxygenated and can progress through mitosis.

Simultaneous administration of BrdUrd and NITP should have answered further questions. The presence of a large amount of hypoxia at 28 hours in one of the samples showed that NITP was a viable marker to combine with BrdUrd for at least one cell cycle. Taking the hypoxic population alone and looking at the distribution throughout the cell cycle there does appear to be a pattern (Figure 7.14). These cells are not necessarily in cycle but have DNA content equivalent to individual cell cycle phases. In S phase there was a sharp peak at 4 hours followed by a gradual decrease in hypoxia. The peak could be due to hypoxic cells progressing through S phase but at a reduced rate so they gradually accumulate. The gradual decrease in hypoxia may be because hypoxic cells are no longer entering S phase due to a block at the G1/S border. There was a gradual rise in hypoxia in G2 reaching a peak at 8 hours. This could be due to a block in G2 not releasing hypoxic cells to progress through mitosis. The delayed peak of G2 follows the peak of S phase cells so it may also include cells which have been slowly progressing through S. The fall
in G1 hypoxic cells can be attributed to the accumulation of cells in G2 and S. The peak in G1 hypoxia suggests that cells that have progressed through S (10 hours) and G2 (2 hours) and those that were in G1 (10 hours) have begun to accumulate at the G1/S border. Hypoxic cells may not progress through this restriction point to continue the cell cycle.

Figure 7.15 follows the progress of BrdUrd labelled cells alone (classed as oxic) and those BrdUrd labelled cells which have also incorporated NITP (hypoxic). These cells are actively in cycle as they have incorporated BrdUrd. Both hypoxic and oxic cells follow the same patterns reflecting that cells which have recently become or are chronically hypoxic do not seem to have a slower cell cycle time. There was a trend seen in S phase cells after 12 hours, that a higher proportion of hypoxic cells remained in this phase suggesting that they arrested due to their metabolic deficiencies. The hypoxic BrdUrd labelled cells in G2 remain in very similar proportions to the oxic population in the first four hours, then increased with respect to the oxic proportion. This would suggest that cells becoming hypoxic in late S progress through S and into G2 whereas those becoming hypoxic in early S arrest in S and may block in G2. The peak at 8 hours suggests a G2 block supporting the earlier data and there was a higher proportion of cells that remain in G2. The G2 block may be a consequence of the cell becoming hypoxic in the S phase. In radiation experiments where cells have been irradiated in S there is a G2 block or delay produced while repair of DNA is undertaken. The cell becoming hypoxic during DNA synthesis may increase the probability that replication of DNA is not correct so hypoxia may induce a repair process of its own, to mirror that of radiation, by the same or different mediators. This block in G2 may be so that these cells that have become hypoxic in S phase can be redirected to apoptosis or eventual necrosis.

A G2 block may also be a method of creating a reservoir of cells about to undergo mitosis. *In vitro* modelling has shown that cells do not tend to enter DNA synthesis unless they have a good nutrient and oxygen supply. Gelfant (1977) also showed *in vivo* that mouse epithelium had a
number of cells permanently arrested in G2 which remained there for at least several months. These cells were activated when the tissue was injured to immediately repopulate the area through mitosis, whilst other cells initiated DNA synthesis. A reservoir of G2 cells in unfavourable (hypoxic) conditions could be established so that once normal conditions within the tumour are restored the cells can repopulate the area immediately without the time delay of initiating DNA synthesis or getting G0 cells to re-enter the active cell cycle.

**Histological assessment of hypoxia and proliferation**

The histological sections show that NITP can be widely distributed around the tumour. The patterns of hypoxia vary considerably depending on the structure of the tumours. The three tumours selected show a corded pattern (CaNT), a patchy distribution (SaF) and a random highly hypoxic pattern (Rh). The staining of hypoxic areas would appear to confirm the flow cytometry data of mean values of 11.6% for the SaF and 35.6% for Rh tumours as hypoxia in the Rh tumour is more widespread and at a greater density.

