RESPONSE OF CHINESE HAMSTER SPHEROIDS TO MULIFRACTION IRRADIATION

by

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ABSTRACT

The response of mammalian cells to ionizing radiation has been extensively studied with single cells exposed to acute doses. Little information is, however, available for cells growing in tissues, especially for cells subjected to multiple exposures. Our aim in this thesis was therefore to use a more complex *in vitro* system, three dimensional spheroids grown from V79-171b Chinese hamster lung cells, to determine the role of repair, redistribution and repopulation during multifraction irradiation.

Repair and redistribution effects were isolated by using spheroids under normal culture conditions of 37°C, or at 22°C where repair occurs but cell proliferation is markedly inhibited. As expected, we found that cells surviving an initial 8 Gy dose showed cell cycle dependent fluctuations in radiosensitivity when allowed to progress at 37°C before exposure to a second 8 Gy dose. Sublethal radiation damage was repaired more rapidly at 37°C than at 22°C, and was also affected by proliferation. Due, however, to the small proliferating population in the spheroid system, a large initial dose was required to produce a population with enough synchrony for the expected split dose survival fluctuations throughout the cell cycle to be observed.

When two doses of 6 Gy separated by 4 hours were administered to spheroids, the subsequent cellular radiosensitivity to a third dose remained quite constant for at least 10 hours, indicating a more extended mitotic delay than observed in the two dose experiments. Mitotic delay consequently was not linear with dose, a result apparently dependent upon the fractionation scheme used, and the complexity of the multicell system.

In multifraction schedules where doses of 6 Gy or 8 Gy were administered daily for 6 days, we found, as expected, that repair, redistribution and repopulation all affected cell

viability. However, each effect dominated at different times throughout the experiments. The overall cytotoxicity for each 6 Gy fraction decreased with increasing fraction number, while the 8 Gy fraction survival remained fairly constant. A novel feature of our experimental design, administering each 6 Gy or 8 Gy fraction in 1-2 Gy increments, also allowed evaluation of successive responses to the clinically relevant dose of 2 Gy. Cell survival at that level fluctuated greatly due to a decreasing repair capacity, and an increasing effect of repopulation with fraction number.

Using two radioactive Iridium sources of different activities, high dose rate fractionated exposure was compared to continuous low dose rate irradiation. Also, the linear quadratic model was used to predict the equivalent doses. We found that the model did not provide a good prediction; more repair of radiation induced damage was observed at the lower dose rates than the higher dose rates, an effect which could not be incorporated into this theoretical model.

We conclude that, with fractionated radiation exposures to the spheroid system, repair, redistribution, repopulation and cell killing all contribute to the multifraction responses. Each has varying significance on each fraction. An equal effect per fraction, often implicit in radiotherapy regimens, is therefore only achieved in the fortuitous situation where repair, redistribution, repopulation and cell killing combine in different proportions to result in the same overall survival.

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1. INTRODUCTION

Radiation has both toxic and genetic effects on organisms; these occur at the cellular level and are manifested in the form of DNA damage. Although much is known about the cellular response after one dose of radiation, less is understood about multiple exposures. This is especially true in tissues or tissue-like models.

At the cellular level, there has been much progress in determining the effects of single and multiple doses of radiation as well as low continuous doses. To obtain greater understanding of the responses in tissues, however, a model is required with more complexity than single cells and having more characteristics of tissues. The three-dimensional spheroid model can meet some of these needs.

The spheroid is a mass of cells attached together at tissue-like density, and like its name, is in the shape of a sphere. It grows suspended in a medium which contains all the necessary nutrients. These nutrients, including oxygen, diffuse from the periphery of the spheroid toward the center causing a diffusion gradient which results in different microenvironments throughout the spheroid, and at large spheroid sizes, becomes similar to those found in nodular tumours. The spheroid retains the simplicity of experimentation while the more complex responses of the tumours can be addressed.

In this thesis, the response of cells in spheroids to radiation was examined, with particular emphasis on multiple doses. This included examining the effects of cell phase on survival after radiation, as well as the effects of radiation on cell cycle progression by carefully monitoring the survival of the cells along with their progression through the cycle after 2 or more doses of radiation. Cell survival was measured as the number of irradiated cells that retained the capacity to divide indefinitely and, therefore, grew into colonies. Cytofluorometry

was used to monitor progression of the cells with split dose experiments being used as a method to monitor survival of the cells by partially synchronizing the cells with the first dose and monitoring the survival of the partially synchronized population after the second dose. These two methods together provided insight into the interdependence of cell progression and radiation response as well as the effects of repair and repopulation on the cells.

These results can be useful in understanding the responses of tissue to the many types of radiation that humans are exposed to on a daily basis from our surroundings. They can also be of practical use aiding in the prediction of the responses of tissues to radiotherapy.

To understand all of these effects, it is important first to have a basic knowledge of the response of single cells to radiation, and how the response is dependent upon cell cycle effects, repair of damage, and progression post-irradiation. This understanding would be facilitated with some knowledge about the cell cycle and its phases.

1.1 CELL CYCLE

The cell cycle is defined as the interval between the completion of mitosis in the parent cell and the completion of the next mitosis in one or both of the daughter cells. With recent advances in methodology the cell cycle can be divided into four distinct phases. These include the presynthetic interphase or postmitotic gap (G_1), DNA synthesis phase (S), postsynthetic interphase or premitotic gap (G_2) and mitosis (M).

G_1 is a functional period during which the cells prepare for S phase and which can last up to a few hours. During S phase, the entire DNA content of the nucleus must be replicated completely and precisely. Failure to do so would lead to chromosome breakage at the next mitosis. G_2 is a period of activity in preparation for mitosis and during mitosis the cell divides (Baserga, 1985).

Recent methodology has allowed us to distinguish between these four phases, enabling the determination of the length of each phase. In Chinese hamster lung cells the total cycle length is about 16 hours with the G_1 phase being about 4 hours, the S phase about 10 hours and G_2 and M phase combined being about 2 hours (Durand, 1990).

1.1.1 Cytofluorometry

One method of distinguishing between the cell cycle phases is cytofluorometry. This method is based on the principle that the amount of DNA in normal non-replicating cells is constant (Boivin, Venderly and Venderly, 1948). Although this is not exactly true, somatic cells contain fixed amounts of DNA, usually diploid (2n amount) or tetraploid (4n). Cancer cells and most cells in culture contain an amount which is not a multiple of 2 (aneuploid). Since the cell replicates its DNA during the S phase, G_2 cells will have twice the DNA content of G_1 cells and cells in S phase have an intermediate amount of DNA. Measurement of the DNA content of the cell can, therefore, provide information regarding the cycle phase (Baserga, 1985).

In cytometry, the cells are fixed and stained with a dye specific to DNA which can then be detected, the amount of stain being proportional to the amount of DNA. This amount of stain may be detected either by fluorescence (cytofluorometry) or by absorption of light (cytophotometry).

1.1.1.1 Flow Cytometry

Flow cytometry can be used to measure the DNA content of the cell. Cells can be stained with ethidium bromide, propidium iodide or one of many other dyes which stoichiometrically bind to the DNA. When the stain is excited by a laser, the cells fluoresce with an intensity proportional to their DNA content. A DNA profile is shown in figure 1. The first

peak represents G_1 cells, the second peak G_2 + M cells with the remainder being S cells. The percentage of each can be estimated by computer modelling. This method is fast, non-radioactive and excellent for studying perturbations to the cell cycle but suffers from the limitations that it cannot distinguish between living and dead cells, and that the results of the computer fit are highly dependent on the method used, especially when predicting the S phase population.

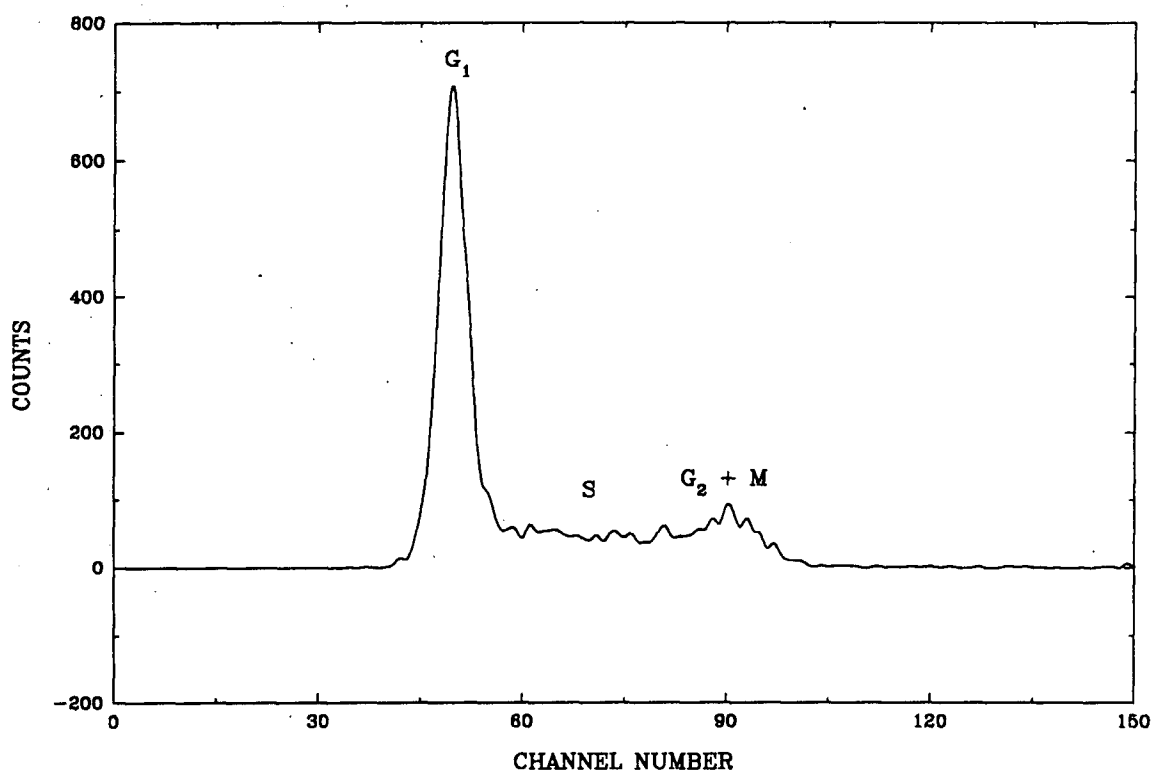

Figure 1. DNA Profile

DNA profile of cell from 3 day old V79-171b Chinese hamster spheroids.

1.2 EFFECTS OF RADIATION ON THE CELL

Radiation produces a spectrum of damage in a cell, with single or double strand breaks in the DNA of the cell being the lesions of greatest biological relevance. This results from the absorption of the radiation energy which is ultimately transferred to electrons. These electrons in turn produce a large number of ionizations and excitations in the DNA which cause chemical dissociations and rearrangements in the bonds between the nucleic acids of DNA. If the DNA is lethally damaged, the cell has difficulty dividing the chromatin equally between the daughter cells during the next mitosis and, therefore could abort during the attempt. For this reason lethal radiation damage is typically expressed during division (Hall, 1988).

An abortive mitosis can occur at up to several cell divisions after the dose is received depending on the length of the cell cycle, the phase of the cell at the time of irradiation and the dose of radiation received. Usually doses of 1 to 10 Gy lead to death after 1-2 divisions, whereas doses greater than 10 Gy may cause death at the first division attempt (Denekamp, 1986).

1.2.1 Radiosensitivity Throughout the Cell Cycle

The radiosensitivity of the cell changes throughout the cell cycle as shown by Sinclair and Morton (1965) with synchronized Chinese hamster cells (figure 2). These cells were synchronized by selecting for mitotic cells. The selection was made by adding trypsin and gently rocking a plastic Petri dish of cells which had been grown overnight. Since cells become round and partly detach from the dish prior to division, mitotic cells were selectively recovered in the supernatant of the cultures. This method resulted in a population of about 75% mitotic cells (Sinclair and Morton, 1963). In figure 2, the selected population was reincubated after inoculation and pulse labeled with tritium-labeled thymidine (^{3}HTdR) for 15

minutes at various time intervals. When cells are synthesizing DNA, as they do in the S phase, ^{3}HTdR is incorporated into the DNA. Non-incorporated thymidine can be washed out and the cells retaining the thymidine, which are now radioactively labelled, can be identified by silver grains in a photographic emulsion (Howard and Pelc, 1953).

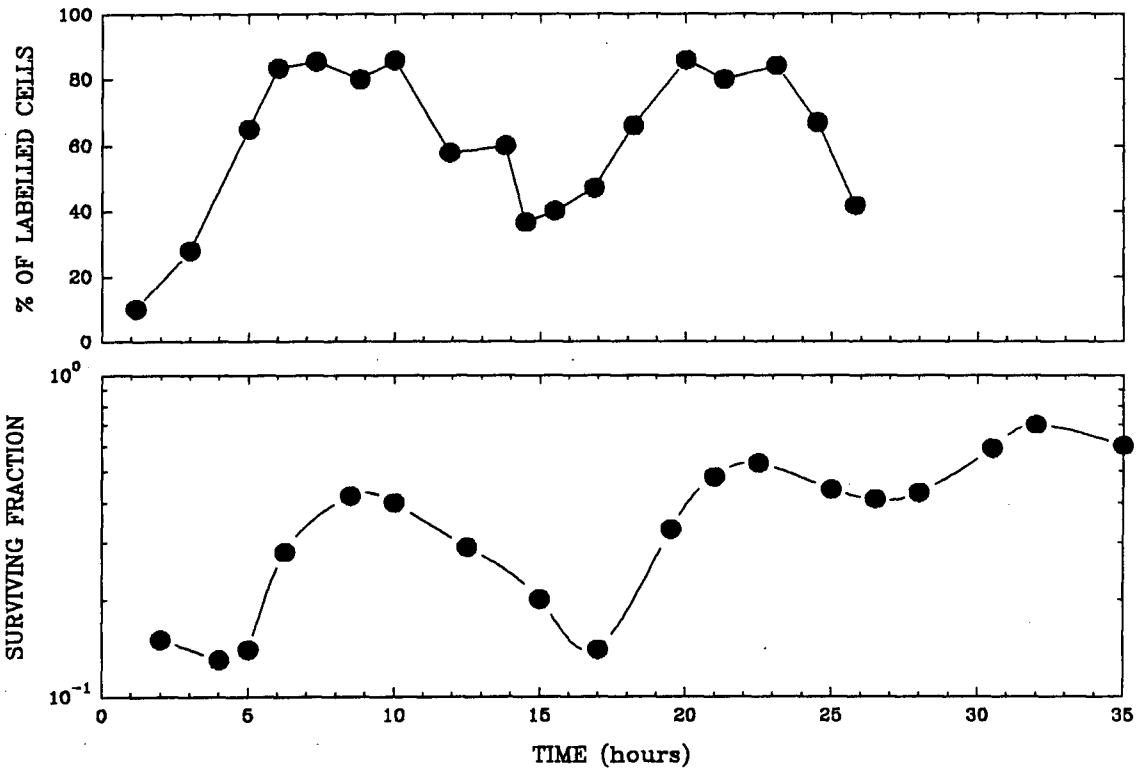

Figure 2. Radiosensitivity of Cells Through the Cell Cycle

a) partially synchronized population of V79 Chinese hamster cells. Percentage of cells labelled with a pulse of tritiated thymidine versus incubation time. b) Colony surviving fraction in partially synchronized hamster cell population irradiated with 6.60 Gy at defined times after reincubation (Sinclair and Morton, 1865).

Figure 2a shows the percent of labelled cells as a function of time after reincubation. At the beginning of the first cycle, few cells were labeled but these increased as the cells entered into the S phase and decreased again during the G_2 period with most of the cells dividing at about 15 hours. Figure 2b shows the response of the same population to a single dose of 6.60 Gy. A peak in survival occurred about 10 hours after reincubation corresponding to the peak in the previous graph in the S phase. A subsequent minimum occurred after 15 hours corresponding to mitosis. These data indicate that the radiosensitivity of the cell varies with cell age, being most resistant during the S phase and most sensitive during M and G_1 phases.

When administering more than one dose of radiation, the surviving population after the first dose depends on the sensitivity of the individual cells. Since the cells in different phases have different survivals, the population distribution changes after each dose, which in turn has a large effect on the cell survival after subsequent doses. It is, therefore, essential to understand how cellular radiosensitivity changes throughout the cell cycle and how radiation can affect the progression of cells.

1.2.2 Mitotic Delay

Radiation can retard the appearance of the next mitosis, a process called mitotic delay (Gray et. al. 1940). It affects surviving and non-surviving cells equally, and the length of the delay is linear with dose. It has also been found that viable cells reestablished the same doubling time after the delay as they had had before (Elkind, Han and Voltz, 1963). This delay was shown to occur in the intermitotic phases of the cycle and therefore must result from damage to or inhibition of the system required to initiate division. *In vitro* studies in rodent cells

show that cells in G_2 phase are subject to large delays in the progression around the cell cycle, whereas S and G_1 cells are less influenced. Quantitatively, the delay is usually expressed as about 10% of the cycle time per Gy (Hall, 1988).

1.3 SUBLETHAL DAMAGE AND RECOVERY

When mammalian cells are first exposed to low ionization density radiations such as X-rays, their survival curve has a characteristic shape on a semi-log plot consisting of an initial shoulder followed by a steeper straight portion. This indicates that damage must be accumulated before a lethal effect occurs, that is, low doses are relatively ineffective in cell killing since damage must be accumulated.

When a population of cells is irradiated, one of three things can occur. 1) An ionizing event may not occur in a critical target site and therefore does no damage to the cell, 2) one or more ionizing events may occur in all necessary critical sites, rendering the cell incapable of division, or 3) a cell may receive an ionizing event in some, but not all, critical sites. The cell in this case does not die but retains its capability of repairing and dividing. This type of damage is known as sublethal damage (Hall, 1988).

1.3.1 Split Dose Experiments

Studies done by Elkind and Sutton et. al. (1965) have shown that with a log-phase population of asynchronous Chinese hamster cells, the surviving cells after a 10-50% survival dose have varying amounts of sublethal damage due to cell heterogeneity.

To test for repair of this damage, a technique was used which involved exposing the cells to two single doses with a defined time interval between them. If the effect of the first dose is not repaired at all, the damage from the two doses would be cumulative, equaling the

damage of a single dose equal to the sum of the two doses. If the two dose survival is greater than an equivalent single dose survival, the damage due to the first exposure must have been repaired to some extent (Elkind and Sutton, 1959). This holds only if the survival after the first exposure does not vary except for repair of damage. From experiments done with synchronized cells, it is known that the survival response as a function of position or age in the division cycle is not constant (Dewey and Humphrey, 1965, Whitmore et. al., 1965, Sinclair and Morton, 1965). Therefore, if cells progress through their cycle between the doses of radiation, the observed survival will not be due simply to repair of sublethal damage. Also, if the time interval is long enough for division to occur, proliferation must also be taken into account.

Fractionation survival curves in Chinese hamster cells, in which survival curves were generated for two doses given with defined time intervals, showed that the shoulder of the curve varied appreciably while the slope remained fairly constant (Elkind, Sutton and Moses, 1961). Immediately after an initial dose of 4.33 Gy no shoulder was present. By 2.6 hours, the shoulder was regenerated to about half its original magnitude. At 5.3 hours, it completely disappeared and by 10.6 hours it had reappeared again and was close to its original size (figure 3).

The explanation for the variation in the shape of the curves generated at the different times is the combined effect of repair of sublethal damage and aging of cells after division delay. Repair led to increases in the subsequent survival while aging caused fluctuations in the radiosensitivity. Both effects were seen under growth conditions which allowed both repair and aging to occur.

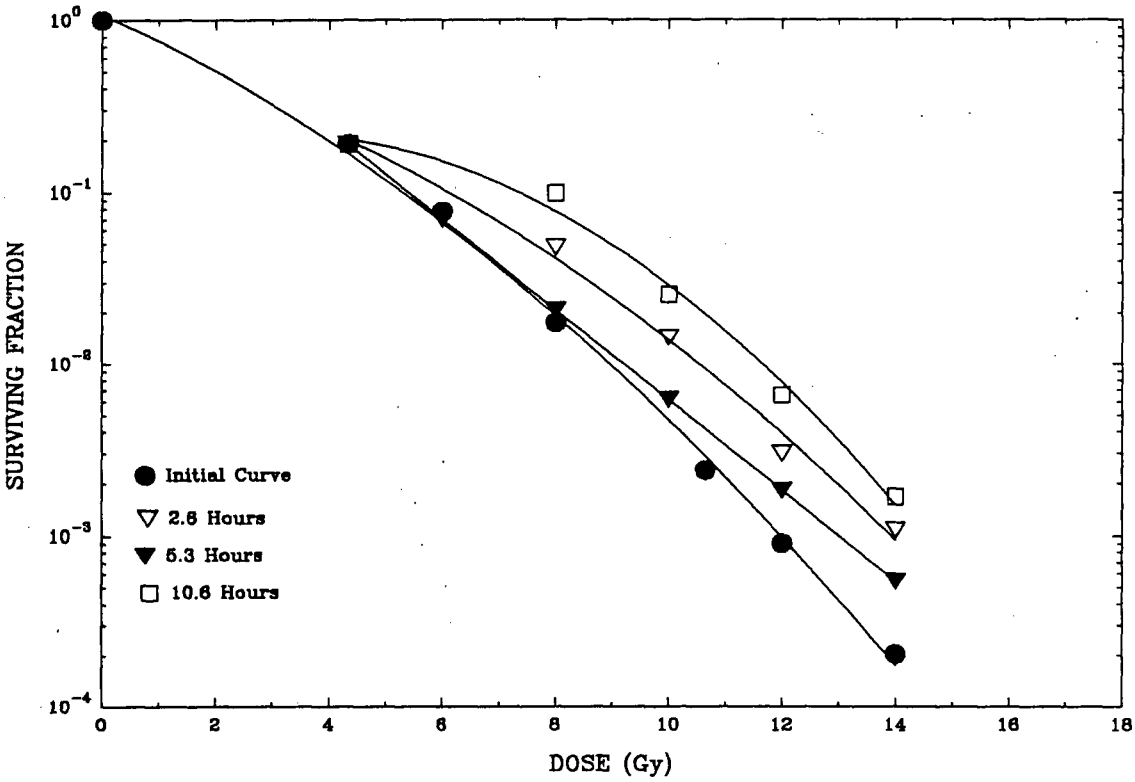

Figure 3. Recovery of Irradiated Log-phase Cells

The survival of cells from an initial dose is compared with curves generated at the time intervals shown after a 4.33 Gy initial dose (Elkind, Sutton and Moses, 1961).