Sequential sections stained for NITP and BrdUrd labelled cells shows that the majority of staining is mutually exclusive although there are pockets where both stains can be found. The CaNT, with its corded structure is an ideal tumour to display chronic and acute hypoxia as shutdown of vessels is easily visualised and compared to chronically hypoxic cells.
CHAPTER 8  GENERAL DISCUSSION

The purpose of these studies was to investigate the tumour environment and improve understanding of cellular interactions. This could lead to the ability to individualise treatment and thus improve therapeutic benefit. Research on two biological factors affecting tumour response, proliferation and hypoxia, has been active for many years. However, recent technological advances mean that further progress can be achieved.

Hypoxia has been known for 40 years to have a deleterious effect on radiotherapy treatment; hypoxic cells are 3 times as resistant to radiation than their well oxygenated counterparts. A number of strategies have been proposed to overcome hypoxia in solid tumours; including hyperbaric oxygen, radiation sensitizers, oxygen or carbogen breathing and blood transfusions of anaemic patients. Many of the trials of chemical radiation sensitizers gave disappointing results for several reasons, including lack of patient selection, small numbers of patients in individual trials, and poor tolerance of some of the radiation-sensitizing drugs. For these reasons the results of these trials have been conflicting such that importance of hypoxia has not been fully appreciated. However, recently Overgaard (1992) has undertaken a literature based meta-analysis of these clinical trials attempting to combat the effects of hypoxia in the diverse sites of bladder, uterine cervix, CNS, head and neck and lung. Of 44 trials comparing different treatments to overcome hypoxia using local tumour control as the end-point, 8 showed no benefit over hypoxic treatment versus radiation alone but 36 gave improved response. Seven, primarily concerned with squamous cell carcinoma of the head and neck, showed a statistically significant improvement. Meta-analysis demonstrates that improvements in therapy can be made if hypoxia is treated and more emphasis is now being placed again on overcoming hypoxia in radiotherapy regimens.

Therapy uses two approaches to the hypoxia problem, increasing oxygenation of the tumour, by such means as oxygen or carbogen breathing, or exploiting the hypoxic environment for selective cell kill by
such means as bioreductive agents. The problem with eliciting information on the benefit of treatments to overcome hypoxia is that not all patients will necessarily have significant numbers of hypoxic cells in their tumours and the value to those with hypoxic tumours will be diluted by the lack of effect on others. Hence, a hypoxic probe would be of great value. However knowledge of re-oxygenation and the biological status of hypoxic cells is also required. We need to know whether hypoxia is acute or chronic. Hypoxic cells proliferate and potentially become re-oxygenated on mitosis, this might present a real problem for radiotherapy.

The National Cancer Institute held a workshop in November of 1992 into "Oxygen in human tumours" and Table 8.1 summarises the present methods of measurement under investigation for clinical use (Stone et al. 1993). The polarographic oxygen electrode is the present method of choice, however, it is invasive, measures extracellular pO₂ and requires accessible tumours. The Eppendorf oxygen electrode has been shown in clinical trials of breast and uterine cervix cancers to predict survival (Vaupel et al. 1991; Höckel et al. 1993). Histograms from this data of the distribution of oxygen tensions display the heterogeneity of the tumour environment. This accentuates the need to know tumour oxygenation on an individual basis but also emphasises the need to know about the oxygen dependence of the drugs or sensitizers being employed.

The SaF cell line was used in these investigations to elicit information on the response of tumour models to two quinone bioreductives, mitomycin C and porfiromycin, currently undergoing clinical trial as adjuncts to radiotherapy. Cell lines demonstrated the respective oxic:hypoxic differentials for mitomycin C and porfiromycin to be 1.7 and 3.5 at a surviving fraction of 0.1. Examination of the survival curves of mitomycin C and porfiromycin showed that both drugs had similar hypoxic cell kill but the oxic:hypoxic differential of porfiromycin was increased by the low oxic cell kill. In terms of treating patients this was a good finding as there would probably be less toxicity to the normal well oxygenated cells from porfiromycin than mitomycin C.
<table>
<thead>
<tr>
<th>Method</th>
<th>Invasive</th>
<th>Resolution</th>
<th>Measure</th>
<th>Tumour</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarographic oxygen electrodes</td>
<td>Yes</td>
<td>50-100 cells</td>
<td>Extracellular pO₂</td>
<td>Accessible</td>
<td>Accurate at low pO₂? Complex technology.</td>
</tr>
<tr>
<td>Comet assay</td>
<td>Yes</td>
<td>Single cells</td>
<td>Radiosensitivity</td>
<td>Accessible</td>
<td>Needs 4Gy now. Assay time ~ 5hr</td>
</tr>
<tr>
<td>(¹²³I)IAZA</td>
<td>No</td>
<td>Whole tumour</td>
<td>Nitroimidazole binding</td>
<td>All</td>
<td>Improve compound? Enzyme levels important?</td>
</tr>
<tr>
<td>'¹⁸F-PET</td>
<td>No</td>
<td>50mg (5x10⁷ cells)</td>
<td>Nitroimidazole binding</td>
<td>All</td>
<td>Perfusion problems? Enzyme levels important?</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>Yes</td>
<td>Single cells (FACS) Biopsy (ELISA, Imm.)</td>
<td>Nitroimidazole binding</td>
<td>Accessible</td>
<td>Antibodies required. Enzyme levels important?</td>
</tr>
<tr>
<td>Cryospectroscopy</td>
<td>Yes</td>
<td>Tumour cord</td>
<td>HbO₂ saturation in vessels</td>
<td>Accessible</td>
<td>Relationship to tissue pO₂?</td>
</tr>
<tr>
<td>Optical spectroscopy</td>
<td>No</td>
<td>10g now, less in future</td>
<td>Average, saturation [HbO₂]</td>
<td>Accessible</td>
<td>Needs validation.</td>
</tr>
<tr>
<td>'³¹P-MRS</td>
<td>No</td>
<td>Whole tumour, 5-8g image</td>
<td>P metabolites</td>
<td>Most</td>
<td>May not give hypoxic fraction across tumour types</td>
</tr>
<tr>
<td>'¹H-MRS (lactate)</td>
<td>No</td>
<td>1g</td>
<td>Lactate concentration, lactate production (with blood flow)</td>
<td>Brain; others?</td>
<td>Correlation of lactate and hypoxia? Needs blood flow data.</td>
</tr>
<tr>
<td>ESR spectroscopy</td>
<td>No</td>
<td>?</td>
<td>pO₂ at site of paramagnetic probe molecules</td>
<td>Accessible</td>
<td>Not yet suitable for routine clinical use.</td>
</tr>
<tr>
<td>Alkaline elution</td>
<td>Yes</td>
<td>10-50mg</td>
<td>Radiosensitivity</td>
<td></td>
<td>2Gy possible, 4Gy more accurate; high technical skills required.</td>
</tr>
<tr>
<td>Phosphorescence imaging</td>
<td>Yes</td>
<td>Small volume at surface</td>
<td>Phosphorescence decay in blood</td>
<td>Accessible</td>
<td>Not yet suitable for clinical use.</td>
</tr>
<tr>
<td>'¹⁹F-MRS</td>
<td>No</td>
<td>1g</td>
<td>Nitroimidazole binding</td>
<td>All</td>
<td>Affected by enzyme differences between tumours.</td>
</tr>
</tbody>
</table>
Closer examination of the oxygen dependence of mitomycin C and porfiromycin showed two distinct patterns. Porfiromycin had two plateaux of survival at low and high oxygenation (Figure 4.5, lower panel) joined by a steep slope between 200 and 3000ppm oxygen. Mitomycin C had a plateau of survival at low oxygenation up to 600ppm oxygen (Figure 4.5, upper panel), a steep slope up to 6000ppm oxygen followed by a shallow, gradual slope to the highest oxygenation used (200,000ppm oxygen). These two patterns of oxygen dependency for the bioreductives highlighted the importance of intermediate levels of oxygenation in the use of bioreductive agents as adjuncts to radiotherapy. The oxygen dependency of radiation (Figure 4.9) showed that at 1000ppm oxygen (0.1% or a pO₂ of 0.6mm Hg) radiation had only 80% of its maximal effect. At this oxygen concentration in these clinical regimes the bioreductives would be expected to kill the remaining cells and by the oxichypoxic differential data porfiromycin would be the bioreductive agent of choice. However, examining the survival data at this oxygen concentration mitomycin C has greater cell kill than porfiromycin (surviving fractions of 0.01 and 0.1 respectively). This highlights the dilemma that although porfiromycin has the greater oxichypoxic differential with similar hypoxic cytotoxicity to mitomycin C, it is mitomycin C that is the more effective bioreductive agent at intermediate oxygen concentrations. Marshall and Rauth (1988) commented that survival at intermediate oxygen concentrations could limit therapeutic utility of porfiromycin/mitomycin C as adjuncts to radiotherapy.