Since growth is strongly dependent on temperature, while repair of sublethal damage is not (Elkind et. al., 1965), it is easy to separate the effects of repair and aging. By lowering the temperature to one at which growth does not occur, the two dose survival could be studied with the effects of repair being isolated.

Elkind et. al. (1965) carried out these experiments with V79-379-A Chinese Hamster cells exposing them to 2.5 MV X-rays (figure 4). Microcolonies were used which contained two to four cells per colony. The split dose curves from irradiation done at 22°C showed only a

progressive increase in survival. This would indicate that the minimum which occurred at 37°C was due to cell cycle progression and when no progression occurred, no fluctuation in survival response was observed.

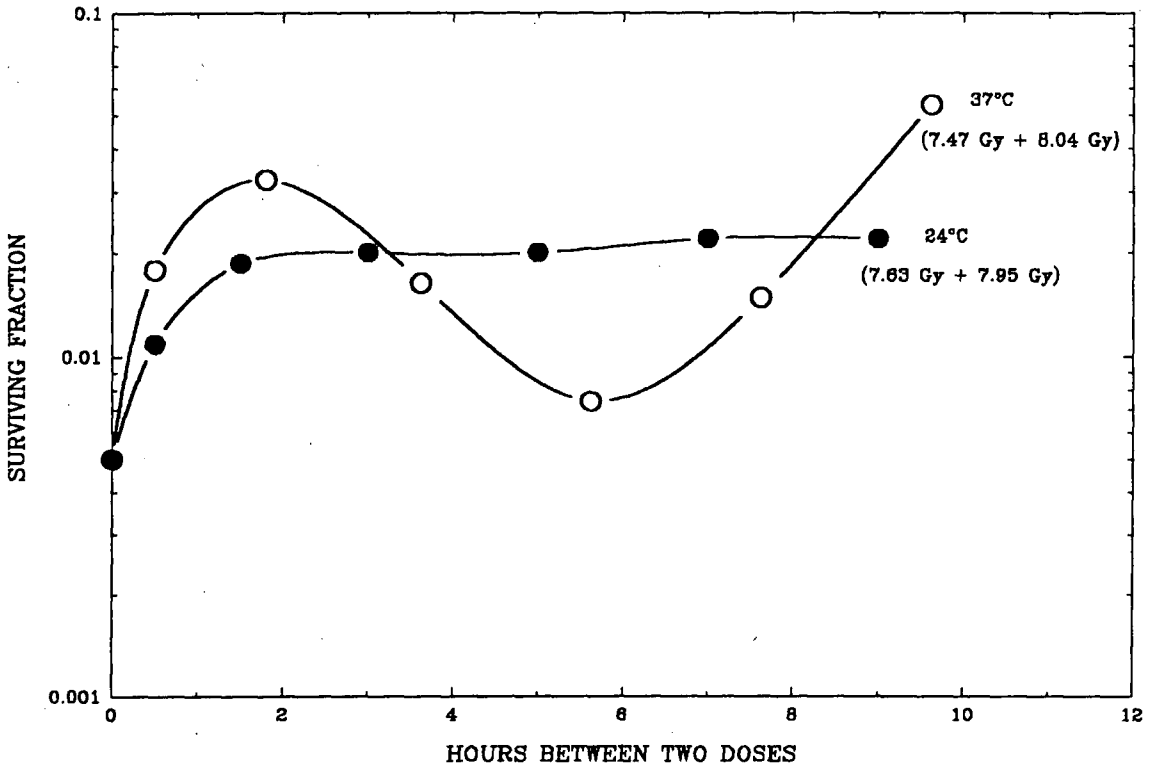

Figure 4. Two Dose Survival of Chinese Hamster Cells

Two dose survival of aerobic Chinese V79-379-A hamster cells exposed to 2.5 MV X-rays and incubated at the temperatures shown between doses. Numbers in parenthesis are first and second doses (Elkind et. al., 1965).

Cell cycle progression not only has an effect on the survival response from two doses of radiation, but may also affect the survival response to multiple doses, that is to fractionated schedules.

1.4 FRACTIONATION

Fractionation is a method of radiation treatment which involves administering several small doses of radiation to the patient as opposed to fewer large doses. Standard fractionation schedules are comprised of a six week radiation period with 5 doses of about 2 Gy each given per week. Research is now being done on non standard fractionation schedules to determine if there are any clinical gains achievable by this method. These schedules can be divided into two categories: hyperfractionation, which utilizes doses of less than 1.8 Gy, and accelerated fractionation which must occur in much less than 6 or 7 weeks. These schedules avoid extending the overall treatment time, which would allow tumours to proliferate excessively, by administering at least 2-3 fractions each day. Due to the accepted minimum time between doses being 6 hours, however, it is not practical to administer more than 3 doses/day (Fowler, 1989).

Over the last 90 years, there have been many fractionation schedules devised but only within the last 10 years has the understanding of the biological factors concerning the delayed proliferation after irradiation and the effect of the dose per fraction been greatly improved. These biological factors apply to the majority of tissues and tumours and with this knowledge, new schedules are being planned that should be more effective.

The most important advance, however, in fractionation radiotherapy was a French discovery that rather small daily fractions over a period of approximately 6 weeks kept both early and late complications in normal tissues within acceptable limits (Coutard, 1932, Baclesse, 1958). These doses were given 6 days each week. It is now known that 6 weeks is long enough to allow accelerated compensatory proliferation in the skin and mucosa to improve acute reactions and that fractions of about 2 Gy keep late reactions from being excessive.

Short schedules of about 16 fractions in 21 days have also proven to be successful (Paterson, 1963). For example, the cells in about half of the head and neck tumours proliferate quickly, doubling in 4-6 days, therefore the shorter time scale reduces this growth. A lower total dose is, however, required to keep acute reactions at a tolerable level (Fowler, 1989).

1.4.1 Linear Quadratic Model

The linear quadratic model (LQM) has aided in the comparison of total doses required to produce the same biological damage in different fractionation schedules, provided that the effect of proliferation can be neglected (Fowler, 1983).

This model was first used by Douglas and Fowler in 1976 to interpret the skin isoeffect doses for fractionated radiation. It assumes that repair, cell cycle progression, mitotic delay, cell proliferation and environmental change caused by radiation are identical for every dose fraction, i.e. equal effect per fraction. The model is described by the equation

$$\ln S = -n(\alpha d + \beta d^2) \qquad (1)$$

where:

S = surviving fraction
n = the number of fractions
d = dose per fraction

This equation has a linear term where the number of lethal events is proportional to the dose, and a quadratic term where two sublesions interact to produce a lethal event. α is the initial sensitivity or initial slope of the survival curve. The term βd^2 indicates that the sensitivity increases with increasing dose. α/β is a measure of the relative importance of the α and β terms which is equal when $\alpha d = \beta d^2$, that is when $d = \alpha/\beta$ (figure 5).

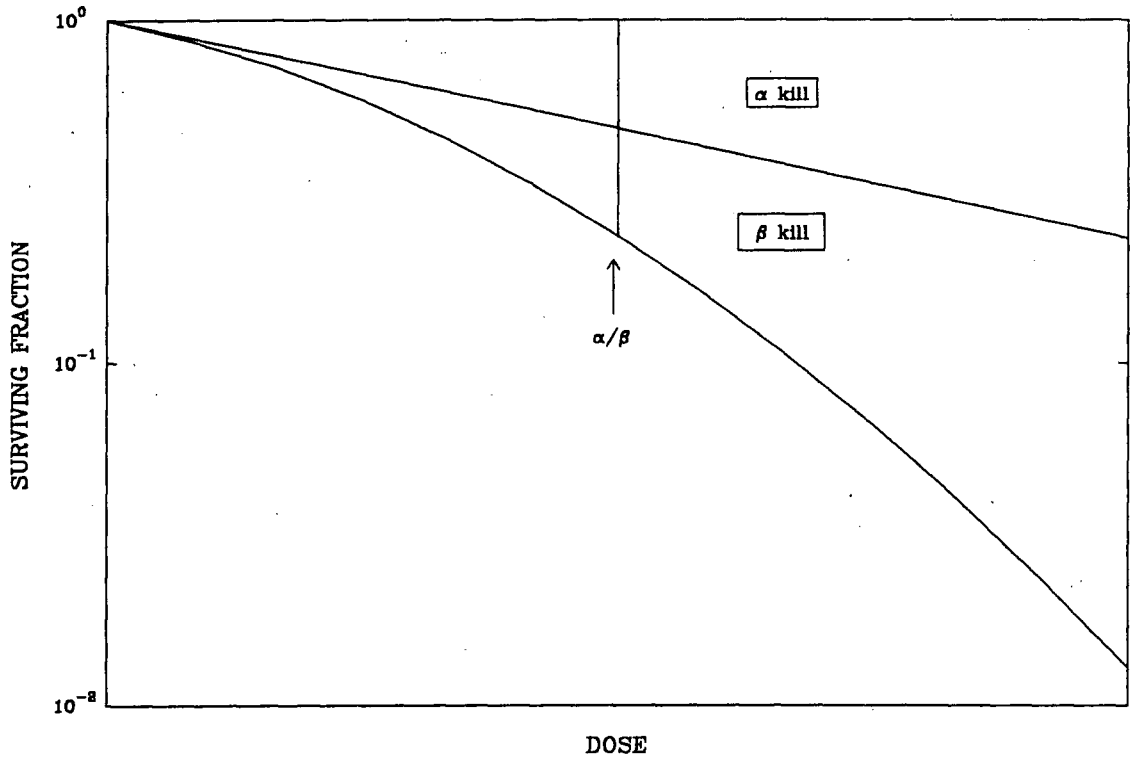

Figure 5. Linear Quadratic Model

Linear quadratic survival curves showing that α/β is the dose at which the components of cell killing from the α and β modes are equal (Thames and Hendry, 1987).

This model also takes repair of sublethal damage into account. Repair is thought to occur approximately linearly with dose and if no repair takes place, the shoulder of the curve would be diminished. As repair occurs, the biological consequences are progressively reduced and the width of the shoulder is increased. Since repair is less probable at higher doses, the curve bends downwards (Thames and Hendry, 1987).

This model can also be used to predict fractionated doses equivalent to a low continuous dose or vice versa. For example, if a 10 Gy per day low continuous dose is given, the equivalent fractionated doses can be calculated as follows:

By the LQM,

$$-\log S = n\alpha d + n\beta d^2 \quad (2)$$

where:

$$d = D/n \quad (3)$$

D = total dose

therefore,

$$-\log S = \alpha D + \beta D^2/n \quad (4)$$

For a continuous dose, β is assumed to be negligible since n is infinite with no time between doses and therefore,

$$-\log S = \alpha D \quad (5)$$

or

$$S = \exp(-\alpha D) \quad (6)$$

If a 10 Gy/day continuous dose is given over 5 days, 50 Gy is administered. If α is estimated as 0.1 Gy^{-1} and β as 0.01 Gy^{-2} then the expected survival after this dose would be 0.00674. From equation 4, the equivalent dose administered in high dose rate fractionated exposures, with enough time between fractions for repair to be complete, would be 17.9 Gy/week. If this dose is given in 5 fractions one can calculate the dose per fraction as follows:

$$\alpha D + \beta D^2 = \alpha nd + \beta nd^2 \quad (7)$$

therefore,

$$D = \frac{nd(\alpha/\beta + d)}{(\alpha/\beta + D)} \quad (8)$$

From this equation, d can be calculated as 6.2 Gy. This indicates that for 5 fractions over 5 days, the equivalent dose to 50 Gy of continuous dose is 6.2 Gy/fraction. Similarly, if 10 fractions are given the equivalent dose is 3.67 Gy/fraction.

Using this calculation, a fairly accurate estimation of dose required per fraction to produce the same effect as an extended exposure to a low continuous dose can be obtained.

1.4.2 Effect of LET on the Survival Curve

To be able to compare the responses of cells to different types of radiation, the linear energy transfer (LET) of the radiation must be taken into account. LET is the energy lost by an ionizing particle per unit path length it has travelled. This varies for each type of radiation, for example, X-rays have low LET and neutrons have high LET.

High LET radiation is less efficient per unit dose than low LET in the inactivation of single molecules because the ionizing particle track is very dense and much of the deposited energy (dose) is "wasted". Less of the high LET damage is, however, repairable and therefore these particles are very effective at killing on a "per particle" basis.

This is summarized in figure 6 where it can be seen that survival curves of cells exposed to X-rays had a larger shoulder than those exposed to neutrons since the damage caused by X-rays was more easily repaired. The difference between these two curves can be described in terms of relative biological effectiveness (RBE). RBE is a comparison between the test radiation and 250 kVp X-rays. When calculated, the survival level must be defined; RBE changes with increasing dose. In the case of figure 6, the RBE was highest at small doses. As can be seen in figure 6a, the RBE was 1.7 for neutrons indicating that a surviving fraction of 0.01 was produced using only 60% of the X-ray dose.

Since the survival curve for cells exposed to neutrons has a smaller shoulder, when extended to a fractionated schedule, the RBE increases. X-ray damage, which can be easily repaired, produces curves which display regenerated shoulders between fractions producing a less efficient effect overall (figure 6b). In this graph the RBE increased to 2.1. Therefore, as the number of fractions increased and the dose per fraction decreased, the neutrons became progressively more efficient relative to X-rays (Hall, 1988). This is of interest when comparing two different types of radiation.

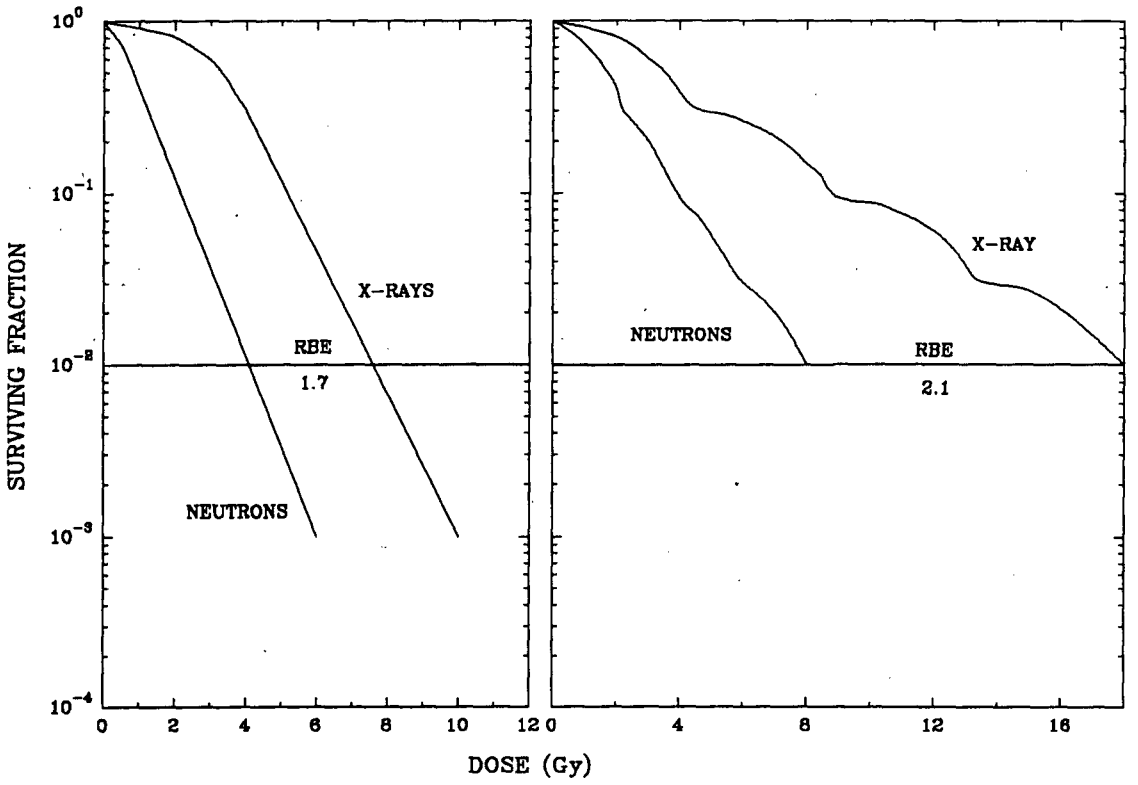

Figure 6. Typical Survival Curves for Mammalian Cells Exposed to X-rays and Neutrons

a) Single doses. In the case of X-rays, the survival curve has a large initial shoulder; for neutrons the initial shoulder is smaller and the final slope is steeper. b) Fractionated doses. The effect of giving doses of X-rays or neutrons in four equal fractions to produce the same level of survival as in (a). This results in an enlarged RBE for neutrons (Hall, 1988).

1.5 TUMOUR MODELS

In the search for improved treatments for cancer, a greater understanding of the properties of tumours is essential. It would be ideal if the overall tumour response could be well defined but unfortunately, tumours consist of several different cell subpopulations which differ with respect to both function and genetics, each with a different response to therapy.

Most experiments conducted with tumours lack a sufficiently informative quantitative endpoint, making it difficult to detect the small degrees of damage resulting from typical increments in therapeutic procedures. There have, however, been significant contributions to the understanding of tumour cell properties and responses to therapy which have resulted from *in vivo* studies such as reoxygenation effects involving vasculature studies. These studies have several limitations including difficult technical procedures and cost (Durand, 1976).

In vitro studies, on the other hand, tend to be less expensive, faster and easier to conduct. Many variables in the system can be controlled independently, allowing distinct causes and effects to be defined. Conventional radiobiology studies *in vitro*, first introduced by Puck and his associates in 1956, use a cell population growing on glass Petri dishes. Although these systems differ in many aspects from the tumour they are modelling, they still have many advantages. Of primary importance is high resolution due to the ability to work with large numbers of surviving cells allowing survival curves to extend over several decades. Single cells also provide insight into many factors affecting tumour cell response such as variation in response throughout the cell cycle, repair of sublethal and potentially lethal damage, and variation in response depending on environmental oxygen and other metabolites.

A significant advance in *in vitro* studies was the development of plateau-phase or non-cycling cell systems (Hahn and Little, 1972). Since tumour cell subpopulations are composed of non-cycling or slowly cycling cells, this system seemed to have advantages over the single cells, including being a more representative tumour model. As in tumour cells, crowding occurs in the plateau-phase cultures causing the cells to have cell cycle parameters similar to tumour cells. For example, as the cell density increases, the cell cycle length increases (Tannock, 1969). Also, a similarity exists between the radiation responses of the cells in

plateau phase and the tumour cells (Belli et. al., 1970). This type of culture was the best two-dimensional model but still lacked many properties of a tumour (Durand, 1976).

1.5.1 Spheroids

A number of three dimensional tumour models have been developed, including both spontaneous systems and cells constrained to grow in a three dimensional symmetry using semi-solid growth media (Dalen and Burki, 1971, Folkman, 1974). Multicell spheroids of Chinese hamster cells, for instance, have been in use for over 20 years. These spheroids have many similarities to nodular tumour growth (Durand, 1990).

Chinese hamster V79-171 cells grown appropriately in suspension, remain attached to each other after division and form spheroids. As they grow, the growth rate decreases, due in part to the elongation of the cell cycle time because of an extended G_1 phase, but mostly due to a decrease in the growth fraction (Durand, 1976). As the size of the spheroids increases, cell death occurs at the center of the spheroid as a result of a lack of oxygen, glucose and other substrates. Accumulation of toxic metabolic waste products also contribute to the central necrosis in the spheroid (Sutherland, 1988). When metabolites and oxygen were maintained at the required level to provide sufficient nutrients for cell growth, the thickness of the viable rim was found to be between 100 and 200 μm (Sutherland and Durand, 1973).

Spheroids which have been grown for less than five days under well fed conditions have no anoxia and no necrotic center. Since anoxic cells are more resistant to radiation, larger spheroids show two populations of cells with different radiosensitivities when used to generate survival curves. With the smaller non-anoxic spheroids this effect is not seen (Sutherland and Durand, 1976).

The decreased proliferative activity in the rim of the spheroids can be compared to the decreasing proliferative index outward from blood vessels as found in tumour cords (Tannock, 1968). There may be more variation in the spheroid microenvironment and therefore in the growth parameters than in those of the tumour. Karyotypic change from diploid to aneuploid which is found in spheroids resembles the transition and development of aneuploidy spontaneously in tumours *in vivo* (Durand, 1990).

The effects of radiation on single cell cultures have been thoroughly investigated, showing evidence for the variation of radiosensitivity throughout the cell cycle, mitotic delay, sublethal damage and recovery. It has been shown, with this single cell model using split dose experiments and survival curve generation along with DNA profiles, that repair and proliferation affects the survival response to radiation.

Using the three dimensional spheroid model, which has more similarities to tumours than the single cell cultures, further investigations can be undertaken. In this thesis, the effects of repair and proliferation were studied in two dose radiation schedules to determine how they are interrelated. This was done with methods similar to those used for the single cell experiments. The experiments were then extended to three dose schedules and ultimately to longer multifraction schedules in which repopulation also played a role. These data were analyzed with the LQM although its validity was questioned.

The information gained was also applied to a practical question in which multifraction dose survival responses were compared to the survival response of a low continuous dose. The responses predicted by the LQM were critically assessed to determine the validity of this model.