More complex tumour model systems, spheroids and subcutaneous tumours, were then used to further define the relationship of hypoxia and bioreductive agents. The internal environment produced by a spheroid is more representative of the solid tumour and external factors can be manipulated for in vitro spheroids to mimic changes in nutrient delivery. Experiments showed that increasing diameter in a spheroid correlated with increasing hypoxia, however this was associated with a larger number of cells not an increasing depth of hypoxia. Growth delay measurements with mitomycin C showed that even at high doses of drug, where the three
dimensional structure of the spheroid broke down, there were still a few surviving single cells which had growth potential and were capable of reforming into a spheroid. The growth delay data again demonstrated that porfiromycin had little effect on well-oxygenated cells, hardly altering the size of spheroids despite producing some cell kill. The less selective mitomycin C caused greater growth delay and even "cures" in less hypoxic spheroids.

*In vivo* spheroids were difficult to manipulate. The size and number of spheroids within the peritoneum could not be determined until after excision when treatment had already been administered. This could affect the dose and exposure of the drug reaching each individual spheroid. The SaF peritoneal model did not produce any unattached spheroids, probably due to the aggressive nature of the tumour, and thus no comparison could be made between vascularised and unvascularised spheroids. Other cell lines, such as the Neo, could be used to assess the effect of vascularisation and hypoxia on chemotherapeutic agents as some spheroids remain unattached and free floating. The peritoneal spheroid model would be useful for investigation of neovascularisation, however it is a poor model for investigation of bioreductive potential.

The more commonly used subcutaneous tumour system is a more satisfactory *in vivo* model. Growth can be readily monitored and the macroscopic effects of drugs visualised. The subcutaneous model also incorporates the biological effects of systemic toxicity and host modifiers to the tumour. Investigation of hypoxia in these tumours was facilitated by their more regular growth, larger size and the ability to discern between tumour and normal mouse tissue on excision. The size of the tumour meant that individual tumours could be assessed for their hypoxia, survival after exposure to bioreductive agents and histological structure. There was no correlation between size of tumour and percentage hypoxia which was surprising, especially for the SaF tumour, which is anaplastic with rapid growth. It may be expected to outgrow its vascular supply and thus contain large regions of nutrient deprivation. However, this did not appear
to be the case either with the SaF or the more highly structured Rh tumour. It may be that hypoxia mediated angiogenesis is taking place due to tumour mediators such as vascular endothelial growth factor (Shweiki et al. 1992; Plate et al. 1992) where low concentrations of oxygen trigger an influx of blood vessels into that area. Histological NITP distribution verifies that the degree of hypoxia varies greatly in similar sized tumours.

One of the more striking findings was the degree of correlation between the surviving fraction after porfiromycin administration and the percentage tumour hypoxia. It would be expected that due to the high oxygen dependency of porfiromycin metabolism the correlation would be greater than that found with mitomycin C. Porfiromycin would have little effect on well oxygenated regions of the tumour, but would be metabolised well where hypoxia is present. Mitomycin C would have a more widespread effect and may be metabolised by the better oxygenated cells. Thus mitomycin C could be metabolised before it reached the cells furthest from the blood vessels i.e. those most hypoxic and therefore highest in bound NITP for measurement. The lack of correlation between surviving fraction after irradiation and percentage tumour hypoxia is more difficult to explain. It is possible that individual variation is greater from whole body irradiation or there were insufficient numbers of mice per dose to show a correlation. The radiation survival curve (Figure 6.8) between hypoxic and air-breathing mice shows that there is only a small difference between survival of totally hypoxic tumours and tumours from air-breathing mice. This and the individual variation in surviving fraction at a particular dose may be the explanation for the lack of correlation.

Proliferation like hypoxia has been studied for many years but recent advances in both methods to assess proliferation plus the renewed desires to try different fractionation schemes by clinicians has brought this aspect to the fore again. Trials are in progress such as Continuous, Hyperfractionated, Accelerated Radiation Treatment, CHART, (54Gy in 36 fractions, 1.5Gy/fraction, 3 fractions/day) where patients have BrdUrd administered prior to treatment to establish the proliferative characteristics
of their tumour. A pilot study has shown there is no profound influence of proliferation on the response of tumours to accelerated radiotherapy although there was a non-significant trend for slowly proliferating \( T_{\text{pot}} \) less than 4.3 days) tumours to do worse (Lochrin et al. 1992). An EORTC phase III trial 22851 in head and neck cancer is comparing conventional fractionation (70-72Gy in 7-8 weeks, 1.8-2Gy/fraction, 35-40 fractions) with accelerated fractionation (72Gy in 5 weeks, 1.6Gy/fraction, 3 fractions/day, 45 fractions with a 2 week break after the first week of treatment). Administration of IUdR is optional and the numbers still small (60) but there is a trend for tumours with a short \( T_{\text{pot}} \) (less than 4.6 days) to do worse in the conventional arm whilst there is no significant effect in the accelerated arm (Begg et al. 1992).

Much information is now available on proliferation of individual tumours, and as expected it shows tremendous variation, but little is known about the interplay between proliferation and hypoxia. This study has highlighted some intriguing and unexpected data concerning the relationship between hypoxia and proliferation. There is a predominance of hypoxic cells in the G2 part of the cell cycle possibly linked to a checkpoint. Cells actively incorporating DNA precursors can be hypoxic. The pulse chase studies show that hypoxia may be involved in a cell cycle regulation process in that metabolically challenged cells are subject to cell cycle block and perhaps, ultimately death, at G2 and G1 checkpoints. Further research could be applied to study whether the consequence of cells stopped at the G2 checkpoint is apoptosis or whether cells become permanently fixed in the G2 phase. The cyclins and other regulatory proteins accumulation profiles in hypoxic cells could be analysed and gene induction at these checkpoints examined.

Development of a simultaneous method of measuring hypoxia, proliferation and DNA content on the flow cytometer shows that these measurements could be accomplished with a single biopsy. Once ethically approved for human studies NIITP could be used in the clinic and a predictive test for these two influential factors established. One drawback
of this test is the clinical use of two biochemicals. Induction of oxygenregulated genes could also be a method of measuring tumour hypoxia. A hypoxia-induced protein could be used as an intrinsic marker. Presently there are several possible intrinsic markers (e.g. heme oxygenase, HSP70 and VEGF) although development and verification of these as markers of hypoxia will take several years. There are also intrinsic markers of proliferation such as Ki67, a protein found in all phases of the cell cycle except early G1 and G0. Identification of intrinsic markers of proliferation and hypoxia would be simpler and less hazardous than the administration of external probes and would probably be less prone to variation. Histological analysis of hypoxia and proliferation could readily be incorporated into the normal routine of clinicians as biopsies are currently taken to grade and assess malignancy of the tumour, so only further staining need be done. Histological analysis is often time consuming and tedious. Thus a flow cytometry method could be established which would be rapid and easily analysed.

Development of predictive tests for factors affecting treatment outcome leads to the tailoring of therapy to suit the particular tumour environment. The individualisation of treatment is becoming a popular concept in solid tumour research as tumours of the same type are often diverse in nature and respond differently to therapy. Another feature in treatment for the individual tumour is to administer drugs which only the tumour metabolises to a cytotoxic species. Bioreductive drugs are especially susceptible to this theory as they are metabolised by specific enzymes under certain environmental conditions. A biopsy could be used to "enzyme profile" the tumour. For example, the bioreductive, EO9, which is mainly metabolised by DT-diaphorase, could be given to patients whose tumours have high levels of this enzyme, whereas those low in dt-diaphorase would have an alternative drug administered.