2. METHODS AND MATERIALS

2.1 SPHEROID GROWTH

All spheroids used in these studies were grown from Chinese hamster V79-171b lung cells. These cells were propagated as monolayers in 100 mm plastic Petri dishes with twice-weekly subcultivation in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Sutherland and Durand, 1976, Durand, 1980). They were grown at 37°C in a humidified atmosphere of 5% CO_2 in air. Subcultivation involved the removal of the medium by aspiration, rinsing the cells with 1 to 2 mL of 0.1% trypsin and incubating with 4 mL of trypsin for 7 minutes. The cells were then pipetted up and down gently to produce single cells. They were then transplanted to new 100 mm plastic Petri dishes containing MEM with 10% FCS. To initiate spheroid growth, asynchronous cells were removed from the plastic Petri dishes by typsinization with 0.1% trypsin. Spinner culture flasks (250 mL size) were then inoculated with $2x10^6$ cells in 200 mL of the same MEM used for single cell cultures but with 5% FCS and stirred at 180 rpm. The gas phase contained 5% CO_2 in air. The spheroids were grown for 3 days before use. This allowed some cells to accumulate in the G_1 phase as in a tumour without allowing anoxia to occur.

2.2 IRRADIATION

After 3 days of growth, the spheroids in each flask were divided into two 100 mL water jacketed spinner culture flasks. The jackets were filled with water of the desired temperature to maintain a constant temperature in the medium during irradiation. The flasks were irradiated horizontally using a Philips 250 kVp X-ray beam (HVL 1.5 mm Cu) as shown in figure

7. The spheroids in the flasks were continuously stirred at 180 rpm during the irradiation with a dose rate of 4.12 Gy/min.

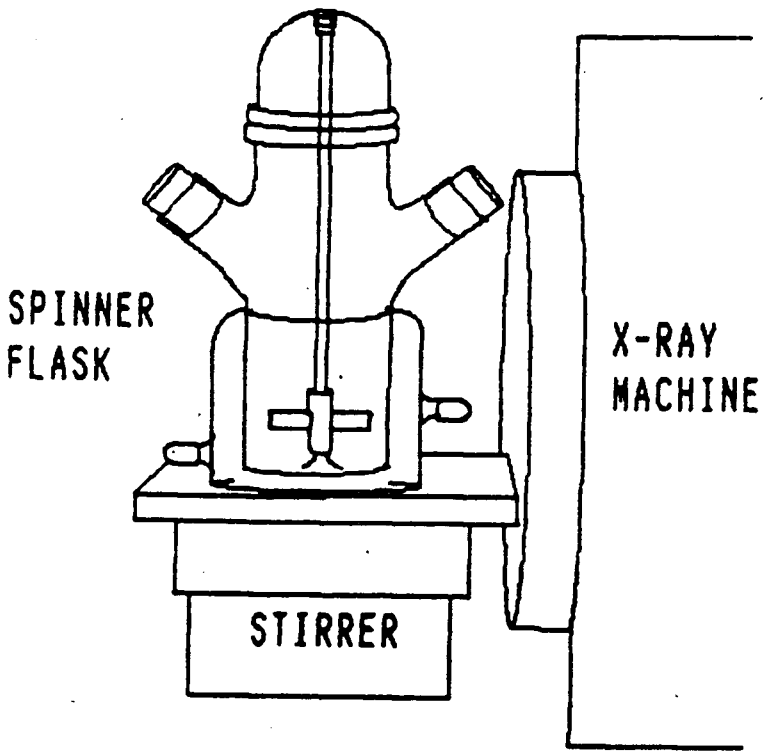

Figure 7. Experimental Configuration for Spinner Flask Irradiations

Configuration used for exposures to 250 kVp X-ray beam for initial doses in split dose experiments and for doses used in generation of survival curves.

In the split dose irradiations, the second dose was administered by removing a 10 mL sample from the flask and placing it in a 10 mL plastic test tube. This test tube was then irradiated as shown in figure 8, using a dose rate of 4.73 Gy/min.

The dose rate for both these configurations was determined using a Victoreen model 500 precision electrometer and the calibration curves are shown in figure 9.

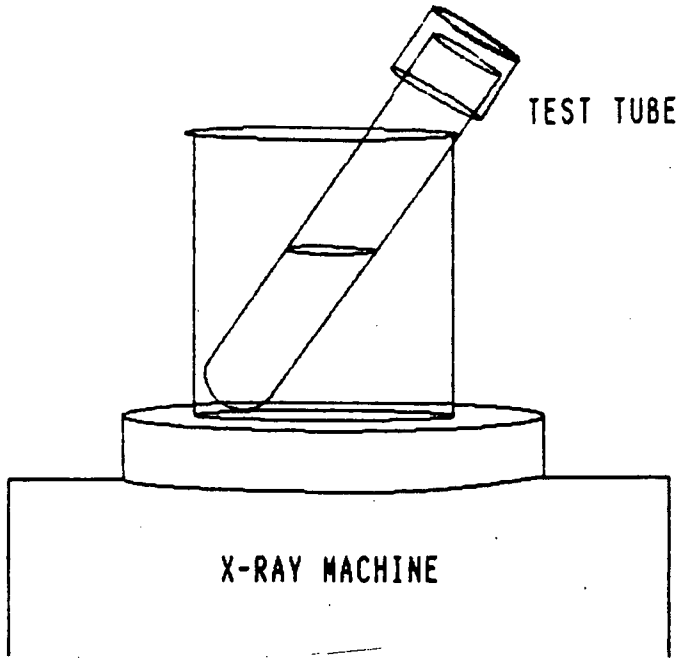

Figure 8. Experimental Configuration for Test Tube Irradiations

Configuration used for exposure to 250 kVp X-ray beam for second dose of two dose split experiments and third dose of three dose split experiments.

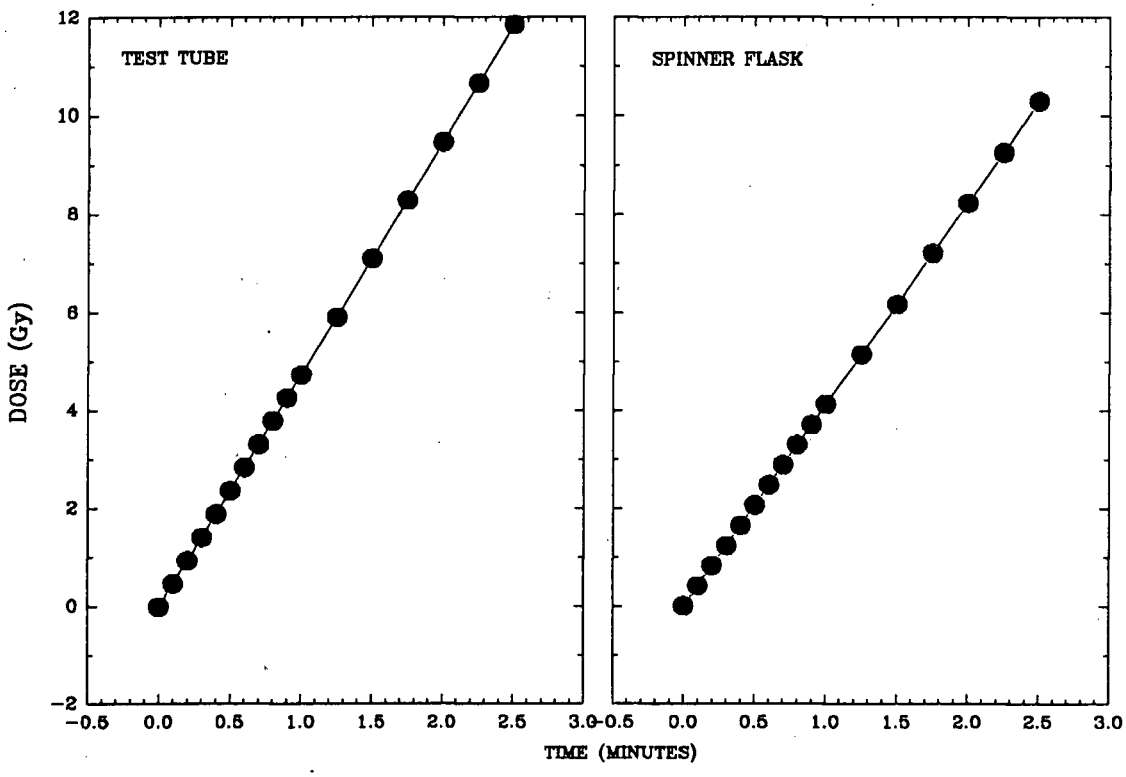

Figure 9. Calibration of Philips 250 kVp X-ray Beam

Dose rate calibration for (a) test tube configuration (figure 8) and (b) spinner flask configuration (figure 7).

2.3 PLATING

After irradiation, the spheroids were trypsinized with 0.25% trypsin. This was done by removing the medium, rinsing the spheroids with 3 mL of trypsin, then adding 5 mL of trypsin. The spheroids were placed in a 60 mm plastic petri dish and agitated gently for 10 minutes. Two mL of MEM were added and the solution was pipetted up and down until the spheroids were disaggregated into single cells. The concentration of cells was determined using a

Coulter counter and the sample was then diluted so that 1 mL of sample contained the required number of cells for plating. Four 100 mm petri dishes containing 9 mL of MEM with 10% FCS were then inoculated with 1 mL of sample for each dose point. These plates were incubated at 37°C and 5% CO_2 for 1 week to allow viable cells to form colonies.

After one week, the medium was poured off and the colonies were stained with 2 mL of cold malachite green stain, then rinsed under cold running water. Colonies containing more than 50 cells were counted and survival was expressed as colonies/cells plated, with a correction made for the zero dose point plating efficiency.

2.4 DNA PROFILES

To determine the percentage of cells in each of the cell phases, DNA profiles were produced by a flow cytometer. Cells which remained from the samples after plating was complete were centrifuged at 1000 rpm for 5 minutes. The medium was discarded and the cells were resuspended in 2 mL of 1% para formaldehyde (PARA). After 15 minutes, the sample was again centrifuged, removing the PARA and adding phosphate buffered saline (PBS). The sample was then refrigerated until staining. To stain the cells, they were centrifuged, the PBS was drained off and 0.1% Triton X-100, a detergent, was added to permeabilize the cells. The cells were centrifuged again and 1.0 mg/mL RNAse was added and allowed to sit for 30 minutes at 37°C. The RNAse treatment stopped cytoplasmic fluorescence due to fluorochrome binding to RNA. The sample was centrifuged once more and 50 μg/mL of propidium iodide (PI) in 0.1% sodium citrate buffer was added. This was left for at least 60 minutes at 4°C before being analyzed by the flow cytometer.

The DNA profiles were analyzed using Multicycle Multiple Option Cell Cycle Analysis purchased from Phoenix Flow Systems. This program uses the method of Dean and Jett

which defines all cells in G_1 to have one "unit" of DNA, those in G_2 and M to have two units and those in S phase to have between one and two units. The S phase cell distribution is fit with a second-degree polynomial; the G_1 and G_2 + M peaks are fit by two Gaussian curves. All curves are fit by non linear least squares techniques (Dean and Jett, 1974).

2.5 EXPERIMENTAL PROTOCOLS

2.5.1 Split Dose Experiments

To determine how quickly repair occurred in spheroids and its dependence on temperature, split dose experiments were performed. These involved irradiating two flasks of spheroids, one at 22°C and one at 37°C, with two X-ray doses separated by a defined time between zero and 10 hours. Initially, doses of 4.86 Gy and 5.58 Gy were used with DNA profiles taken for each time point to monitor the progression of cells around the cell cycle.

The experiments were repeated using 2 doses of 8 Gy and for 3 doses of 6 Gy keeping the flasks at 22°C for 4 hours between the first two doses when 3 doses were given. This allowed the cells to repair with no progression occurring.

2.5.2 Survival Curves

To further test if complete repair was occurring, survival curves were generated for spheroids at 22°C and 37°C. An initial curve up to 8 Gy was produced with a second fraction of 8 Gy given at defined time intervals. This allowed the shapes of the survival curves to be examined, specifically the shoulder widths and slopes to indicate the amount of repair occurring.

This was extended to 3 doses of 6 Gy. Again, the first two doses were given four hours apart with the cells remaining at 22°C for that time interval; the third dose was given after a defined time interval with the spheroids either at 22°C or at 37°C.

2.5.3 Fractionation Experiments

Three day old spheroids were irradiated once a day for eight days with doses of 6 and 8 Gy/fraction. These doses were given in increments of 1 or 2 Gy to generate survival curves for each day. The spheroids were maintained at 37°C throughout the experiment. After the 3rd day of growth, the spheroids were fed each day by changing the medium and replacing the gas phase with 5% CO_2 in air.

2.5.4 Brachytherapy

To compare a fractionated schedule with a continuous low dose irradiation, experiments were done with a brachytherapy unit as shown in figures 10 and 11. In this unit a seed of radioactive Iridium (^{192}Ir) could be sent down a catheter, stopping at positions around the flask of spheroids and for time intervals which were preprogrammed. These positions and times were calculated to give doses of 4.25, 4.75, 5.25, 6.50 ,7.50 and 8.50 Gy. The first three doses were administered twice daily and the last three only once a day. Samples were plated, as before, after each dose.

For a low continuous dose of about 10 Gy per day, a catheter with ^{192}Ir seeds was set up around the flask in the same configuration as for the high doses, such that the spheroids would receive approximately 10 Gy each day. This emulated the treatment used in the clinic when a radioactive seed is surgically implanted at the site of the tumour. Samples were removed after each 10.0 Gy dose and plated. The medium in both fractionated and

continuous radiation groups was replenished to keep the volume constant throughout the experiment.

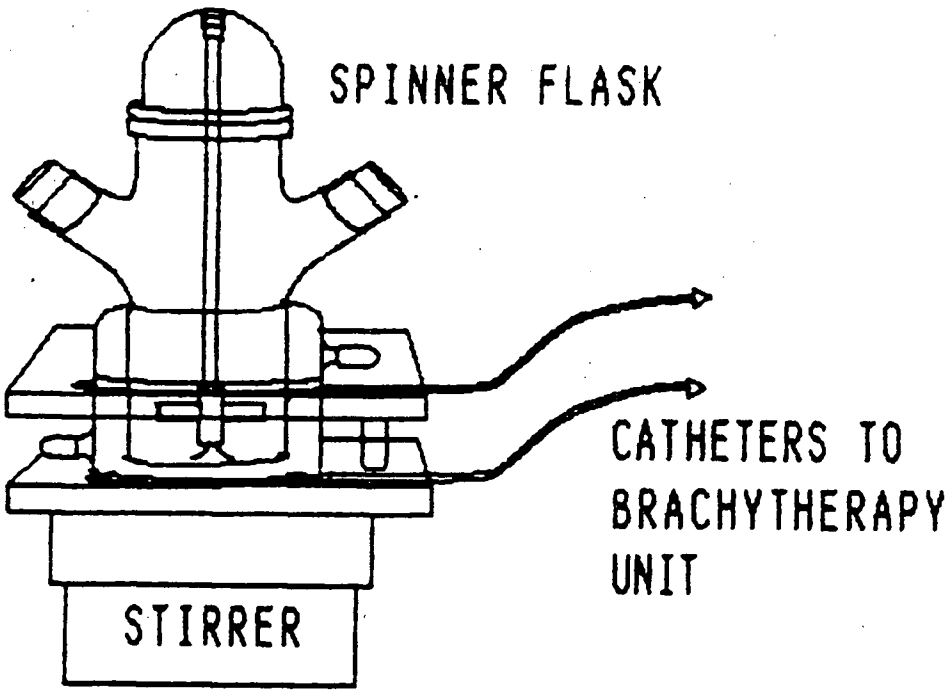

Figure 10. Experimental Configuration for Irradiations Using ^{192}Ir Source.

Spinner flask used for both high dose ^{192}Ir irradiation using a brachytherapy unit and low dose rate continuous ^{192}Ir irradiations using radioactive seeds (side view).

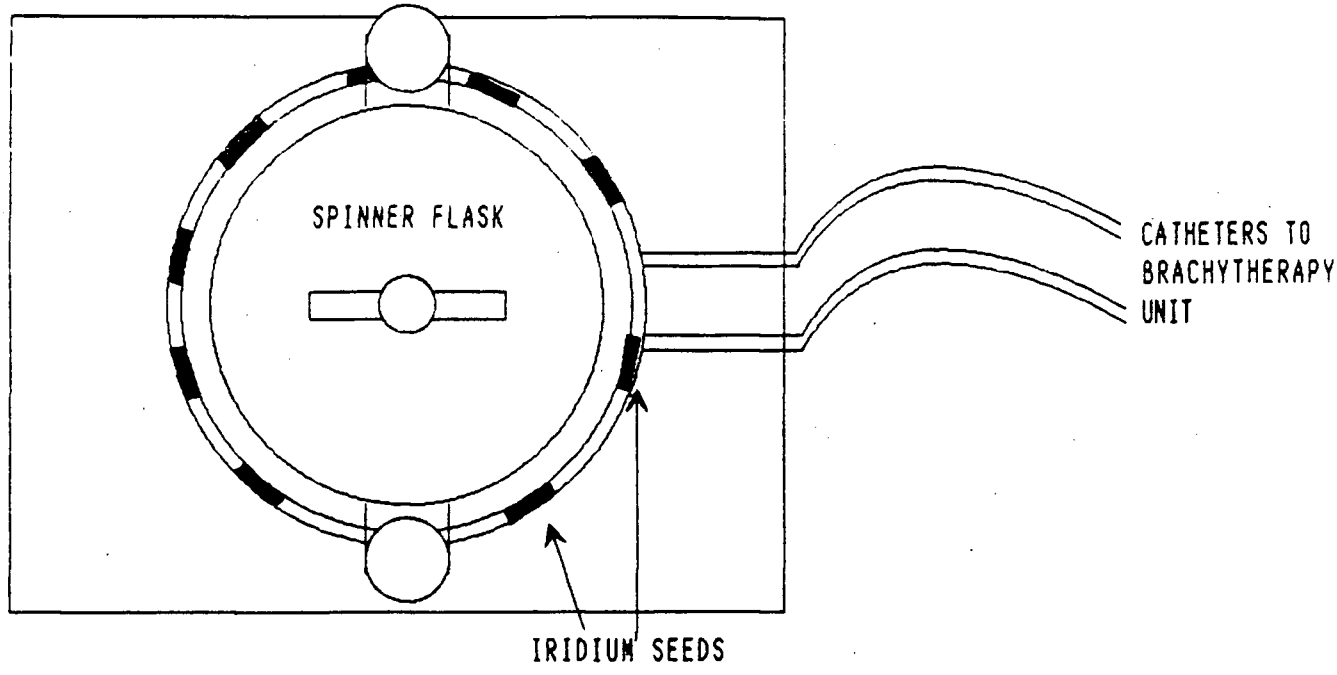

Figure 11. Experimental Configuration for Irradiations Using ^{192}Ir Source.

Spinner flask used for both high dose ^{192}Ir irradiation using a brachytherapy unit and low dose rate continuous ^{192}Ir irradiations using radioactive seeds (top view).

3. RESULTS

Using the techniques described in the previous section, the effects of repair, repopulation and redistribution on the response of the cells in the three dimensional spheroid model were examined, with the intent of providing further insight into the responses of tumour cells to radiation.

These experiments will be described in detail in this section, with limited speculations as to the causes of these results. The results will then be integrated and a more complete description of the spheroid response to multifraction radiation presented in the Discussion.

3.1 TWO DOSE SCHEDULE

Two dose experiments specifically provided evidence concerning the effects of repair and redistribution on the survival response of cells in the spheroid to radiation. We compared the experiments at 37°C and at 22°C to indicate the difference in responses with and without proliferation.

3.1.1 Split Dose Experiments

The effects of the cell cycle on the response of spheroids to radiation were investigated in split dose experiments where cell cycle status was monitored using cytofluorometrically produced DNA profiles. Radiation effects on cell cycle progression were also measured.

Initially, we used two doses of 4 Gy both at 37°C and at 22°C. The results are shown in figure 12; very little change occurred in the surviving fraction as a function of time between doses for both temperatures. There was a slight initial increase in the survival but little

structure was seen after this increase. This shows a lack of synchronization in the cells before the second dose of radiation.

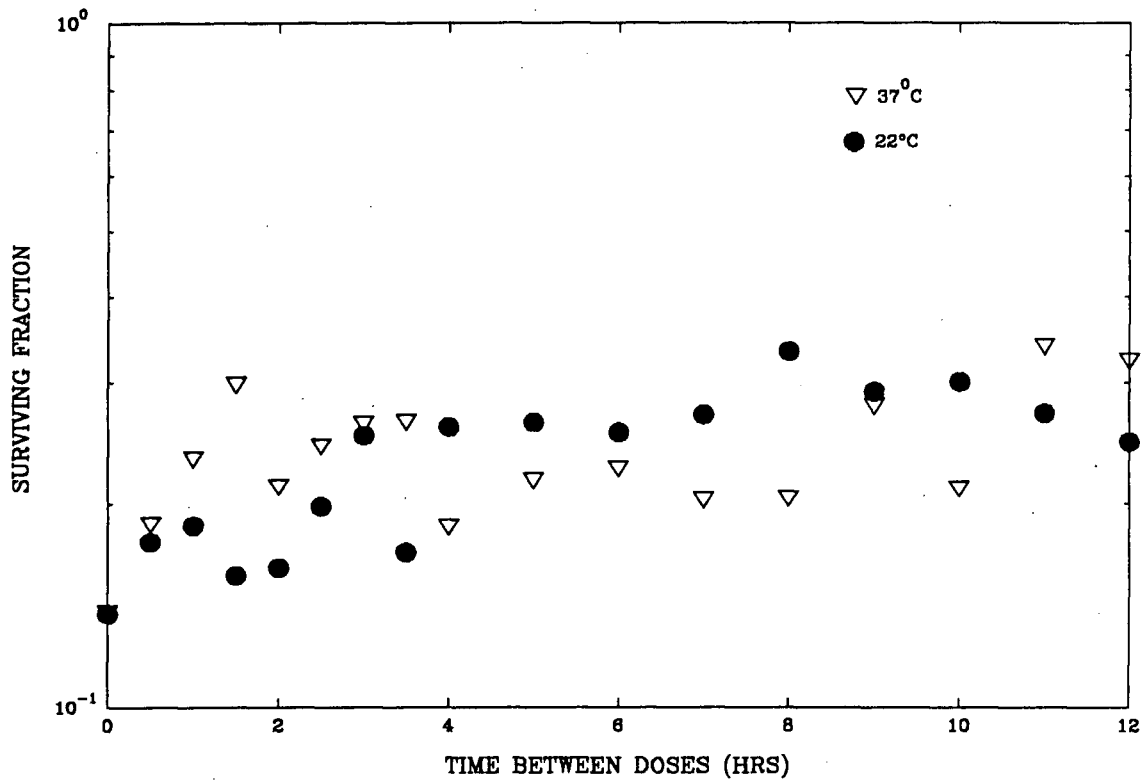

Figure 12. Split Dose Curves of cells from V79-171b Chinese Hamster Spheroids

Survival after 2 doses of 4 Gy separated by defined time intervals during which spheroids were held at either 22°C or 37°C.