One way forward in improving the response of solid tumours to treatment may be to provide the clinician with a number of relevant predictive indicators. A single biopsy routinely taken for histological
diagnosis could be used to show degree of hypoxia, proliferative status and enzyme profiling. These prognostic indicators could be used as a basis for the clinician to determine the type of treatment of most benefit to that patient.
Appendix 1: Suppliers of reagents and materials

2.1 Tissue Culture

<table>
<thead>
<tr>
<th>Product</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute alcohol</td>
<td>BDH</td>
</tr>
<tr>
<td>Agar (Tissue culture grade)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Alginate sodium salt</td>
<td>Sigma</td>
</tr>
<tr>
<td>Benzylpenicillin sodium BP</td>
<td>Britannia</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>Dimethylsulphoxide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Fetal Calf Serum</td>
<td>Biological Industries</td>
</tr>
<tr>
<td>L-Glutamine 200mM</td>
<td>Integra Biosciences</td>
</tr>
<tr>
<td>HEPES 1M</td>
<td>HyClone</td>
</tr>
<tr>
<td>Microcarrier beads; Cytodex 1 &amp; Cytodex 3</td>
<td>Sigma</td>
</tr>
<tr>
<td>Minimum Essential Medium Eagle's con / sus concentrate</td>
<td>Integra Biosciences</td>
</tr>
<tr>
<td>Peanut Oil</td>
<td>Sigma</td>
</tr>
<tr>
<td>PBS</td>
<td>Oxoid</td>
</tr>
<tr>
<td>Sodium Bicarbonate solution 7.5%</td>
<td>Integra Biosciences</td>
</tr>
<tr>
<td>Streptomycin Sulphate BP</td>
<td>Evans</td>
</tr>
<tr>
<td>Trypsin/EDTA</td>
<td>Integra Biosciences</td>
</tr>
</tbody>
</table>

**Enzymes:**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase Type IV</td>
<td>Sigma</td>
</tr>
<tr>
<td>Deoxyribonuclease I</td>
<td>Sigma</td>
</tr>
<tr>
<td>Protease Type XI</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

**Drugs:**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitomycin C</td>
<td>Kyowa</td>
</tr>
<tr>
<td>Porfiromycin</td>
<td>Agift from the National Cancer Institute, USA</td>
</tr>
<tr>
<td>Saline solution</td>
<td>Antigen Pharmaceuticals</td>
</tr>
<tr>
<td>Sterile water for injection</td>
<td>Antigen Pharmaceuticals</td>
</tr>
</tbody>
</table>

2.2 In vivo techniques

<table>
<thead>
<tr>
<th>Method</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metofane</td>
<td>C-Vet</td>
</tr>
</tbody>
</table>

2.3 Flow Cytometry

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Aminoactinomycin D (A9400)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Goat serum (S2007)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Hydrochloric acid (Analar grade)</td>
<td>BDH</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Propidium iodide (P4170)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Tween 20 (polyoxyethylenesorbitan)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bam H1</td>
<td>Boehringer Mannheim Biochemica</td>
</tr>
<tr>
<td>Exo III</td>
<td>Boehringer Mannheim Biochemica</td>
</tr>
</tbody>
</table>
Incubation buffers:-
Calcium Chloride
Citric acid
Magnesium Chloride
2-mercaptoethanol
Nonidet P-40
Sodium Chloride
Sucrose
Tris-HCl
Triton X-100

Antibodies:-
Mouse monoclonal to human bromodeoxyuridine (M744)
Mouse monoclonal to bromodeoxyuridine (BR3) (MD5200)
Rat monoclonal to bromodeoxyuridine
Biotin conjugated anti-rat IgG (whole molecule)
developed in goat (B7139)
Biotin conjugated anti-rabbit IgG (whole molecule)
developed in goat (B7389)
FITC conjugated anti-mouse IgG (whole molecule)
developed in goat (F2012)
FITC conjugated anti-rabbit IgG (whole molecule)
developed in goat (F0382)
FITC conjugated anti-rat IgG (whole molecule)
developed in goat (F6258)
R-phycocerythrin conjugated Streptavidin (R438)
R-phycocerythrin conjugated anti-mouse IgG (R480)
Theophylline antiserum developed in rabbit (T2524)

35µl nylon mesh
Swinnex filters

2.5 Immunohistochemistry

Avidin biotin complex (ABC)
Biotinylated anti-mouse IgG developed in rabbit (E354)
Biotinylated anti-rabbit IgG developed in swine (E353)
3,3'-Diaminobenzidine tablets (10mg)
DPX
Human AB serum
Hydrogen peroxide (30%)
Mayers haematoxyline
Methanol
Tris buffered saline
Xylene

Poly-l-lysine slides

John Staniar & Co.
Millipore
Raymond Lamb
REFERENCES


BEGG A.C., McNALLY N.J., SHRIEVE D.C. & KARCHER H. A method to measure the DNA synthesis and the potential doubling time from a single sample. Cytometry 6, 620-626 (1985a)


BROOKS R.F. "Regulation of the eukaryotic cell cycle." in Assessment of cell proliferation in clinical practice. (Eds) P. Hall, D.A. Levison and N.A. Wright. 1-26 (1992)


DINGWALL C. The accumulation of proteins in the nucleus. *TIBS* 10, 64-66 (1985)


DOBROWSKY W., DORROWSKY E. & NAUDÉ J. "Mitomycin C (MMC), 5-fluorouracil (5-FU) and radiation: clinical experiences." in Bioreductive drugs, sensitizers, oxygen and radiotherapy. (Ed) W. Dobrowsky. Facultas-Universitätsverlag 159-166 (1993)


DRAETTA G., LUCA F., WESTENDORF J., BRIZUELA L., RUDERMAN J. & BEACH D. Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell* 56, 829-838 (1989)

DULHANTY A.M. & WHITMORE G.F. Chinese hamster ovary cell lines resistant to mitomycin C under aerobic but not hypoxic conditions are deficient in DT-diaphorase. *Cancer Res.* 51, 1860-1865 (1991)

DURAND R.E. Multicell spheroids as a model for cell kinetic studies. Cell Tissue Kinet. 23, 141-159 (1990a)


FRACASSO P.M. & SARTORELLI A.C. Cytotoxicity and DNA lesions produced by mitomycin C and porfiromycin in hypoxic and aerobic EMT6 and Chinese hamster ovary cells. Cancer Res. 46, 3939-3944 (1986)


HIRAOKA M. & HAHN G.M. Changes in pH and blood flow induced by glucose, and their effects on hyperthermia with or without BCNU in RIF-1 tumours. *Int. J. Hyperthermia* **6**, 97-103 (1990)


HORSMAN M.R., CHAPLIN D.J. & OVERGAARD J. Combination of nicotinamide and hyperthermia to eliminate radioresistant chronically and acutely hypoxic tumour cells. *Cancer Res.* 50, 7430-7436 (1990b)


IYER V.N. & SZYBALSKI W. Mitomycins and porfiromycin: Chemical mechanism of activation and crosslinking of DNA. *Science* 145, 55-58 (1964)


248


NEDERMAN T. & TWENTYMAN P. "Spheroids for studies of drug effects." in Recent Results in Cancer Res. (Eds.) H Acker, J Carlsson, R Durand and RM Sutherland. Springer-Verlag 84-102 (1984)


OVERGAARD J. Importance of tumour hypoxia in radiotherapy: a meta-analysis of controlled clinical trials. Radiotherapy Oncol. 24, S64 (1992)


SIEGEL D., GIBSON N.W., PREUSCH P.C. & ROSS D. Metabolism of mitomycin C by DT-diaphorase: Role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. Cancer Res. 50, 7483-7489 (1990)


SORRENTINO V. Growth factors, growth inhibitors and cell cycle control. Anticancer Res. 9, 1925-1936 (1989)


SZYBALSKI W. & IYER V.N. Crosslinking of DNA by enzymatically or chemically activated mitomycins and porfiromycins, bifunctionally "alkylating" antibiotics. Federation Proc. 23, 946-957 (1964)


TSURIMOTO T. & STILLMAN B. Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases, alpha and delta. EMBO J. 8, 3883-3889 (1989)


VERWEIJ J. & PINEDO H.M. Mitomycin C: mechanism of action, usefulness and limitations. Anti-Cancer Drugs 1, 5-13 (1990)


WHITE R.A. & MEISTRICH M.L. A comment on: method to measure the duration of DNA synthesis and the potential doubling time from a single sample. Cytometry 7, 486-490 (1986)


SUPPLEMENTARY REFERENCES