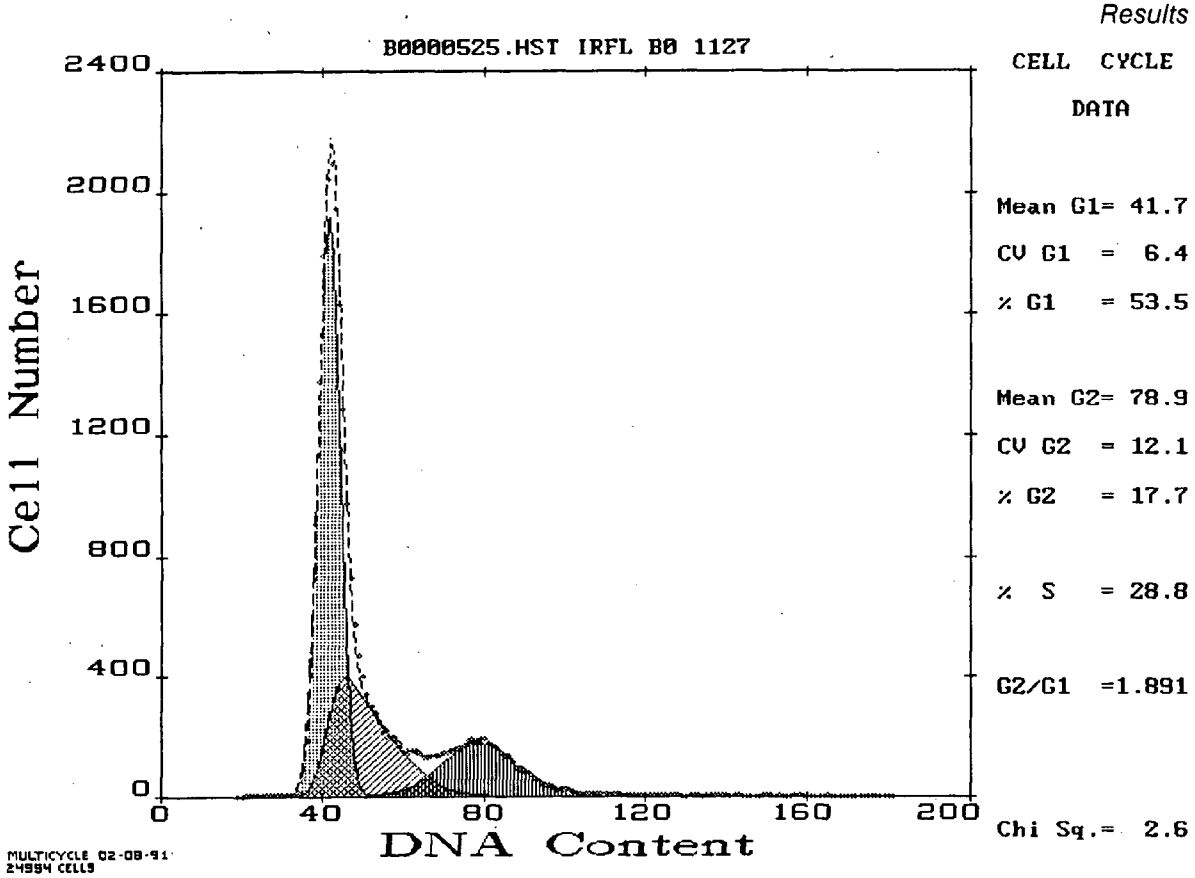

Figure 13. Analyzed DNA Profile

Analyzed DNA Profile of initial population of cells from V79-171b Chinese hamster spheroids using Dean analysis.

A DNA profile taken for the first time point is shown in figure 13. This shows that the initial G_1 fraction in these 3 day old spheroids was about 50% with about 30% of the cells being in S phase. In order to see any structure in the split dose response, the cells must be partially synchronized after the first dose. This synchronization allows the effect of a cell population with a homogeneous radiosensitivity to be monitored as opposed to a combined effect of a heterogeneous population of varying survival responses. This means that a greater fraction of the sensitive G_1, G_2 and M phase cells must be killed leaving a larger proportion of resistant S phase cells. To accomplish this at least 70% of the total cells population must be

killed with the first dose leaving only a 30% surviving fraction composed of an enriched population of S phase cells. Figure 14 shows that a 4 Gy dose reduced the survival to 40% to 50% leaving a heterogeneous population including G_1, S, G_2 and M phase cells. These cells varied in their response to radiation as they progressed through the cycle, which smoothed out the structure one would have expected to see at higher doses.

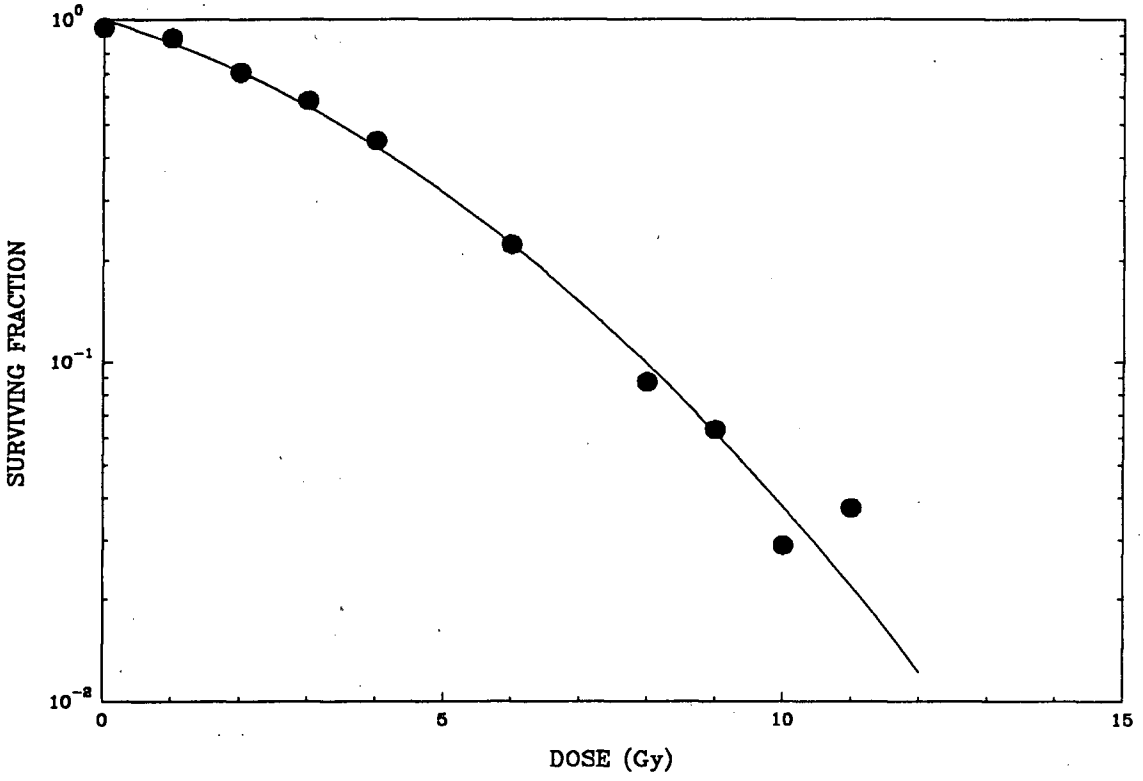

Figure 14. Survival Curve for Cells from V79-171b Chinese Hamster Spheroids

Typical survival curve for cells from 3 day old V79-171b Chinese hamster spheroids irradiated at 37°C by 250 kVp X-rays. After irradiation, the cells were disagregated and plated for colony formation.

In single cell experiments, if the inherent sensitivity were the same in spheroids as it is in monolayers, the cell cycle dependent fluctuation in survival would be greater than in spheroids because of a smaller initial G_1 population. The initial single cell population would, therefore, be more radioresistant than the initial population of the spheroid and, after an initial synchronizing dose, the increased resistance would be even greater. Because of the accumulation of cells in the G_1 phase in spheroids, the size of the initial dose is of great importance. The increased G_1 population is characteristic of a tumour population, therefore, it is instructive to carry out this study on the complex spheroid model.

From these results, it was decided that two doses of 8 Gy should be administered since an 8 Gy dose reduces survival to less than 10%. This would give a more homogeneous population of S phase cells after the first dose so that the fluctuation in sensitivity of the surviving population could be followed as it progresses through the cell cycle.

Figure 15 shows the results from the split dose experiment which best demonstrated the variance in radiosensitivity throughout the cell cycle. In this case, cells kept at both 37°C and 22°C between irradiations showed increased survival for the first two to three hours as a result of repair occurring. The cells kept at 22°C rose to a lower maximum survival than those at 37°C, indicating that cells at 37°C repair to a greater extent. Also, cells held at 37°C repair faster as indicated by the steeper initial rise. Progression of the cells had a role in this increased survival rate since the cells which progressed through S phase had the opportunity to repair during DNA replication. After the first maximum, the survival of cells from spheroids at 22°C remained at a more constant level. The survival of cells from spheroids at 37°C fluctuated due to the progression of the cells through the cell cycle and as the cells moved to a more sensitive G_2 phase a dip in survival occurred followed by an increase as the cells underwent division. This effect was observed as a result of the initial dose leaving a partially

synchronized population of predominantly S phase cells. From this result it was determined that the cells changed sensitivity throughout the cell cycle as was found in the single cell system. The temperature at which the cells were held not only affected the progression of the cells, which affected the radiosensitivity of the population, but also affected the rate of repair.

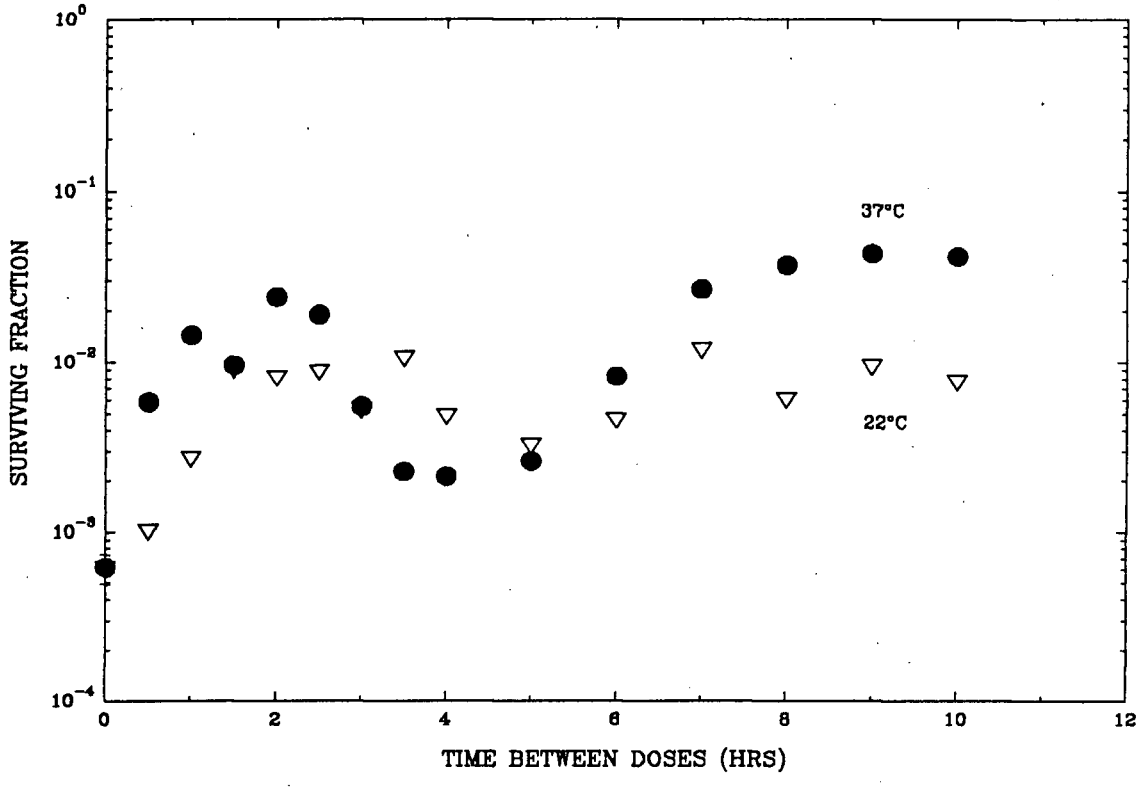

Figure 15. 8 Gy Split Dose for Cells from V79-171b Chinese Hamster Spheroids

Two dose survival with spheroids held at either 22°C or 37°C for a defined time interval between doses.

Due to the inability of flow cytometry to differentiate between living and dead cells, synchronization of solely the living cells could not be observed in the cell cycle analysis shown in figure 16. It is, however, obvious that, unlike the cells at 22°C, both the living and dead cells

at 37°C were in fact progressing through the cell cycle and there was a definite block occurring in the G_2 phase.

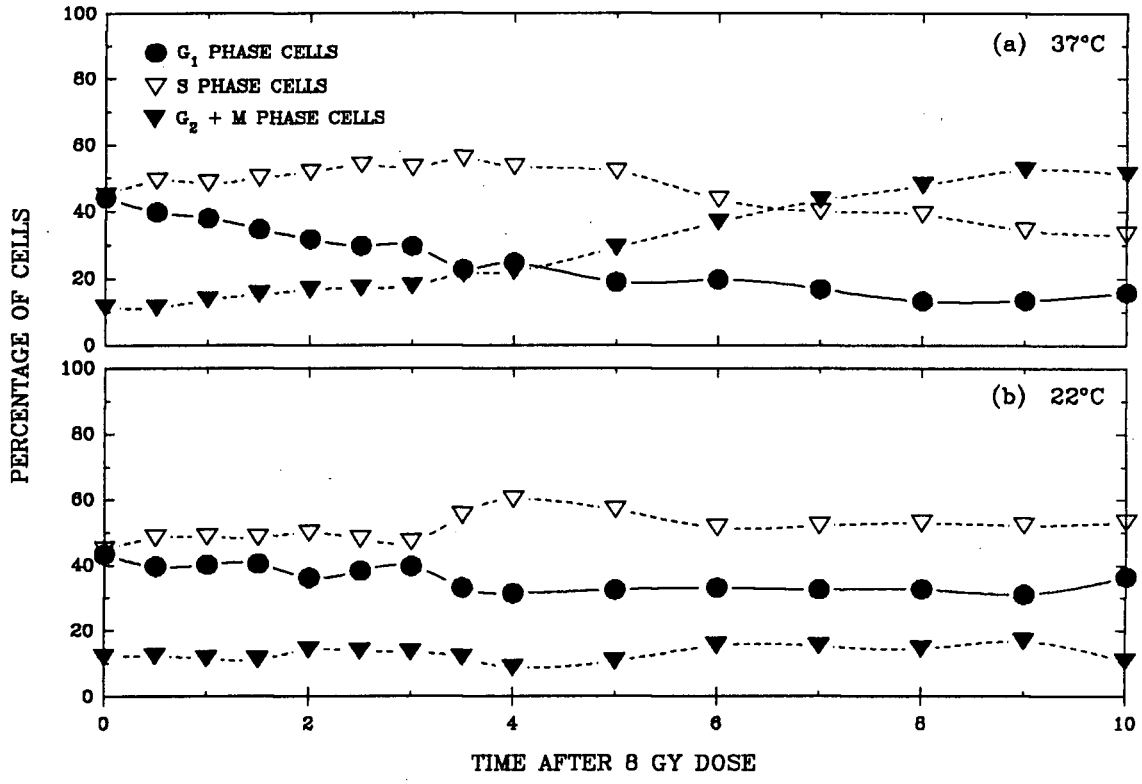

Figure 16. Data from DNA Profiles

Percentage of cells from V79-171b Chinese hamster spheroids in each cell cycle phase at defined time intervals after an initial X-ray dose of 8 Gy. (a) At 37°C an accumulation in G_2 is observed due to a G_2 block. (b) At 22°C no G_2 block is evident.

3.1.2 Survival Curves

Figure 17 shows the initial survival curves for 12 Gy administered incrementally with second curves generated from doses given after an 8 Gy initial dose with a 2, 4 or 6 hour time

interval. These curves were generated so that the change in survival curve shape could be observed. The shape, specifically the shoulder width and the slope, is an indication of whether the change in survival is a function of repair or whether other factors such as cycle dependent radiosensitivity are involved. An initial curve was generated followed by a second curve at defined times after the first with the spheroids being kept either at 22°C or at 37°C.

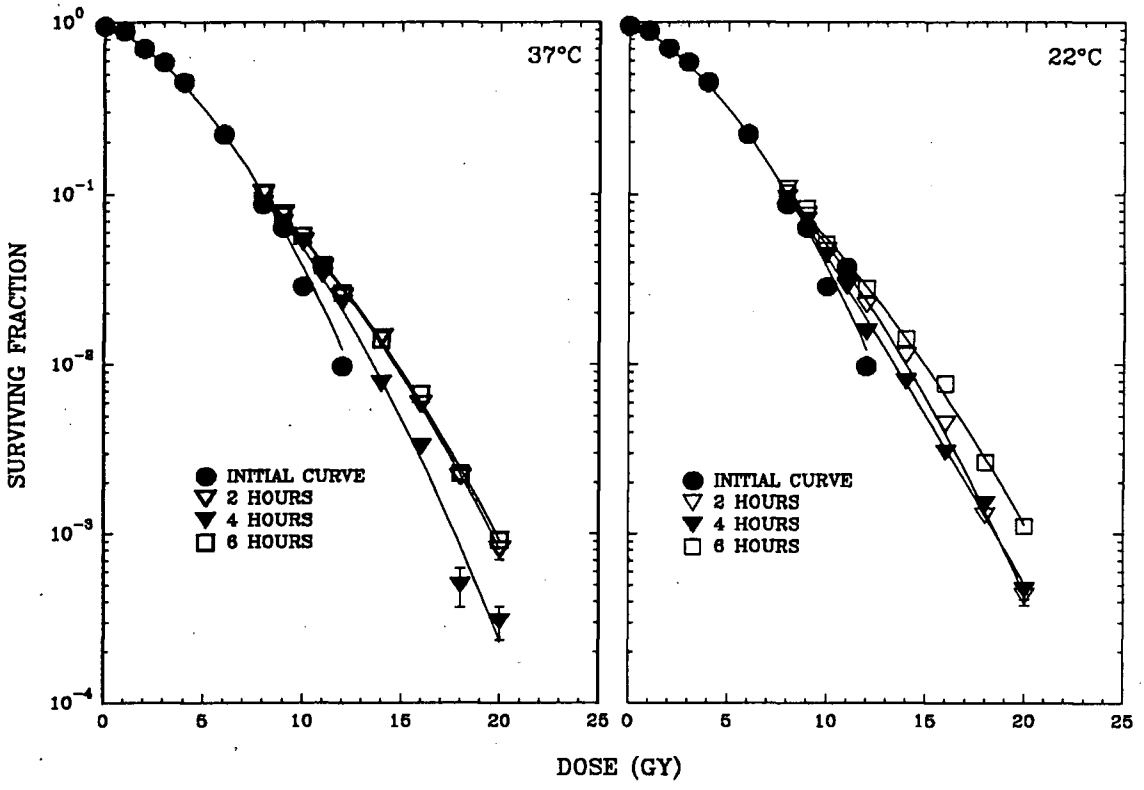

Figure 17. Survival Curves for Two Dose Experiment

Initial curve plus second curves after 8 Gy X-ray dose and 2, 4 or 6 hour time intervals at (a) 37°C or (b) 22°C.

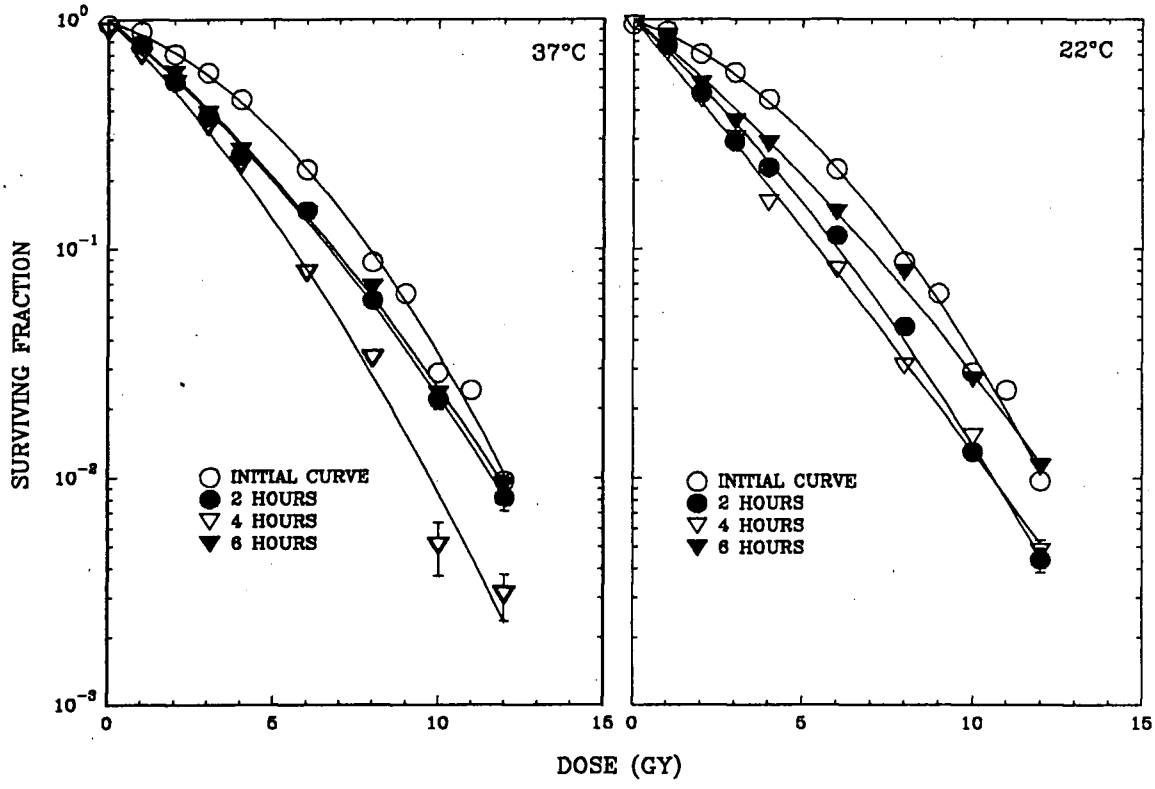

Figure 18. Normalized Two Dose Survival Curves

The data from figure 17 normalized so that cells surviving the initial 8 Gy fraction are plotted at 100% survival (a) 37°C and (b) 22°C.

Figure 18 shows the second set of survival curves normalized so that cells surviving the 8 Gy fraction were defined as representing 100% survival. From this figure it is more readily noted that the shoulders of the second curves were substantially decreased relative to the initial curve. Relative to the curves generated at 37°C, the curves 2 and 6 hours after the initial dose were not significantly different while at 4 hours, survival was considerably reduced. This agrees with the split dose curve in which the survival reached a maximum 2 hours after the initial dose and fell to a minimum after 4 hours as the cells entered the G_2 phase. By 6 hours,

the survival had increased to that of the 2 hour curve as the cells started to divide. There was, however, little difference in the shape of the shoulders in these three curves indicating that all repair had occurred by 2 hours after the initial 8 Gy and the change in the survival was due to a variation in radiosensitivity. Survival curves at 22°C produced 2 and 4 hours after the initial dose showed no significant difference while the survival after 6 hours had increased. The lower survival, at 22°C, was due to a decrease in the shoulder region more than in overall slope. It seems that repair may have been occurring even more slowly than initially thought, with the shoulder being regenerated to that of the curves produced at 37°C only by 6 hours. This may be due to repair occurring more slowly at 22°C.

Using the LQM, a prediction was made as to what the expected survival curves would be according to the sensitivity of the cell population. By making a few assumptions and using the α and β values from some of the experimental curves, predictions were made for cell populations of different sensitivities. Assuming that the resistant S phase cells had a survival of about three times (figure 2) that of the rest of the population after an 8 Gy dose, along with the facts that the initial population was made up of about 30% S phase cells and that 8 Gy produced an overall survival of approximately 10%, the LQM was used to estimate the survivals expected for population distributions in which cells from V79-171b spheroids were subjected to the same radiation and in which repair was complete.

After the first dose of 8 Gy, the surviving population would be expected to be comprised of approximately 60% S phase cells. The surviving population is at its most resistant state here, and its survival response to the second dose is best represented by the curve produced with the cells at 22°C having 6 hours between exposures. These cells have completely repaired but have not progressed and therefore have retained the large S phase

population as they approach the time for the second dose. α and β values were estimated from this curve.

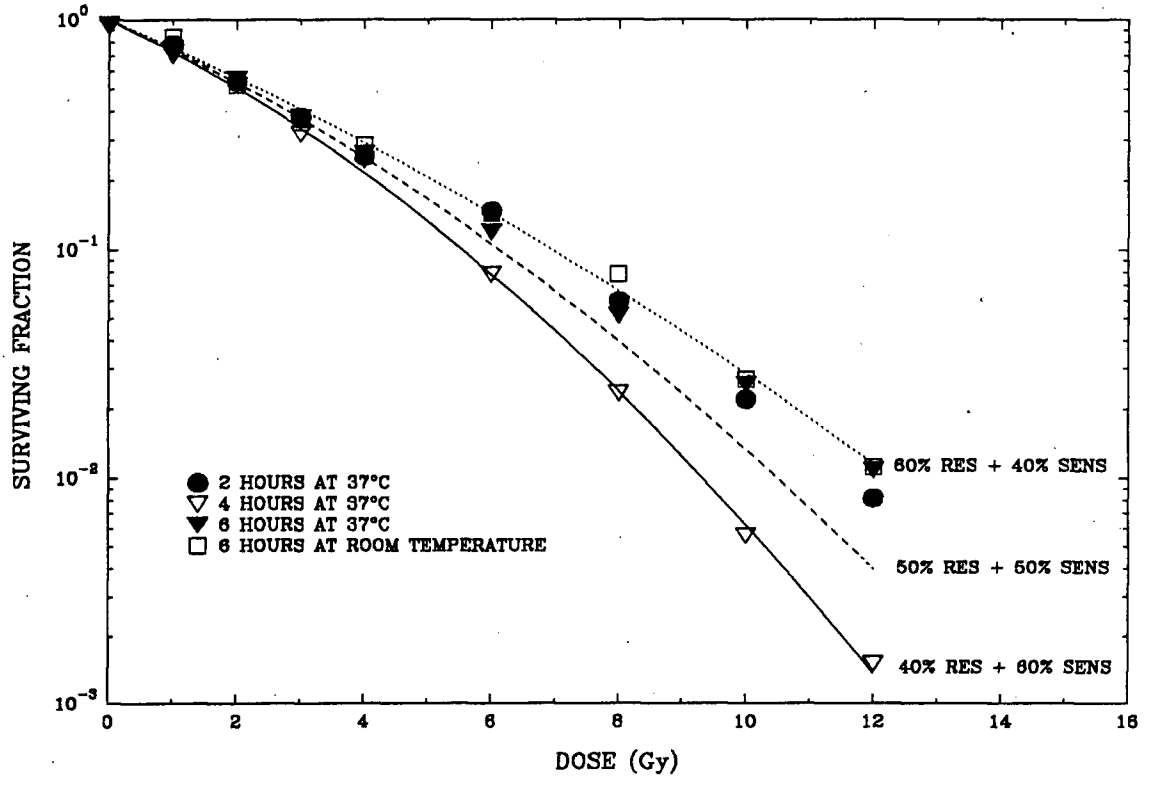

Figure 19. LQM Predicted Curves and Experimental Results

LQM (lines) of calculated survival curves of cells from V79-171b Chinese hamster spheroids after initial 8 Gy X-ray dose. Experimental data (symbols) of curves in which repair was complete.

If one also assumed that the most radiosensitive surviving population occurred when about 40% of the cells were in S phase, the curve generated 4 hours after the initial dose and incubated at 37° would be the most representative. Since the resistant population was comprised of 40% S phase cells, presumably when these progressed to a sensitive phase of

the cycle, the 60% which was sensitive would have progressed to a resistant phase of the cycle. Using the α and β values from this sensitive population and those from the resistant population allowed a prediction of the response for any population in which repair was complete (see appendix A).

Figure 19 shows these predicted curves for several populations along with all experimental data of curves in which repair was deemed to be complete. This shows that the experimental results were realistic as they could be satisfactorily fit by the LQM.

A representation which emphasizes the relative responses at 22°C and 37°C is shown in figure 20. Here, the survival curves fit by the LQM at 37°C have been divided by those at 22°C. This clearly shows that there was much more survival at 37°C two hours after an initial 8 Gy dose than at 22°C which, as shown with the two dose split dose curves (figure 15), indicates that repair occurred faster and to a greater extent at 37°C. After 4 hours, however, a greater survival was found at 22°C because the cells at 37°C progressed into the more sensitive G_2 phase while those at 22°C had not progressed but had remained in the resistant S phase. After 6 hours, not much difference was noted as the cells at 37°C had now started to divide (figure 2), increasing their survival.

This evidence suggests that cell cycle progression has a significant effect on the radioresponse of cells in spheroids. Redistribution affects the radiosensitivity of the cell population as cells in different phases have varying radiosensitivities with S phase being most resistant. Progression also contributes to repair. It has been found that cells which are progressing repair faster than cells which are quiescent (Wheeler et. al., 1988).

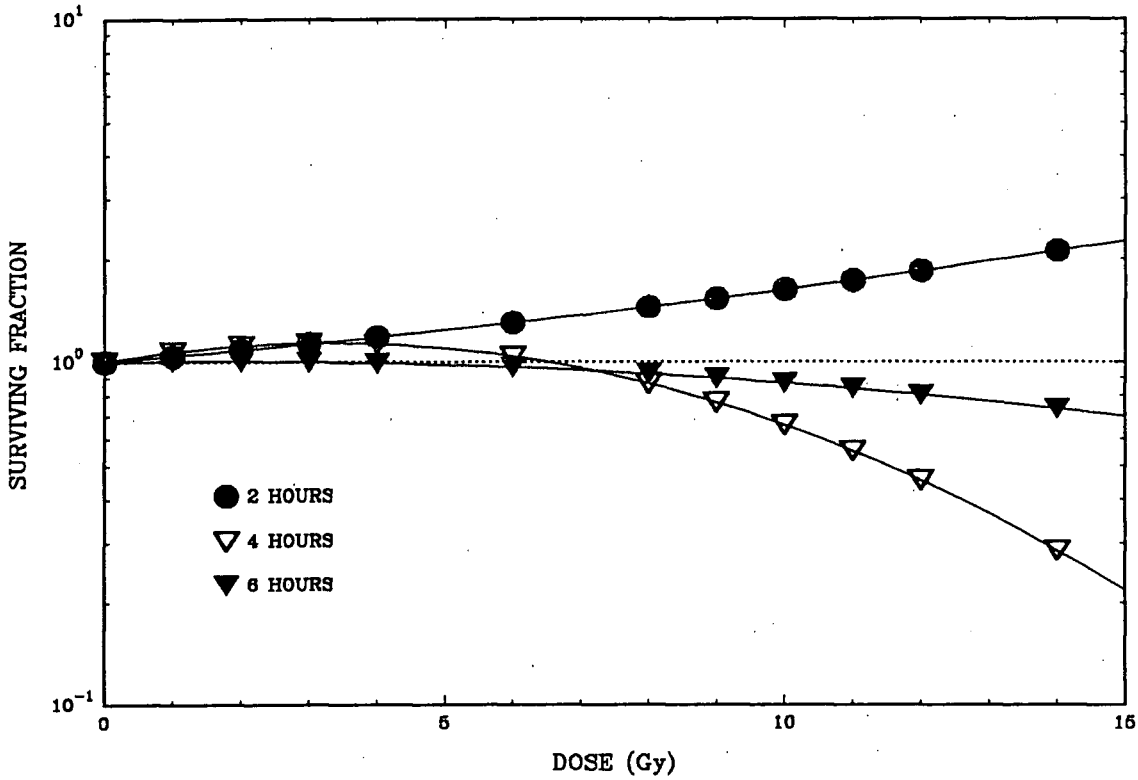

Figure 20. Two Dose Survival Curves at 37°C Relative to 22°C

Calculated second dose survival curves from two dose survival curves of cells from V79-171b Chinese hamster spheroids held at 37°C between irradiations divided by survival curves of cells held at 22°C for defined time intervals after an initial 8 Gy X-ray dose.

3.2 THREE DOSE SCHEDULE

3.2.1 Split Dose Experiments

Figure 21 shows the split dose curves for cells irradiated with two doses of 6 Gy separated by 4 hours at 22°C followed by a third dose of 6 Gy after defined time intervals of incubation at 22°C or 37°C.

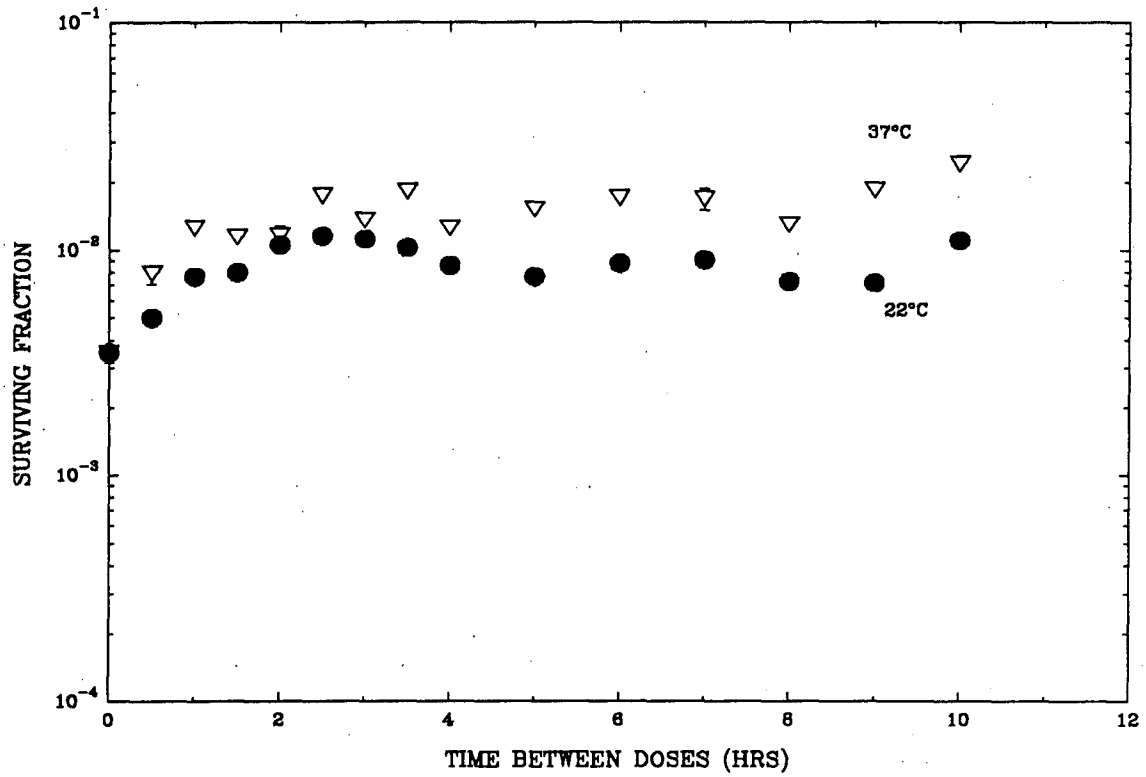

Figure 21. Three Dose Survival for Cells from V79-171b Chinese Hamster Spheroids

The first two 6 Gy X-ray doses were given 4 hours apart with spheroids held at 22°C. The third 6 Gy dose was given at increasing time intervals with spheroids held both at either 22°C or 37°C.

The curve for the cells kept at 37°C rose more quickly and to a higher survival than that of cells kept at 22°C. Little progression related fluctuation in survival was found at either temperature. This may be due to the fact that the 12 Gy of accumulated radiation was sufficient to cause a substantial delay in proliferation such that the effects of the cell cycle progression were not noticeable in the initial 10 hours. Such a delay would cause the observed result, however, only if the decrease in survival due to increased radiosensitivity up to the G_2 block was of similar magnitude to the increase in survival due to increased repair. While our experimental design did not allow this to be examined definitively, our results lead to the speculation that the delay may not be linear with dose for the cells in the spheroid, unlike single doses on single cell systems. An extension to this argument is that a threshold dose may exist over which the delay may be greatly increased.

Another mechanism which may have had an affect on the survival is recruitment. In spheroids, cell growth is arrested in the G_1 phase resulting in an increased G_1 population. As the cells in the spheroid die, the arrested G_1 phase cells may be recruited into the cell cycle and continue to proliferate. This introduces another synchronized population which is progressing through the cell cycle and which initially has a higher radiosensitivity than the S phase cells but cycle through a resistant phase within the G_1 phase before entering S phase (Chapman et. al., 1970). This population is, however, a sensitive population when arrested in G_1 and will be preferentially killed by the initial dose therefore having little effect on the survival of the second dose of radiation. Recruitment should therefore have little, if any, effect on the number of viable cells at short times after a large dose of radiation.

3.2.2 Survival Curves

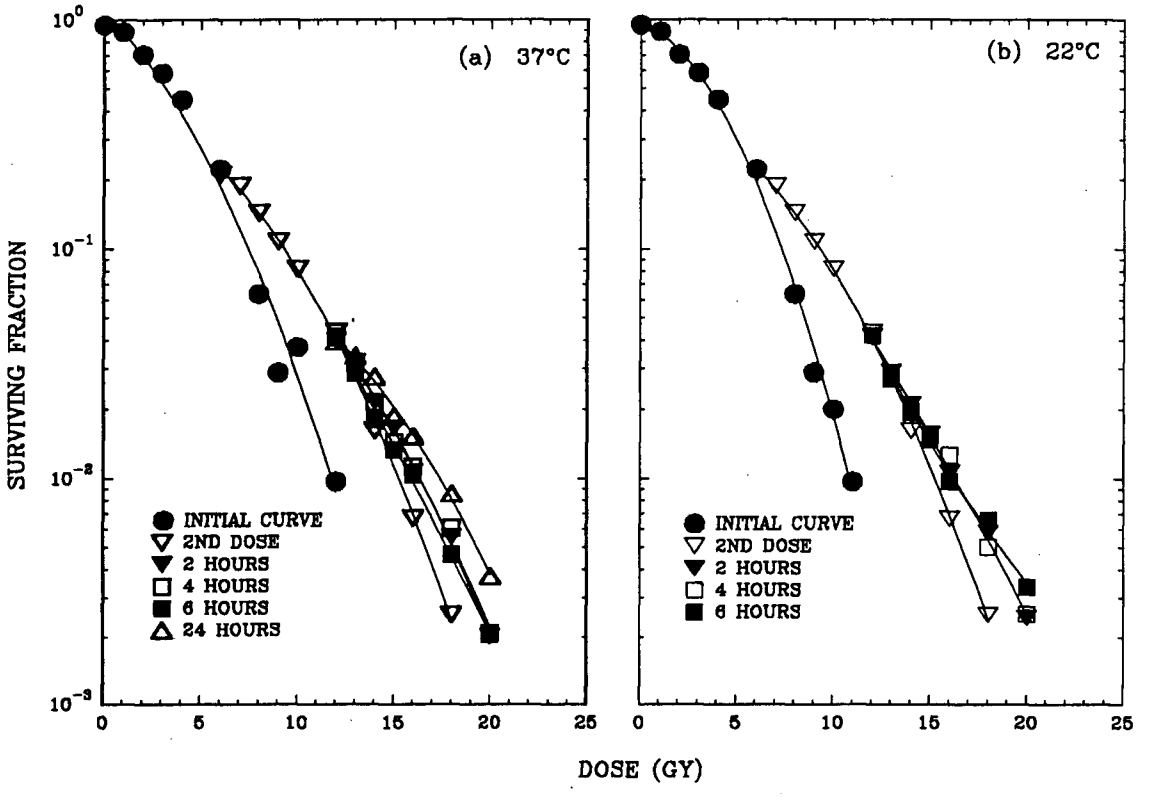

Figure 22. Survival Curves for Three Dose Experiments

Initial curve plus second curve of cells from V79-171b Chinese hamster spheroids irradiated 4 hours after initial 6 Gy X-ray dose with spheroids held at 22°C. Third curves were produced at defined time intervals after the second 6 Gy dose with spheroids held at either (a) 37°C or (b) 22°C.

Figure 22 shows survival curves resulting after 2 doses of 6 Gy given 4 hours apart with the spheroids held at 22°C, followed by a third dose of 8 Gy administered incrementally at defined time intervals with the spheroids held either at 22°C or 37°C. Little difference was seen with any of the curves until 24 hours after the second 6 Gy dose. This supports the

suggestion that the proliferation was reduced for at least the first 10 hours again due to the large cumulative dose of 12 Gy.

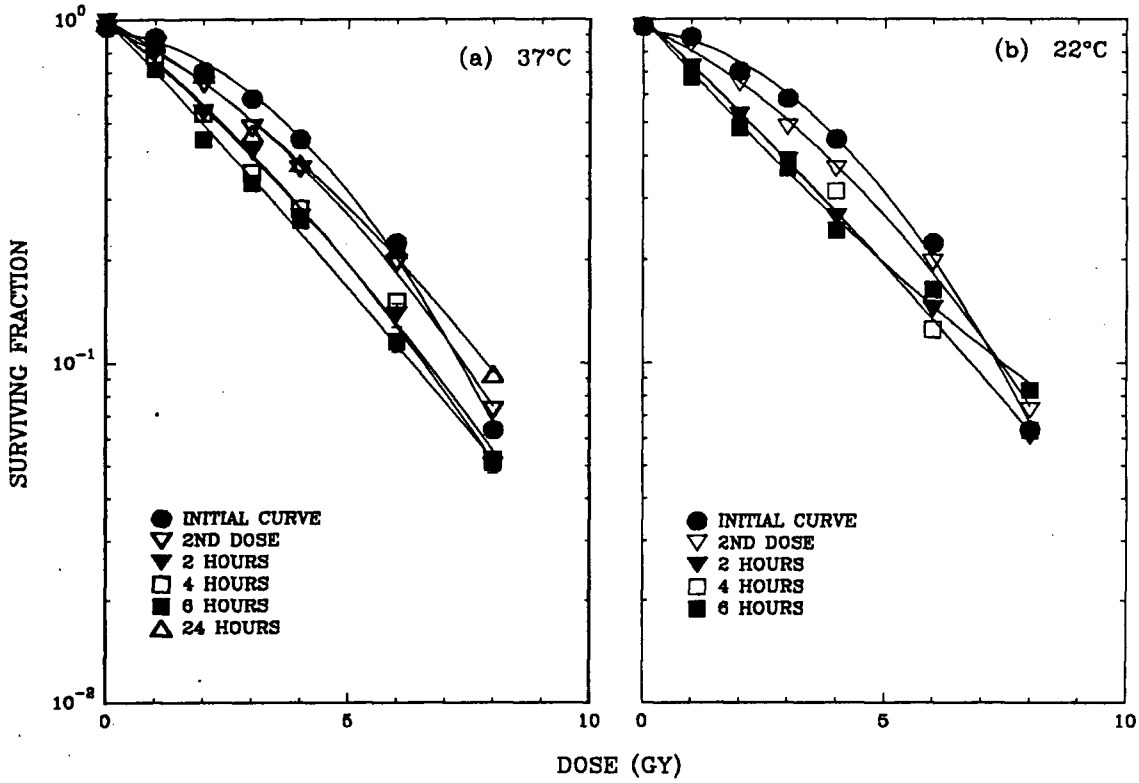

Figure 23. Normalized Survival Curves from Three Dose Experiment

Survival curves of cells from V79-171b Chinese hamster spheroids from the three dose experimental curves with each set of curves normalized to 100% survival with spheroids held either at (a) 37°C or (b) 22°C.

Figure 23 shows the survival curves resulting after the first and second 6 Gy fraction (normalized to 100% survival) compared to that for previously unirradiated cells. The curves after the second fraction have a smaller shoulder than the initial curve, or that after the first

fraction. This is the combined result of repair not being completed as a result of the reduced proliferation, and the progression of the cells into G_2 where they are blocked in a more sensitive phase of the cell cycle.

Again at 22°C, no difference between the survival curves at 2, 4 and 6 hour time intervals after the second fraction was observed, and expressing the data at 37°C and 22°C as a ratio (figure 24) confirmed the small amount of change between them.

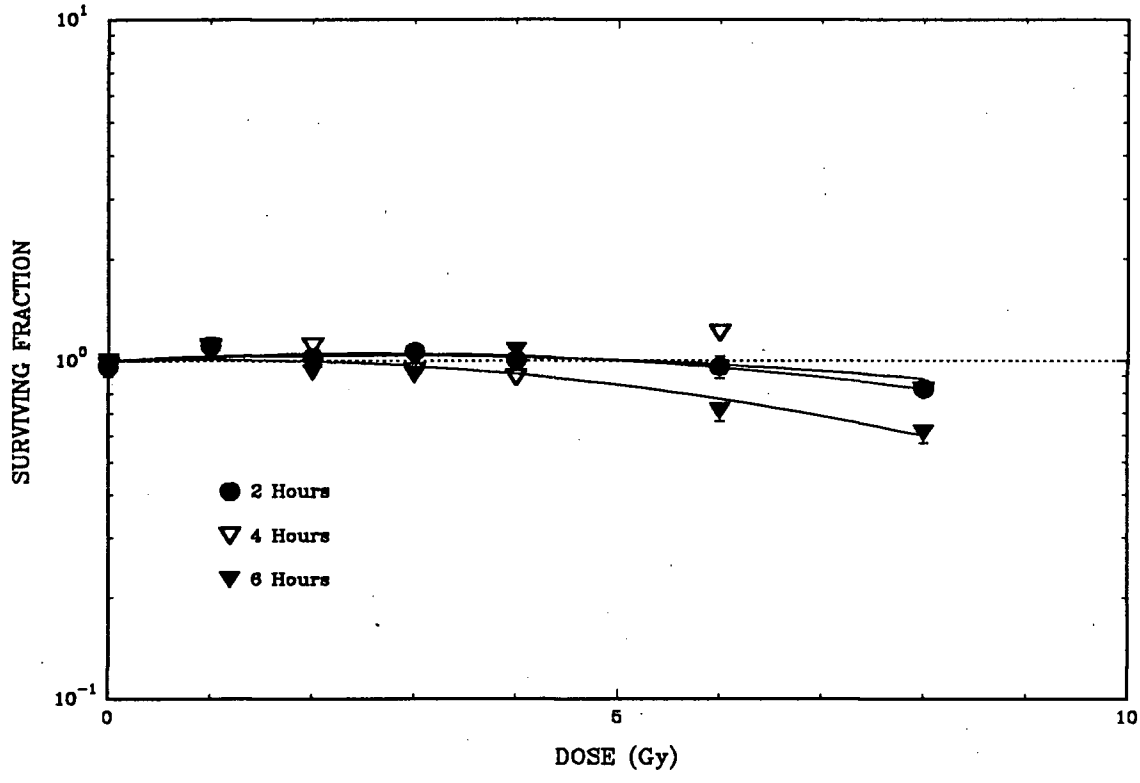

Figure 24. Third Dose Survival of Cells for V79-171b Chinese Hamster Spheroids 37°C Relative to 22°C

Calculated survival curves after two 6 Gy fractions for spheroids held at 37°C divided by those held at 22°C for the defined time intervals.

From these experiments, it appears that the radiation induced delay was large enough that no effect of progression was observed at 37°C, so the response was very similar to that of the cells at 22°C.

3.3 FRACTIONATION

By extending the multifraction schedule to 6 days, the cells in the spheroids can progress around the cell cycle several times allowing the effects of proliferation to be observed. Administering several fractions also tested whether or not the cells would be capable of repairing themselves indefinitely.

Fractionated doses can be administered in several protocols. In one method, the total exposure for each fraction is given in one dose as in the split dose experiments described in the previous sections. Alternatively, a "fraction" can be administered as cumulative small increments of dose, thus allowing the generation of a survival curve during each fraction. For example, in the subsequent experiments in which 6 Gy or 8 Gy per day was given for 6 days, the actual exposures were four 1 Gy doses and either one or two 2 Gy doses administered in immediate succession. These produced five or six survival points for each 6 Gy or 8 Gy fraction thus allowing a survival curve to be drawn. With this method the shapes of the survival curves at each time help interpret what factors have a role in the survival response of the cells at that point in time.

The data from this method of fractionation can be represented in several ways. One representation involves normalizing the "pre-treatment survival" of each curve to the "post-treatment survival" of the previous fraction. This representation factors out repopulation and thus shows only the radiation induced kill. Alternatively, the pre-treatment survival of each 6 Gy or 8 Gy fraction can be normalized to 100%. This allows the comparison of the shoulders and

slopes of each curve. The raw data can also be shown with no normalization. These curves then show the effects of repopulation after each fraction.

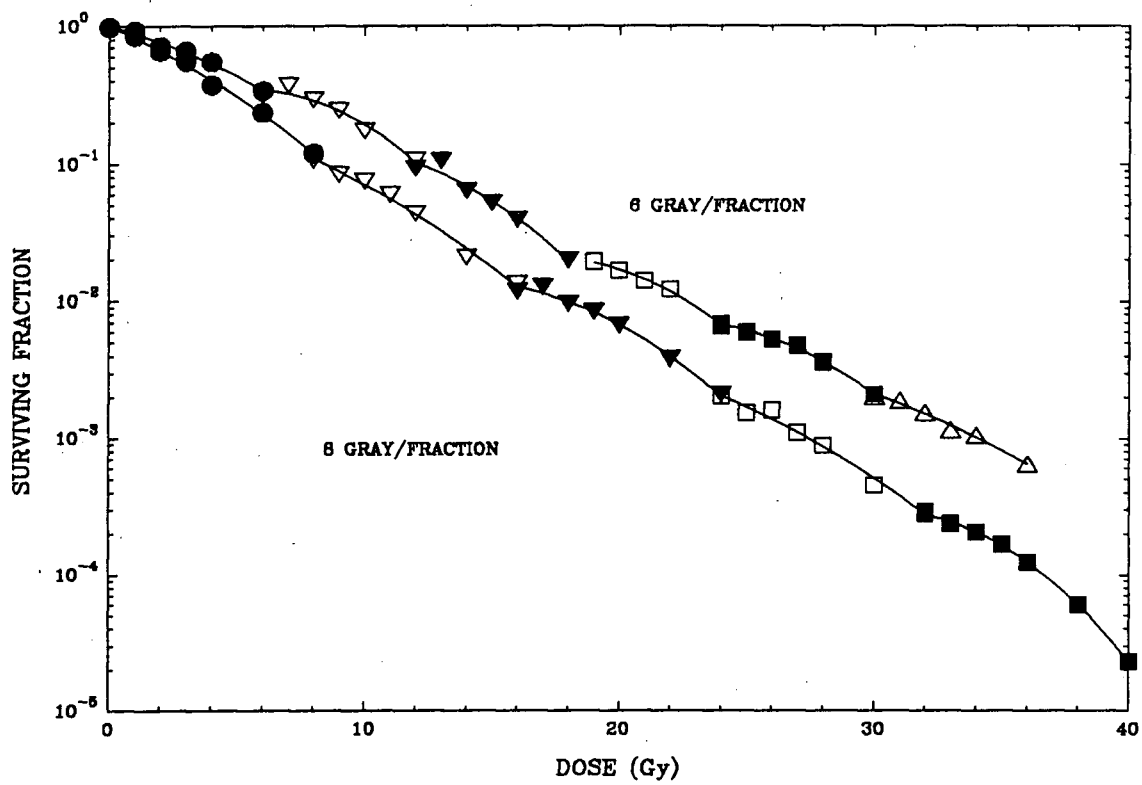

Figure 25. Survival Curves During Daily Multifraction Exposures.

Multifraction exposures of 6 Gy/day or 8 Gy/day cumulative exposures for 6 days administered in 1 Gy and 2 Gy increments. Each day's curve was normalized to the post-treatment survival of the previous fraction.

The results from the 6 day fractionation schedule for both 6 and 8 Gy/fraction are shown in figure 25. Only five of the 8 Gy fractions are shown since the survival from the sixth fraction was very low. The survival curve for previously unirradiated cells was divided by the plating efficiency resulting in a zero dose point plotted at a survival of 100%. The pre-

treatment survival for subsequent curves was normalized to the post-treatment survival of the previous fraction. Plotted in this manner, it is evident that, as expected, the 8 Gy fractions caused greater killing as can be seen by the larger overall slope of the 8 Gy/fraction curve.

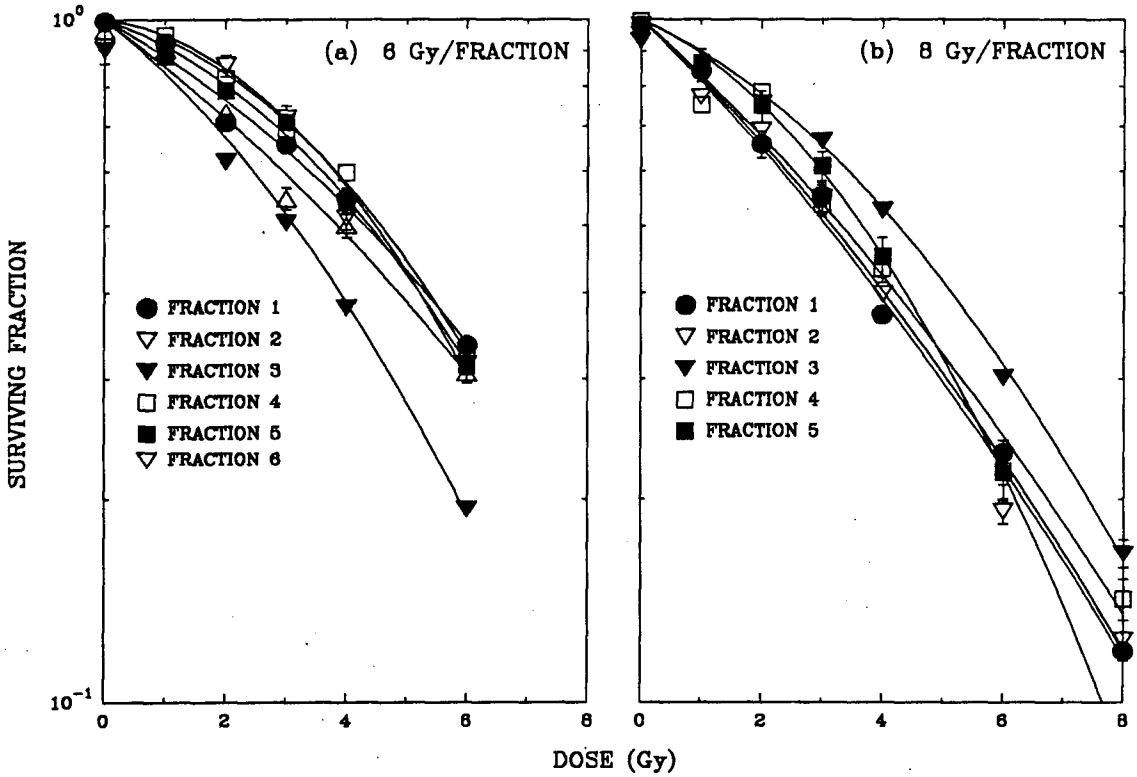

Figure 26. Normalized 6 Day Fractionated Curves

6 day fractionated curves with (a) 6 Gy/fraction and (b) 8 Gy/fraction with 1 fraction given daily. All fractions have pre-treatment survival normalized to 100%.

To compare the relative radiosensitivity after each 6 Gy or 8 Gy fraction, all the survival curves were normalized to an initial survival of 100% as shown in figure 26. Following the first 6 Gy fraction, the survival curve showed a survival increase relative to that of the control cells, which would have been a result of the first 6 Gy fraction selectively killing the more sensitive

cells leaving a more resistant population. Since there was a 24 hour interval before generating the subsequent survival curve, repair would also have been complete before delivering the second 6 Gy, as shown by the split dose experiments previously discussed, and there would have been sufficient time for the cells to overcome the division delay which from the 6 Gy dose should have been about 6 hours. Since the survival after the first 6 Gy dose increased from that of the cells of the initial curve, it seems possible that most of the cells had progressed through mitosis and back into the S phase of the cycle resulting in this more resistant population.

It was expected that after the second 6 Gy fraction the cells of the survival curve would have a decreased survival from those after the first fraction but still higher than after the third 6 Gy fraction response. This was predicted because the repair capacity might have been decreasing with each fraction. After the second 6 Gy fraction, however, there was an unexpected result: much lower survival. This may have been, in part, a result of a decrease in the cells' ability to repair. Also, the surviving cells resulting from the first two 6 Gy fractions would have been synchronized and the time between fractions may have been enough for the cells to progress to a more sensitive phase of the cycle, leaving the cells subject to the third fraction in an exceptionally sensitive state. Again, a delay would have been induced by the previous fractions but these cells would have had time to overcome this delay and been able to progress through division. Due to the accumulation of damage, the delay experienced before the third 6 Gy fraction may have been larger than that before the second fraction. This would have resulted in less progression, perhaps leaving the cells in a more sensitive phase at the time of the third fraction, resulting in a lower survival.

The survival again increased after the third 6 Gy fraction relative to the survival after the second fraction and continuously decreased down to the sixth fraction. This indicates that

the cells' ability to repair decreased with fraction number as the initial shoulder of the curve decreased from the fourth to the sixth fraction. Also the effects due to the cell cycle progression seemed to have decreased as there was no longer any large fluctuation between the curves due to varying sensitivity in the cell population.

From these data it seems that as more fractions of radiation are administered to a population of cells, their ability to repair sublethal radiation damage decreases.

In figure 26b, where daily fractions of 8 Gy were administered, the same effect is observed except that the shoulder increased after the second 8 Gy fraction. This may indicate that the increased dose caused a longer delay that kept the cells from progressing into a sensitive part of the cycle. The higher dose per fraction would have also been more effective at producing a population with greater synchrony which would show a higher surviving fraction if it happened to be in the resistant part of the cycle.

After the third 8 Gy fraction the survival decreased relative to that after the fourth fraction due to accumulation of damage and lack of repair. Again, this supports the suggestion that the cells' capacity to repair decreases as the radiation dose accumulated.

If the raw data for the survival curves are plotted as shown in figure 27 it can be seen that, in the 24 hour interval between the 6 Gy or 8 Gy fractions, recovery has occurred. This recovery is the increase in survival that occurs between doses either due to repair or to repopulation. After the first fraction there was an increase in survival, although this was more emphasized in the 8 Gy fractions, which was due to selection and repopulation. Sublethal damage repair would not cause this result but, instead, would affect the shoulders of the curves as previously discussed. Potentially lethal damage repair would have this effect but it has been shown that no potentially lethal damage occurs in this cell line (Durand and Sutherland, 1973).

After the second 8 Gy fraction, the increase in survival was negligible. This indicates that cell killing has already increased to the extent that it overcomes any recovery due to proliferation between fractions.

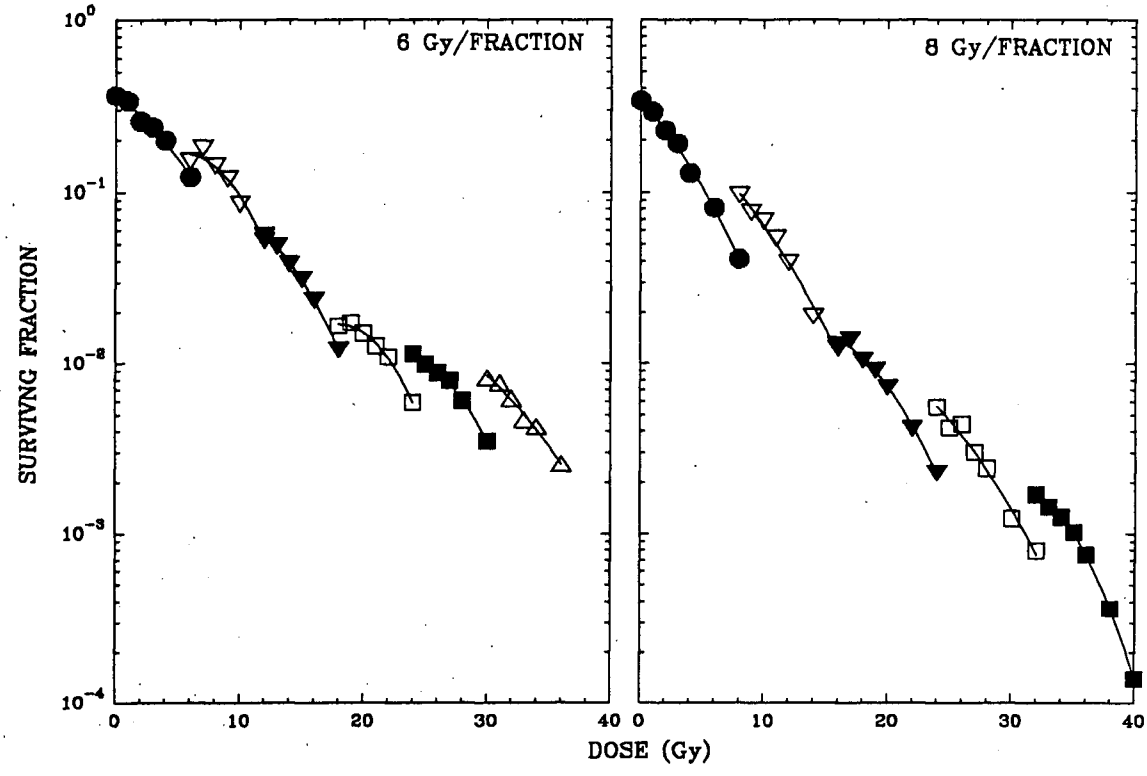

Figure 27. Unnormalized 6 Day Fractionated Curves

(a) 6 Gy/fraction and (b) 8 Gy/fraction given once daily with data not normalized by plating efficiency.

The intervals between subsequent fractions showed more recovery indicating that proliferation had increased due to a compensatory mechanism and, therefore, was having a large effect on the inter-fraction recovery.

These data again are consistent with the idea that the repair capacity of the cells decreases with increasing dose accumulation but also that proliferation increases which may be due to a compensatory mechanism being induced.

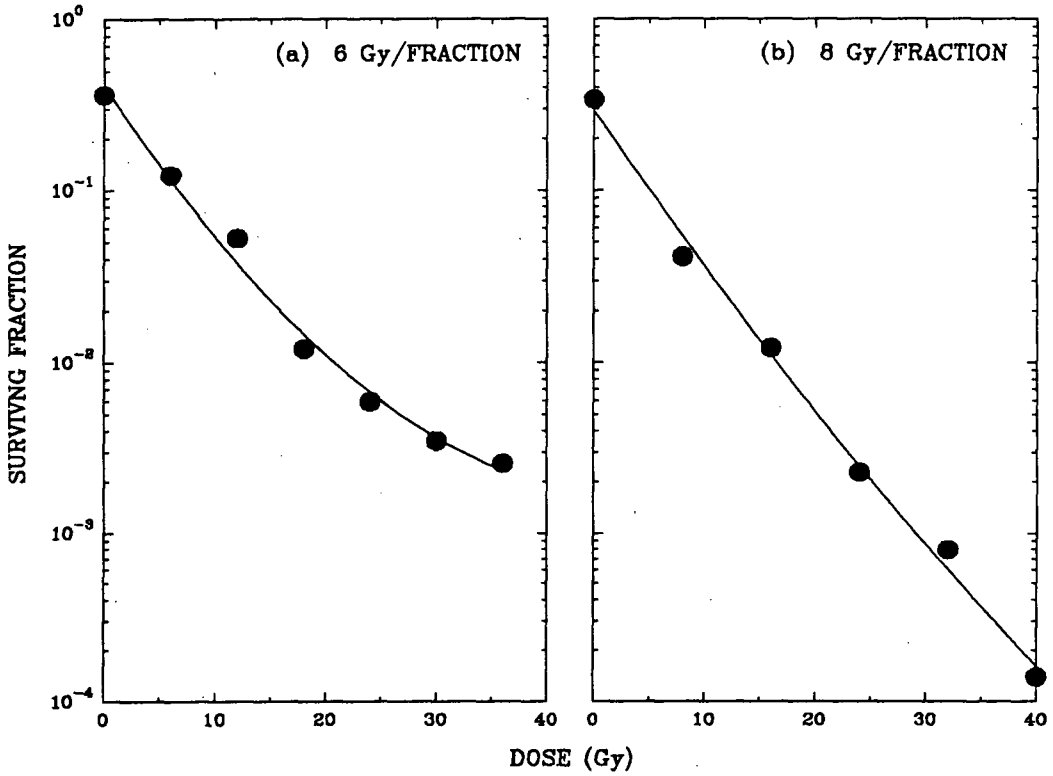

Figure 28. Unnormalized Overall Response of 6 Gy and 8 Gy Fractions

(a) 6 Gy/fraction and (b) 8 Gy/fraction survival response of each fraction showing cumulative effect of each 6 Gy or 8 Gy exposure.

Another feature of interest illustrated in figure 28 is the overall effect of the 6 Gy or 8 Gy fractions. For the 6 Gy fractions, the net effect decreased for each fraction (slope decreases). This was due to the proliferation that occurred between fractions but was not so prominent

with the 8 Gy fractions, where a more linear relation was evident. This indicates that the effect per fraction is more constant at a higher dose per fraction due to the killing being great enough to overcome any recovery resulting from repopulation.

In radiotherapy, this is of importance because if the doses are too low or if too much time is allowed between fractions, the effect of the radiation reduces with each fraction, and potentially has little benefit in the later fractions. It is important either to keep the doses high or the time between fractions low enough so that the recovery between fractions does not overcome the tumour cell kill by those fractions.

For the 8 Gy doses, although the effect per fraction appeared to be constant, the shape of the survival curves at each treatment indicated that repair, repopulation, redistribution and cell killing had varied effects. For instance, the cells of the curves with large shoulder widths indicated more repair than the cells of the curves with small shoulder widths. Survival after fractions 2, 3 and 4 showed large recovery between fractions due to repopulation. Therefore, although the overall toxicity remained constant, the factors controlling that survival were different for each fraction, some being more affected by repair, others by redistribution or by repopulation. These factors would have, of course, each contributed to each fraction but in different amounts with the major contributor varying for each fraction.

From this it can be concluded that in fractionated therapy it is important to achieve the proper balance between the dose per fraction and the time between fractions to achieve an equal or, at least, non-decreasing effect per fraction to ensure that the patient is receiving at least the required dose. Even when a balance can be found, the equal effect per fraction is not necessarily a result of the cells responding in the same way to each fraction of dose. It is more due to a balance in which the varying effects of repair, redistribution and repopulation combine

together to give the same overall effect. As the number of fractions increases, the effect of repair decreases while the effect of repopulation increases due to compensation. Throughout all of this, the effect of redistribution fluctuates as the cell cycles through sensitive and resistant phase of the cell cycle.

In radiotherapy however, it is the overall effect per fraction which is of importance and it seems that with 1 fraction per day in the spheroid system using X-rays, fractions of 8 Gy provide a more constant effect per fraction. For practical considerations, each system would have to be independently analyzed to determine the ideal doses and scheduling to achieve equal effects per fraction.

3.3.1 Two Gray Response

In radiotherapy, it is often convenient to implicitly assume that the response to 2 Gy fractions remains constant regardless of the number of fractions. Our experimental design of administering the 6 Gy and 8 Gy fractions in small increments allowed us to look at the response in each fraction to the initial 2 Gy dose increment to determine whether the response did, in fact, remain constant. To test this, the 2 Gy response from experiments in which cumulative doses of 6 Gy or 8 Gy were administered daily was plotted in different representations to see if the data supported this belief.

Figure 29 shows the 2 Gy response observed when 6 Gy was administered daily. In figure 29a the data are shown as a ratio of the previous post-treatment survival. This shows the survival after 2 Gy with respect to that seen the previous day indicating the response with repair, repopulation and cell killing effects all taken into account. The relative surviving fraction was about 1.2 after the first 6 Gy fraction and decreased to approximately 0.7 after the second 6 Gy fraction. This occurred as proliferation was not sufficient to overcome the

damage. After the third 6 Gy fraction, the proliferation increased and overcame the damage resulting in a relative survival up to about 1.8.

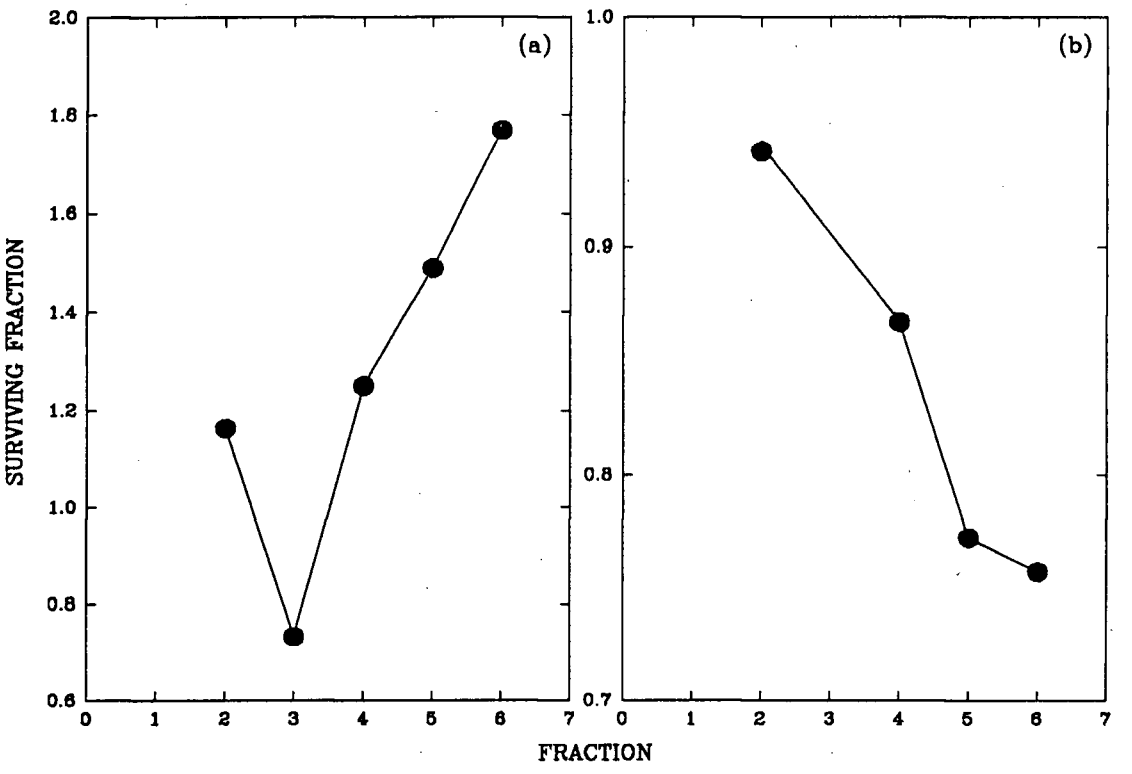

Figure 29. Two Gray Responses for 6 Gy Fractions

(a) 2 Gy survival of each 6 Gy fraction relative to post-treatment survival of previous fraction (b) 2 Gy survival of each 6 Gy fraction relative to pre-treatment survival of same fraction.

To exclude the effects of proliferation and determine how repair affected the 2 Gy response, each 2 Gy survival was shown as a ratio of the pre-treatment survival as in figure 29b. The relative survival after the first fraction was greater than that for the initial 2 Gy dose due to the increased radioresistance of the cells because of selective killing by the first dose.

As more radiation was accumulated, the cells lost their ability to repair and the survival reduced again approaching that of the initial fraction.

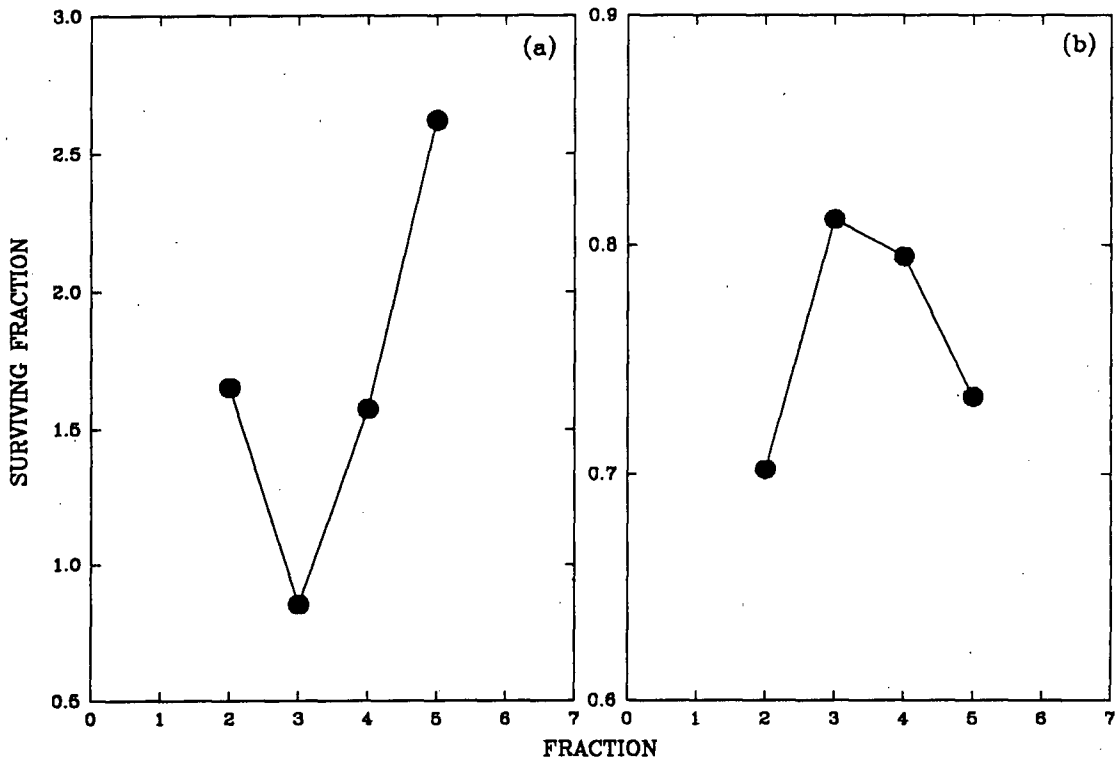

Figure 30. Two Gray Responses for 8 Gy Fractions

(a) 2 Gy survival relative to the post-treatment survival of the previous fraction (b) 2 Gy survival relative to the pre-treatment survival of the same fraction.

Figure 30 shows the 2 Gy response for cells exposed to 8 Gy daily. In figure 30a the response was similar to that for the 6 Gy fractions but the proliferation seemed to have had a much larger effect with the relative 2 Gy response rising to almost 3. The proliferation was apparently not slowed down as significantly and perhaps a compensatory mechanism was also triggered. The data normalized to the pre-treatment survival, however, showed a similar

response to that of the 6 Gy per day data although the survival was slightly lower due to less repair occurring with the greater dose per fraction.

Taken together, figures 29 and 30 provide the following insights: the 2 Gy response was not, in fact, constant when a number of fractions were administered. Radiation induced synchronization of the cells increased the absolute survival of the 2 Gy dose after the first 1 or 2 fractions. The relative survival, however, was increased after the first fraction due to repair, decreased after the second fraction when repair was decreased and increased for subsequent fractions as compensation occurred and proliferation increased. There were, therefore, many factors affecting the 2 Gy dose response resulting in a survival that fluctuated for each fraction depending on accumulated dose, time between fractions, cell proliferation and recruitment rates.

3.4 BRACHYTHERAPY

In cancer therapy, a new method of treatment is being introduced to the clinics using high dose rate brachytherapy. This treatment allows radioactive seeds to be sent down catheters which can be threaded into openings such as the esophagus or trachea. These catheters can also be surgically implanted into a tumour region or placed along the surface of the skin. The driver for the radioactive seed can then be programmed to move to any point in the catheter for any amount of time to distribute the required dose. This would be used in a treatment schedule to replace the method presently used in which the seed itself is surgically implanted in the patient. This new method reduces the exposure to the radiologists, as well as reducing inconvenience and discomfort to the patient.

To convert the low continuous dose schedules to fractionated schedules it is important to find a way of converting the conventional low dose treatments to fractionated treatments.

To test whether this could be accomplished using the LQM, a survival curve was generated for spheroids using an ^{192}Ir source which, with this configuration, gave a 10 Gy/day continuous dose. This curve was compared to curves generated from fractionated doses calculated by the LQM.

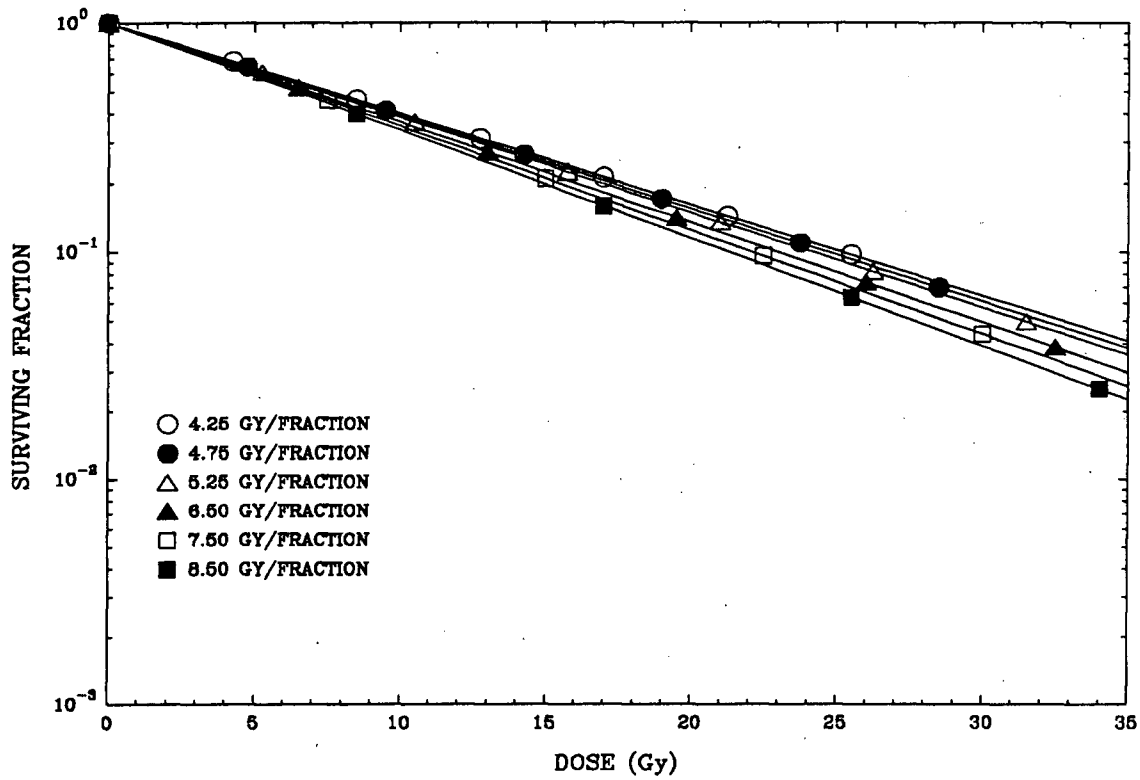

Figure 31. Linear Quadratic Model Prediction

LQM prediction of survival curves for fractionated doses shown.

Curves were predicted using the LQM and α and β values from a survival curve produced by X-rays, as described in section 1.4.1, to show what result would be expected if the LQM were a good estimation. Although the LET of X-rays and ^{192}Ir are not exactly the

same, there is little difference allowing the LQM prediction to be used in at least a qualitative sense. ^{192}Ir is predominantly a β particle emitter with a half life of 74.2 days. The β particles emitted have energies of .24, .54 and .67 MeV. The expected fractionated curves are shown in figure 31 and were predicted to be straight lines with little difference between the doses chosen for this experiment.

For experimental measurements the spheroids were maintained at 22°C throughout the experiment to keep the cells from proliferating. This way, the effects of repair could be studied with no redistribution or repopulation effects having any contribution.

Little difference was detected between the survival curves of 4,25, 4.75 and 5.25 Gy/fraction when two fractions were given per day, but the curves were not found to be linear as shown in figure 31. Similarly, when only one fraction was given each day, the resulting survival curves from each fractionation protocol were not significantly different from each other. Comparison between 4.25 Gy/fraction given twice daily and 6.5 Gy/fraction given once daily showed greater killing at low doses with the one fraction per day than with 2 fractions per day. These curves had a larger difference between them than predicted by the LQM which was the first indication of the LQM not being a perfect predictive model. In the 2 fraction/day curve there was a larger shoulder width than in the 1 fraction/day curves, indicating that at low doses, more repair can occur than with a larger fraction given once daily. This agrees with the findings with the 6 day, 6 or 8 Gy/fraction curves in section 3.3 which indicated that as the dose was accumulated, the ability to repair decreased. With the one or two fractions per day, the repair capacity decreased with accumulated dose making the effect per fraction greater for higher fraction numbers. Since these experiments were done at 22°C, no proliferation was occurring so there was no recovery due to repopulation between fractions.

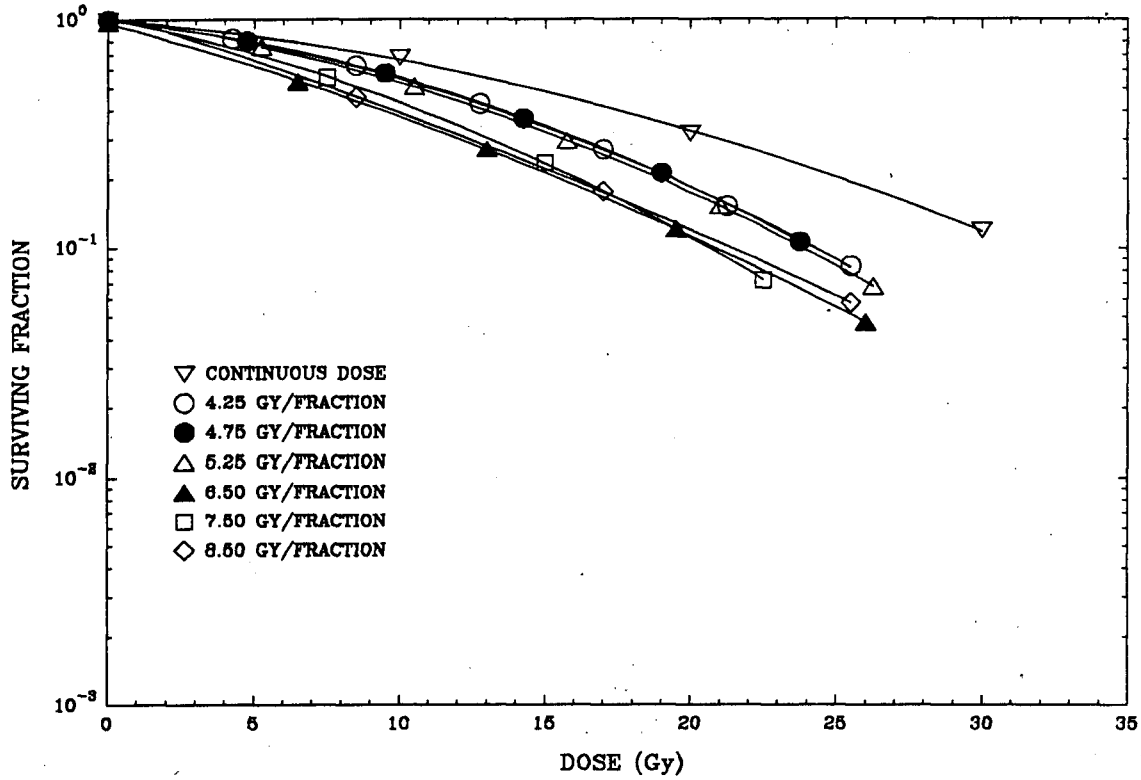

Figure 32. Survival Curve for 1 and 2 Fractions of ^{192}Ir Per Day

Survival curves of V79-171b Chinese hamster cells from spheroids held at 22°C exposed to fractionated doses shown. 4.25, 4.75, and 5.25 Gy fractions were given twice daily and 6.5, 7.5 and 8.5 Gy fractions were given once daily. Also shown is the continuous ^{192}Ir radiation survival curve for continuous exposure over 4 days.

The low continuous dose, also shown in figure 32 which by the LQM should have produced the same survival as the fractionated doses, showed a much lower killing. That curve had a large shoulder region which, along with the 1 and 2 fraction per day curves, indicates that as the fraction number increases and the dose per fraction decreases, the shoulder of the curve increases more than predicted by the LQM. This discrepancy between

the low continuous dose and the fractionated curve supports the idea that damage accumulated at a low dose rate may be more repairable than at a high dose rate. The LQM is also not a perfect model with which to make these predictions. There may, however, have been a problem with the dosimetry such that the dose administered was not as calculated resulting in survival curves which did not match those predicted.

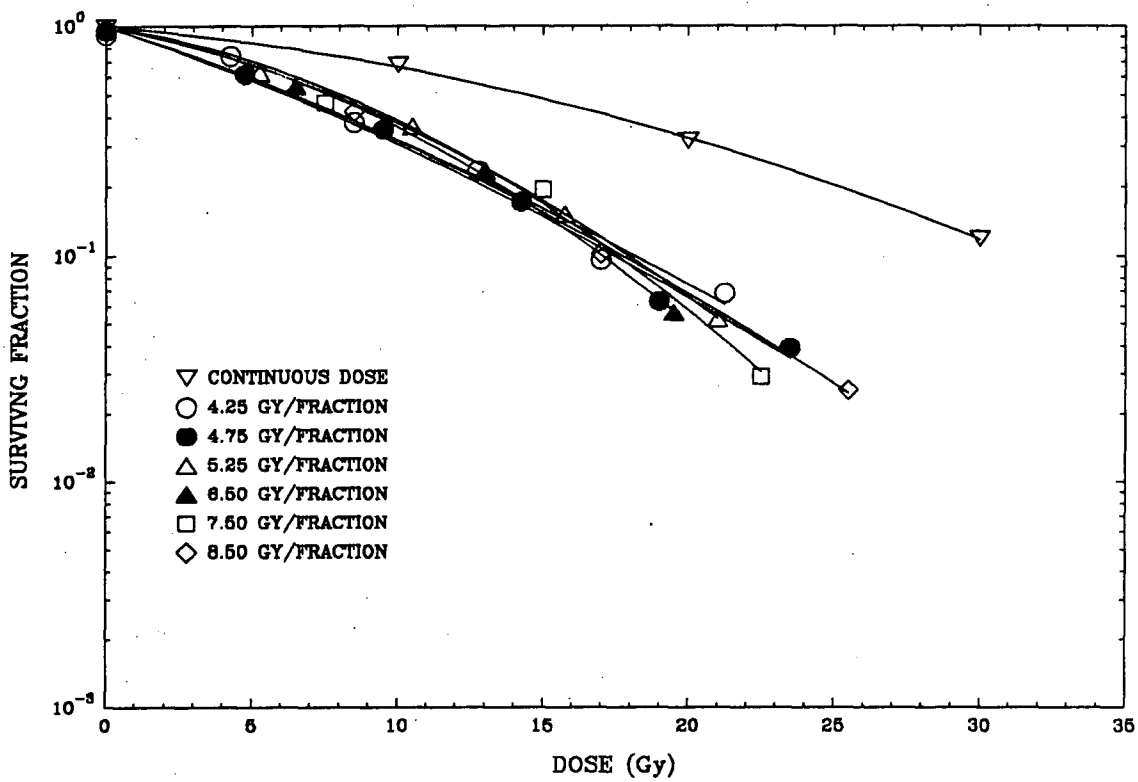

Figure 33. Survival Curve for 1 and 2 Fractions of X-rays per Day

Survival curves of V79-171b Chinese hamster cells from spheroids held at 22°C exposed to fractionated doses shown. 4.25, 4.75, and 5.25 Gy fractions were given twice daily and 6.5, 7.5 and 8.5 Gy fractions were given once daily. Also shown is the continuous ^{192}Ir radiation survival curve taken over 4 days.

The experiments were repeated using an X-ray source for irradiation to examine the effects of different types of radiation. Again, little difference was observed between the doses and again the survival curves were not linear (figure 33). These curves were also very different from the low continuous ^{192}Ir dose but this was less surprising because of the different types of radiation. Again, the 1 and 2 fraction/day curves did not agree as well as predicted by the LQM but were closer than with the ^{192}Ir as expected since the α and β values used for the prediction were taken from X-ray data.

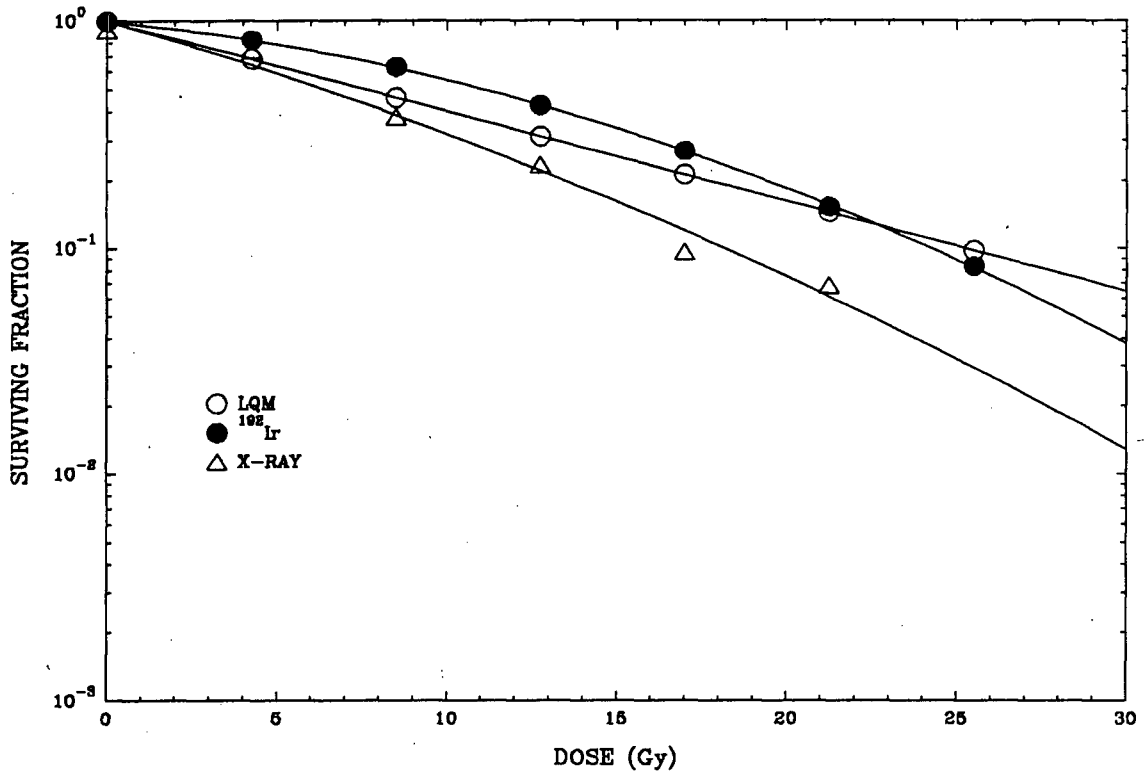

Figure 34. Comparison Between X-rays, ^{192}Ir and LQM

Comparison between the survival curves resulting from two fractions of 4.25 Gy per day from X-rays and a ^{192}Ir source along with the predicted curve from the LQM.

Looking solely at the 4.25 Gy/fraction administered twice daily in comparison with the model data (figure 34), the ^{192}Ir curve had a larger shoulder region than the X-rays whereas with 6.5 Gy/fraction and only 1 fraction per day (figure 35) it seemed to be the opposite. Also with 1 fraction per day the ^{192}Ir dose seemed much closer to the model curve than for the 2 fractions per day.

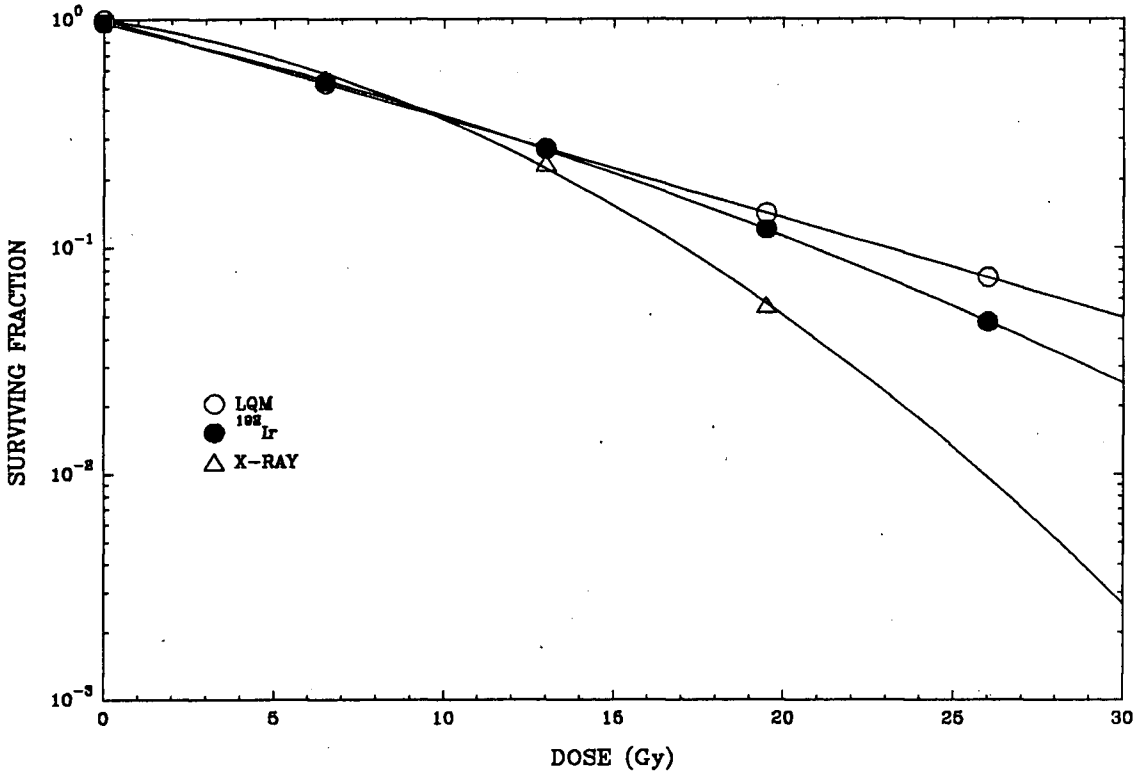

Figure 35. Comparison Between X-rays, ^{192}Ir and LQM

Comparison between the survival curves resulting from one fraction of 6.55 Gy per day from X-rays and a ^{192}Ir source along with the predicted curve from the LQM.

4. DISCUSSION

For the experiments performed to be useful in the prediction of the responses of tumours to multifraction therapy, each of the characteristic responses must be discussed separately with an attempt to unite them once each effect is understood.

The results of repair, redistribution and repopulation have been examined in the spheroid system, attempting to isolate each factor either by eliminating the others or by comparing experiments in which different combinations of factors were in effect. In the latter case, the comparison allowed the different results to be attributed to different causes by normalization or factoring out one or more effects.

Each experiment will be discussed along with the factors it examined with a discussion of each factor and its effect being surmised. Finally, a discussion of how these factors interrelate and an extrapolation to tumours will be made along with an association to clinically relevant dose schedules.

This will then be extended to another type of radiation, namely that from radioactive Iridium with the dose rate effects being discussed.

4.1 TWO DOSE EXPERIMENTS

The two dose experiments which included both split dose experiments and 2 fraction survival curves along with DNA profiles were used to examine the effects of repair and redistribution. By conducting the experiments at both 22°C and 37°C, the effects of redistribution were factored out since minimal progression or proliferation occurred at 22°C.

By comparing the two dose experiments, it is obvious that there was much more fluctuation in the response at 37°C than there was at 22°C; since the only difference between

these cells was redistribution, it can be concluded that the radiation response fluctuates throughout the cell cycle. Since this result was seen with two doses of 8 Gy but not 4 Gy, the cells must be sufficiently synchronized to see this effect since the cells in different phases have different sensitivities to radiation.

Another effect that temperature had on cell response related to the rate of repair. Repair occurred slightly faster at 37°C which seems logical as the metabolism of the cell is higher at this temperature and all of its functions should be occurring faster. Repair also occurred to a greater extent in the proliferating cells. This may be due to the more compact or condensed higher order chromatin structure that is characteristic of the quiescent state (Wheeler et. al., 1988).

4.2 THREE DOSE EXPERIMENTS

The three dose experiments were executed to determine whether the same effects seen after 2 doses occurred after 3 doses, namely, if a fluctuation in radiosensitivity existed with defined time intervals between the second and third doses. It is important to discover whether the fluctuation in radiosensitivity occurs regardless of the number or magnitude of preceding doses or whether it is unique to a two dose scenario.

In the 3 fraction experiments there was no apparent fluctuation in radiosensitivity at the time of the third dose either at 22°C or at 37°C. This indicated that the 12 Gy dose accumulated in the first two doses may have been sufficient to cause a delay longer than the 10 hour period over which measurements were made. Thus, in the spheroid system, the mitotic delay may not necessarily be linear with dose and there may exist a threshold dose over which the mitotic delay increases more drastically with increased dose. The mitotic delay may also be affected by the radiation being administered in fractionated doses.

It would be of interest to extend this 3 fraction split dose experiment to longer time intervals to see if the fluctuation in sensitivity becomes apparent after a sufficient time interval to overcome the mitotic delay.

4.3 FRACTIONATION

The 6 day fractionation schedule with a 6 Gy or 8 Gy dose given once daily provided a more clinically relevant study which allowed the cells in the spheroids enough time to progress through mitosis between fractions allowing the effects of repopulation to be observed. It also provided an indication as to whether the cells could repair after several fractions of radiation and whether the variation in radiosensitivity throughout the cell cycle continues to have an effect after several days of therapy.

The most obvious and expected difference between the 6 Gy and 8 Gy fractions is that the 8 Gy fractions were more effective at killing than the 6 Gy fractions.

Looking at the individual survival curves generated after each 6 Gy or 8 Gy fraction, it can be seen that their shapes changed for each 6 Gy or 8 Gy fraction indicating that different factors were predominantly affecting each survival response. With the 6 Gy fractions, the survival curve measured subsequent to an exposure of 6 Gy had an increased survival relative to previously unirradiated cells. The relatively wide shoulder indicates a more resistant population than initially, and suggests that repair had completed in the 24 hour interval after the first 6 Gy dose. The increased radioresistance indicates that, as was found in the 2 fraction split dose experiments, the prior 6 Gy fraction selectively killed the more sensitive cells resulting in a more resistant population which had completed one full cycle back to S phase during the 24 hour interval.

The more sensitive curve seen after the second 6 Gy fraction shows how proliferation can cause the cells to cycle into a sensitive phase of the cycle resulting in an unexpected increase in radiosensitivity. As expected, subsequent progression led to more resistance after the third 6 Gy fraction, resulting in a survival curve showing higher relative survival than after the second fraction. Less variation in radiosensitivity, however, seemed apparent after the fourth and fifth fractions; the survival curves produced at those times suggested somewhat greater radiosensitivity as would be expected with a loss of ability to repair.

The effects of progression-dependent fluctuating radiosensitivity seemed to be reduced in the survival curves produced after the 8 Gy daily fractions. The survival curves showed an increased resistance after the first and second 8 Gy fractions suggesting some degree of selection for more radioresistant cells, but the survival curves produced after each of the third and fourth 8 Gy fractions indicate increasing radiosensitivity as expected, again showing the loss of the cells' ability to repair.

These results stress the importance of understanding how cell progression is affected by radiation. If it could be reliably predicted both where and when the cells are in their most sensitive state, radiotherapists could take advantage of their increased sensitivity. Unfortunately, since tumours are comprised of such varying cell populations, this seems unlikely but it would be interesting to study how accurately one could predict the fluctuation in sensitivity of a partially synchronized tumour cell population after an initial dose which could selectively kill the sensitive cells.

Another important factor to take into account in radiotherapy is the amount of recovery (repair and repopulation) that occurs in the cells between doses. It was found both with the 6 Gy and 8 Gy fractions that this recovery between fractions was not constant (figure 28). Survival increased significantly between the first two fractions, remained constant between the

second and third fractions, and again increased significantly for the remaining inter-fraction intervals. Therefore, it can be deduced that the recovery between the first two fractions was due mainly to selection and repopulation. Since the cell killing increased after the second dose, and the repopulation rate had not yet maximized, the recovery after the second dose was negligible. After 3 doses, the cells had either accumulated 18 Gy or 24 Gy, depending on the dose/fraction, and it seems that this was a sufficient dose or time for a compensatory repopulation mechanism to occur as the recovery between the fractions again became significant.

This information, along with the data from the 3 fraction split dose experiments, leads to the conclusion that, with the spheroid system, 12 Gy of accumulated dose given in two 6 Gy fractions may be sufficient to cause a significant mitotic delay while after 16 Gy given in two 8 Gy fractions, compensation occurs and repopulation increases, adding more support to the suggestion that the mitotic delay in spheroids may not be proportional to dose when the cells are subjected to fractionated radiation.

Looking at the overall effect per fraction, the 6 Gy/fraction doses had a reduced effect per fraction at the higher fractions while the 8 Gy/fraction doses showed a more constant effect per fraction. Although, in the latter protocol, cell kill appeared to remain constant for each 8 Gy fraction, this was due to varying contributions from several factors, namely repair, redistribution, repopulation and cell killing. Each fraction was affected differently by these factors with the overall effect remaining constant. The same occurred with the 6 Gy/dose fractions although, in this case, the overall effect was not constant as the repopulation seemed to exceed the amount of cell killing occurring.

In radiotherapy, it is advantageous to achieve an equal effect per fraction to avoid having to alter the timing of the fractions or the fraction sizes. As the ideal dose size and timing

varies from system to system, each system should be independently studied, devising a unique treatment schedule for each patient.

4.3.1 Two Gray Response

Since complete survival curves were generated after each fraction during the multifraction experiments just described, the sensitivity of the cell to the therapeutically relevant dose of 2 Gy could also be assessed. It was of particular interest to determine whether this response remained constant with the number of fractions as hoped by many radiotherapists.

For both the 6 Gy and 8 Gy per fraction protocols, radiosensitivity at 2 Gy was not constant (figures 29 and 30) and, in fact, varied considerably. The relative response with repopulation factored out was determined to be dependant on the selection of radioresistant cells by previous doses as well as the repair occurring between fractions. We reached this conclusion because the 2 Gy survival after the first fraction of both 6 Gy and 8 Gy was high; the initial 6 Gy or 8 Gy dose had selectively killed the more sensitive cells and repair had occurred. The relative 2 Gy responses after subsequent 6 Gy or 8 Gy fractions decreased steadily as the selection process became less emphasized and as the cells lost their capacity to repair.

When survival after 2 Gy was expressed as a ratio of the post-treatment survival of the previous 6 Gy or 8 Gy fraction to take into account repopulation between doses, this ratio decreased between the second and third fractions and then increased between subsequent fractions. As discussed earlier, this was the result of a decrease in repair capacity coupled with an increase in repopulation due to a compensatory mechanism. This increase between the later fractions was more emphasized with the 8 Gy fractions indicating that the higher doses may have induced a more significant compensation.

Since 2 Gy doses are used in clinical fractionation schedules, it is of interest not only to discover that the 2 Gy response does not remain constant with each fraction, but also, to note how greatly it varied. A useful study would be one in which 2 Gy doses are administered twice daily for about a week as would occur in a clinical fractionation treatment. This would give a better indication of the consistency of the 2 Gy response in a typical fractionation schedule.

4.4 BRACHYTHERAPY

From the experiment conducted using the brachytherapy unit in the cancer clinic, it was found that the continuous low dose rate survival curve had a higher survival than either the 1 or 2 fractions per day curves for both the ^{192}Ir and the X-rays. The ^{192}Ir data suggest that low dose rate radiation induced damage is more repairable than at a high dose rate, with the most damage being repaired when a low continuous dose rate is used and the least when one fraction of higher dose is administered daily. The LQM does not, therefore, provide a good model for predicting the results of fractionated schedules from continuous low dose treatments since it does not take into account the different repairability at different dose rates. The predicted isoeffect doses resulted in greater killing indicating that a lower dose per fraction than was predicted would be necessary to obtain the same result as the low continuous dose.

4.5 SUMMARY

Our experiments on the three dimensional spheroid model have provided many insights into radiation effects in that system.

In fractionated exposures we found that, even if an equal overall effect per fraction is obtained, it is due to repair, redistribution, repopulation and cell killing each having varying

significance at the time of each fraction. In essence, the competing factors can tend to cancel each other. However, in the first 2 or 3 fractions, repair and redistribution had the largest effects on the response while in the later fractions, repopulation became the dominating effect. We also found evidence to suggest that the mitotic delay may not be linear with dose. After 12 Gy, the delay increased significantly, however, after about 16 Gy, a compensatory mechanism was activated causing the proliferation rate to increase with dose.

Dose rate also affects the response of cells to radiation. Cells damaged by a high dose rate have a low repair capacity while damage caused by a low continuous dose may be more easily or more completely repaired.

All of these factors must be taken into account when predicting a treatment schedule for a cancer patient. It is important to understand how the cells in the tumour will recover between fractions whether due to repair or repopulation, and also to understand how the time intervals between treatments will affect the sensitivity of the cells as they progress through the cell cycle. We have, in this thesis, provided more information on the response of cells in spheroids to radiation with the effects of repair, repopulation and redistribution being better defined. This knowledge can now be extrapolated to tumours, hopefully being of some assistance in the prediction of radiation response.

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APPENDIX A

Assuming that 1) the initial population of cells in a 3 day old spheroid is comprised of about 30% S phase or resistant cells and 70% sensitive cells, 2) that an 8 Gy dose reduces the surviving fraction to about 10%, and 3) that the surviving fraction of the resistant cells is 3 times greater than the sensitive cells at that dose, then the population distribution after an 8 Gy dose can be estimated.

Initially, $$S_i + R_i = 1.00 \quad (1)$$

where: S_i = initial sensitive population
R_i = initial resistant population

After 8 Gy dose, $$S_f + R_f = 0.10 \quad (2)$$

where: S_f = final sensitive population
R_f = final resistant population

And,
$$S_i = 0.70$$
$$R_i = 0.30$$
$$S_f = 0.70(1 - x) \quad (3)$$
$$R_f = 0.30(1 - y) \quad (4)$$

where: x = fraction of sensitive cells killed
y = fraction or resistant cells killed

So,
$$3(1 - x) = 1 - y$$
$$3 - 3x = 1 - y$$
$$x = .67 + .33y \quad (5)$$

Substituting equations 3, 4 and 5 into equation 2:
$$0.70(1 - 0.67 - 0.33y) + 0.3(1 - y) = 0.1$$
$$0.70 - 0.47 - 0.23y + 0.3 - 0.3y = 0.1$$
$$0.43 = 0.53y$$
$$y = .81$$

Therefore,
$$R_f = 0.06$$
$$S_f = 0.04$$

Therefore, the resulting population is comprised of 60% resistant cells and 40% sensitive cells.

Assuming that the survival curve resulting from being held at 22°C for 6 hours after the initial 8 Gy dose is the most representative of this resistant population, the α and β values would be 0.276 Gy^{-1} and 0.0150 Gy^{-2} respectively. Also assuming that the most resistant population would be comprised of about 60% resistant cells and 40% sensitive cells and that the most representative survival curve would be the ones held at 37°C for 4 hours, the α and β values would be 0.301 Gy^{-1} and 0.0207 Gy^{-2} respectively.

According to the LQM, the equation for the resistant curve would be:

$$S = 0.6\exp(-\alpha_r D - \beta_r D^2) + 0.4\exp(-\alpha_s D - \beta_s D^2) \quad (6)$$

$$= 0.6S_r + 0.4S_s$$

and $$-\ln S = 0.267D + 0.00798D^2$$

where: S_r = survival of resistant cells
S_s = survival of sensitive cells

and the equation for the sensitive curve would be:

$$S = 0.4\exp(-\alpha_r D - \beta_r D^2) + 0.6\exp(-\alpha_s D - \beta_s D^2) \quad (7)$$

$$= 0.4S_r + 0.6S_s$$

and $$-\ln S = 0.301D + 0.0207D^2$$

From equations 6 and 7, survivals at a range of doses were determined. Defining the survival from equation 6 as X and from equation 7 as Y, S_r and S_s were calculated at various doses from the following equations:

$$0.6S_r + 0.4S_s = X \quad (8)$$
$$0.4S_r + 0.6S_s = Y \quad (9)$$

Therefore,

$$S_r = 3X - 2Y \quad (10)$$
$$S_s = 3Y - 2X \quad (11)$$

The surviving fractions calculated from these equations were fit by the LQM to determine the α and β coefficients for as sensitive population and a resistant population. The resulting equations for population containing 100% sensitive and 100% resistant cells are:

$$\ln S_s = -0.221D - 0.0899D^2 \quad (12)$$

and

$$\ln S_r = -0.193D - 0.0154D^2 \quad (13)$$

respectively. From these two equations, the response of any population distribution can be predicted for cell in which repair is complete.